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An inhibitor of sequence-specific proteolysis that targets the substrate rather than the enzyme

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Abstract

Background: Traditional protease inhibitors target the active site of the enzyme. However, since most proteases act on multiple substrates, even the most specific protease inhibitors will affect the levels of a number of different proteins. However, if substrate-targeted inhibitors could be developed, much higher levels of specificity could be achieved. In theory, compounds that bind the cleavage site of a particular substrate could block its interaction with a protease without having any effect on the processing of other substrates of that protease.

Results: A model system is presented that demonstrates the feasibility of substrate-targeted inhibition of proteolysis. A peptide selected genetically to bind a 14-residue epitope that encompasses the cleavage site of human pro-IL-1β was shown to inhibit interleukin-converting enzyme (ICE)-mediated proteolysis of model substrates containing the 14-mer target sequence. However, the peptide had no effect on the cleavage of other ICE substrates with different amino acids flanking the minimal cleavage site.

Conclusions: This study demonstrates the feasibility of substrate-targeted inhibition of proteolysis. More potent compounds must be developed before substrate-targeted inhibitors can be used routinely. Nonetheless, this novel strategy for protease inhibition seems promising for the development of extremely selective molecules with which to manipulate the maturation of many important pro-hormones, -cytokines and -proteins.

Keywords: Cleavage site; Protease inhibitor; Sequence-specific proteolysis; Substrate-targeted inhibitor

1. Introduction

A major goal in chemical biology is to develop synthetic molecules capable of manipulating particular biological pathways. A particularly important target in this regard is the post-translational modification of proteins, since phosphorylation, ubiquitinylation, glycosylation, proteolysis and a variety of other events have enormous effects on the activities and levels of many critical regulatory factors. A major issue in this area is specificity. For example, while many protein kinase inhibitors are known, a traditionally difficult issue in this field has been to obtain compounds that are highly selective for a given kinase, since the active sites of many of these enzymes are quite similar. This often requires extensive optimization or multiple cycles of combinatorial chemistry. Furthermore, even in the case where an inhibitor has extremely high specificity for a given enzyme, it will nonetheless affect more than one event. This is because the vast majority of enzymes that mediate the post-translational modification of other proteins operate on multiple substrates. This fact places a fundamental limit on the biological specificity that one can achieve using an enzyme-targeted inhibitor.

An alternative strategy would be to manipulate the post-translational modification of proteins with compounds that recognize the substrate rather than the enzyme. Most proteases, protein kinases, etc. have relatively small recognition sequences. Thus, if one could design molecules able to recognize not only these core sites, but flanking residues as well, it might be possible to employ these species as 'epitope protecting groups' capable of shielding a specific protein from a given modification enzyme without affecting the processing of other proteins by the same enzyme. This concept is illustrated in Fig. 1 in the context of a substrate-specific protease inhibitor.

The challenge in implementing this strategy is to identify molecules capable of recognizing linear epitopes in the

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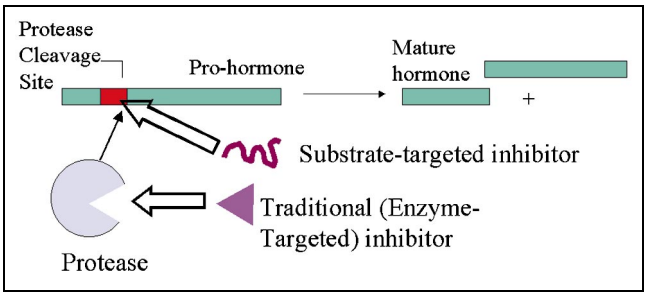

Fig. 1. Schematic representation of the two types of proteolysis inhibitors discussed in this study. Traditional protease inhibitors are targeted to the enzyme itself (usually the active site). A substrate-targeted inhibitor would bind an epitope in the substrate that includes the site of enzymatic cleavage.

target protein that include the processing site. While this can be done using monoclonal antibodies, there is little literature regarding peptide epitope recognition by relatively low molecular weight, synthetic molecules [1–5], particularly in aqueous solution [6,7]. Recently however, we introduced a two-hybrid-like genetic selection system that may be generally useful for the selection of epitope-binding peptides [8]. This assay, modeled after a scheme originally developed by Hu and co-workers for the study of leucine zipper interactions [9,10], involves reconstitution of lambda Repressor activity via a peptide–peptide interaction. Specifically, the native C-terminal dimerization domain of lambda Repressor is replaced by either a peptide library or a specific target epitope. In the absence of dimerization, lambda Repressor DNA-binding domain (DBD) does not bind tightly to operator sequences or repress transcription efficiently. Two different compatible plasmids that express these fusion proteins are transformed into *Escherichia coli* and these cells are then challenged with lethal levels of lambda phage. Cells that survive the phage challenge generally have reconstituted Repressor activity. After the removal of undesired 'false positives' (such as library-encoded homooligomeric peptides [11]) library-encoded peptides (LEPs) that bind to the target epitope and thus reconstitute Repressor DBD dimerization, can be identified.

In a previous report, we identified a 15-residue LEP able to bind a 14-amino acid epitope nearly identical to a sequence found in human pro-interleukin-1β (IL-1β) [8]. IL-1β is a cytokine that is secreted primarily by activated monocytes and macrophages in response to infection or injury [12]. Like many cytokines and hormones, human IL-1β is translated as an inactive precursor that must be matured proteolytically to generate the bioactive species. This cleavage reaction, which transforms a 31.5-kDa proprotein to the 17-kDa mature cytokine, is mediated by an interleukin-converting enzyme (ICE; also know as caspase 1) [13,14]. Given the critical role played by IL-1β in inflammation as well as other biological processes, there has been tremendous interest in the development of ICE inhibitors to block maturation of the pro-hormone. However, ICE has a number of other known substrates, including IL-18 [15,16] and IFN-γ-inducing factor [17]. ICE also autocatalyzes its own conversion from a zymogen to the heterodimeric, active protease [18,19]. Thus, the ICE-mediated cleavage of pro-IL-1β seemed an interesting system in which to probe the feasibility of substrate-targeted inhibition of post-translational modification. We demonstrate here that a LEP isolated in the genetic screen is indeed capable of inhibiting ICE-mediated cleavage of model substrates containing the human pro-IL-1β processing site. The same LEP had no affect on the ICE-mediated cleavage of a substrate containing a cleavage site corresponding to that of the ICE zymogen. This validates the general idea that relatively low molecular weight, synthesizable molecules can be employed as substrate-specific manipulators of the post-translational modification of proteins.

2. Results

As discussed above, we recently described a simple genetic selection system that allows peptide libraries to be screened for molecules able to bind protein epitopes (i.e., other peptides) with excellent specificity [8]. One such complex discovered using this system was comprised of a 15-amino acid residue LEP KARKEAELAAATAEQ (called LEPB since it was the second of four isolated from a genetically encoded library) and a 14-amino acid target epitope NEAYVHDGPVRSLN. This epitope was designated ICS, since it is almost identical to residues 110–123 of the immature form of human IL-1β, which includes the ICE cleavage site (after Asp116). To test the concept of substrate-targeted protease inhibition, LEPB was assayed for its ability to block ICE-mediated cleavage of the ICS epitope and its specificity for this sequence relative to other ICE substrates.

To carry out this experiment, two model protein substrates were made using standard molecular cloning and protein expression techniques. One, called **Matched**, includes an N-terminal glutathione *S*-transferase (GST) protein connected to a C-terminal green fluorescent protein (GFP) protein via a linker that includes the ICS (NEAYVH**D**GPVRSLN). The second, called **Mismatched**, is comprised of an N-terminal GST linked to a C-terminal maltose-binding protein (MBP) via a linker that includes the site in pro-ICE that undergoes cleavage during zymogen maturation [7], GVVWFK**D**SVGVSGN. The Asp residue adjacent to the ICE cleavage site in each sequence is highlighted in bold and underlined. This zymogen sequence has little homology with ICS other than the Asp residue recognized by the enzyme.

The **Matched** and **Mismatched** substrates were mixed in equimolar amounts in the same tube with or without LEPB, then purified ICE enzyme was added. Fig. 2 shows the results of this experiment. The apparent molecular

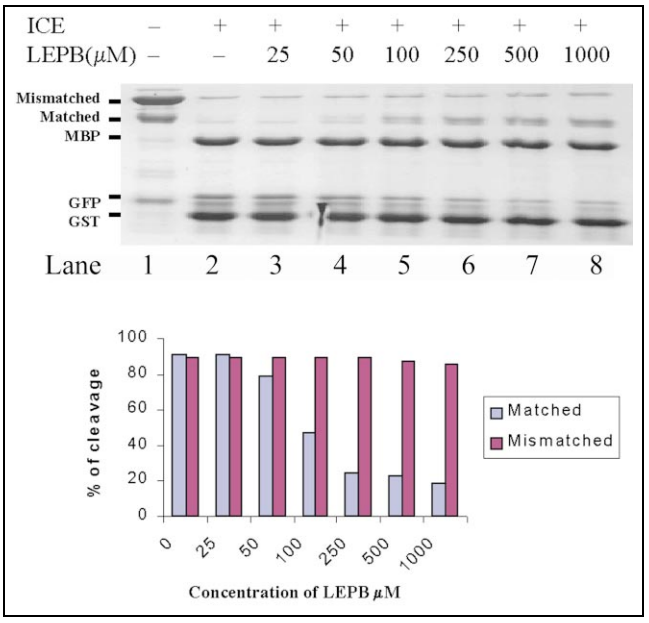

Fig. 2. Substrate-specific inhibition of ICE-mediated proteolysis by LEPB. Varying concentrations of LEPB were included in a reaction containing two model substrates, both of which are cleaved by ICE. One (**Matched**) contains the pro-IL-1β cleavage site that LEPB was selected to bind. The other (**Mismatched**) contains the pro-ICE cleavage site. **Matched** = **GST**NEAYVHDGPVRSLN**GFP**; **Mismatched** = **GST**-GVVWFKDSVGVSGN**MBP**. The bands present in the first lane other than those corresponding to **Matched** and **Mismatched** are due to low levels of proteolytic fragments (from the *E. coli* extract) in the purified preparations. LEPB = KARKKEAELAAATAEQ.

mass of the **Matched** substrate is approximately 60 kDa and that of the **Mismatched** protein is 85 kDa, as shown in lane 1. Lane 2 shows the result of adding purified ICE to a mixture of the two substrates in the absence of inhibitor. As expected, proteolysis of the ≈60 kDa **Matched** protein yielded GST protein (27 kDa) and GFP protein (29 kDa) containing fragments. ICE-mediated cleavage of **Mismatched** produced GST protein (27 kDa) and MBP (50 kDa) containing fragments. Both substrates were processed with approximately equal efficiency.

Addition of synthetic LEPB to the reaction had markedly different effects on **Matched** and **Mismatched** cleavage. In the latter case, LEPB had no inhibitory effect on proteolysis even at concentrations up to 1 mM (Fig. 2, lanes 3–8). However, for the **Matched** protein substrate, significant inhibition of cleavage was observed starting at 50 μM LEPB and reaching saturation around 250 μM. This is most evident by inspection of the **Matched** band intensity at increasing LEPB concentrations and the concomitant decrease in the band corresponding to the GFP fragment-containing product. At 250 μM LEPB, only approximately 20% of the **Matched** protein substrate was cleaved under the conditions employed, compared with approximately 90% cleavage in the absence of the peptide. This differential rate of cleavage can also be observed in a time course experiment (Fig. 4). The data in Fig. 4 reiterate that the two substrates are processed with similar kinetics by ICE in the absence of inhibitor molecules.

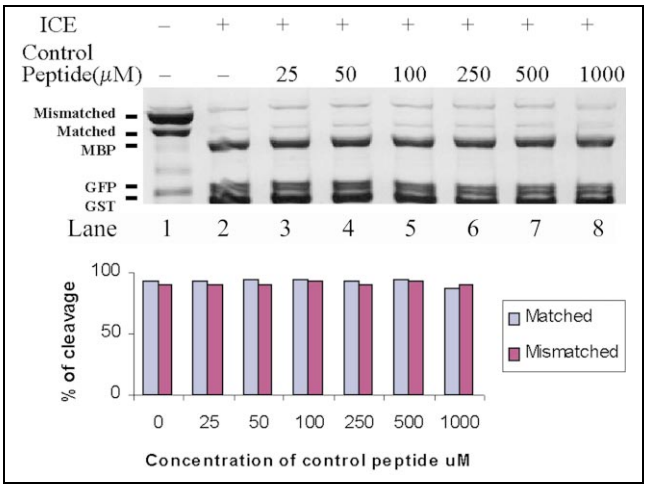

Fig. 3. A control peptide not selected to bind either substrate does not inhibit ICE-mediated proteolysis. A 19-amino acid peptide, picked randomly from the library from which LEPB was isolated, was employed in place of LEPB in the same experiment described in Fig. 2. 19-mer = GGWSGGCGRTSAVSSASFP.

A 19-amino acid peptide, GGWSGGCGRTSAVSSASFP, selected randomly from the library, had no effect on the ICE-mediated cleavage of either substrate at concentrations ranging from 25 μM to 1 mM (Fig. 3). Finally, a 15-amino acid peptide AEAALARKETAKEQA, which is a randomized version of LEPB, also had no effect on ICE-mediated cleavage of either the **Matched** or **Mismatched** substrate at concentrations up to 1 mM (data not shown). These results argue that the 15-residue LEPB is acting as a sequence-specific, substrate-targeted inhibitor of the **Matched** processing by ICE.

The only data that seemed at odds with the proposed mechanism of action of LEPB was the IC_{50} of approximately 100–150 μM. Previously, we had reported that the K_D of the LEPB/ICS complex is approximately 2 μM [8]. If LEPB inhibition of **Matched** cleavage indeed was the result of the peptide binding the cleavage site and shielding

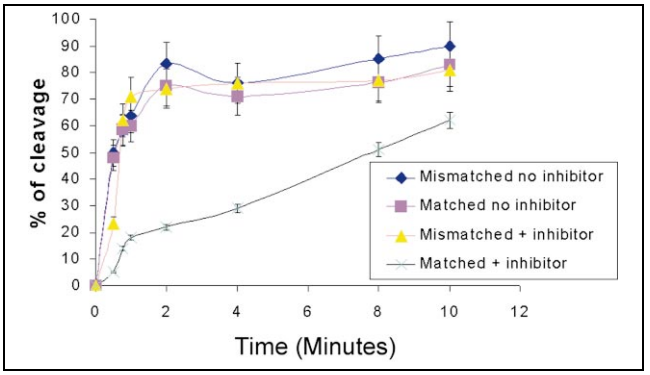

Fig. 4. Time course for the ICE-mediated proteolysis of **Matched** and **Mismatched** in the presence or absence of 250 μM LEPB or the 19-mer peptide.

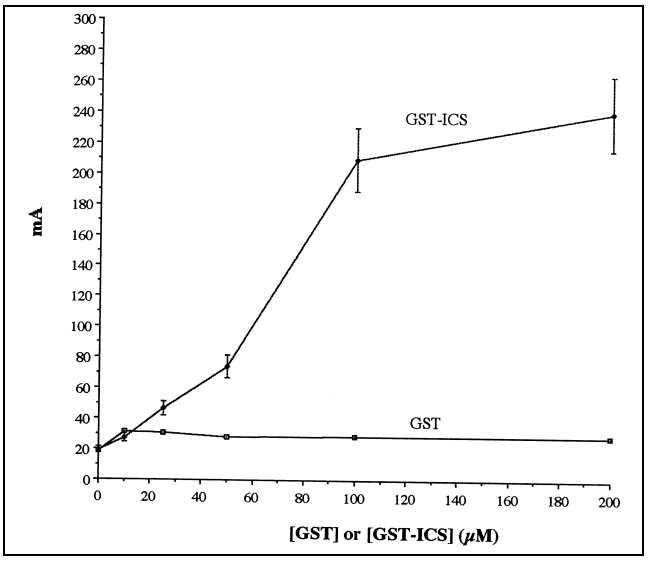

Fig. 5. Association of LEPB (20 nM) and a GST–ICS fusion protein monitored by fluorescence polarization. The data indicate that the K_D of the LEPB–ICS complex under these conditions is on the order of 70–120 μM.

it from ICE, then one would expect that the IC_{50} would roughly mirror the K_D of the peptide–peptide complex. The buffer conditions and temperature employed in the protease assay and the previous K_D measurement were not identical and different fusion proteins were employed in each study. However, this seemed a rather large difference to explain away based simply on these differences.

To probe this issue in more detail, we employed a fluorescence polarization experiment [20] to measure directly the K_D of the LEPB–ICS complex under the conditions of the proteolysis assay. LEPB was synthesized with an N-terminal fluorescein label and this molecule (20 nM) was then titrated with an ICS-containing fusion protein (GST–ICS) in the cavity of a Beacon 2000 fluorescence polarization instrument. The data obtained (average of three experiments) are shown in Fig. 5. The readings at very high GST–ICS concentrations were less reproducible than the values obtained at lower concentrations, possibly due to light scattering, so a specific dissociation constant cannot be gleaned from these data. However, it is clear that the K_D is much higher than the 2 μM measured previously and appears to be somewhere in the range of 70–150 μM, more consistent with the results of the ICE inhibition assay. Similar experiments conducted at different peptide concentrations gave identical results (data not shown), indicating that the conditions chosen represented equilibrium binding. Furthermore, the use of LEPB-related peptides that also contained several residues present in the genetically selected fusion protein (i.e., the 'hit' in the original assay) also provided essentially identical results (data not shown), arguing that context effects are not terribly important. Based on these data, we therefore conclude that all of the available data are consistent with LEPB acting as a substrate-targeted inhibitor of proteolysis that acts through a sequestration mechanism.

3. Discussion

As discussed in Section 1, enzyme-targeted inhibitors of protein-modifying factors have an intrinsic limit in their specificity. This is a simple consequence of the fact that most modifying enzymes have multiple substrates. All of these processing events will be affected to some extent by an enzyme-targeted molecule. Substrate-targeted inhibitors, on the other hand, have the potential for much higher specificity since they could, in theory, retard the rate of a single processing event without affecting other reactions mediated by the same enzyme. Another application would be to block a processing event for which the enzyme is unknown or which can be mediated by more than one enzyme. Substrate-targeted inhibitors could be extremely useful tools for probing the biological manifestation(s) of a particular post-translational modification event. It is also possible that the same strategy could have therapeutic applications, for example if a single enzyme mediated both 'good' and 'bad' reactions from the standpoint of a desired therapeutic outcome.

To the best of our knowledge, this study provides the first example of substrate-targeted inhibition of proteolysis by a synthetic molecule (see [21] for the use of antibodies in this type of application). A peptide selected genetically to bind a linear epitope almost identical to human pro-IL-1β [8] was indeed able to inhibit ICE-mediated proteolysis of the cleavage site contained in this sequence without affecting another ICE-mediated reaction in the same tube. As expected therefore, the epitope-binding peptide was able to use the unique information inherent in the different sequences flanking the site recognized by the enzyme to distinguish between substrates. Various control peptides not selected to bind the cleavage site had no effect on any of the reactions studied. If this approach proves to be general, and more efficient inhibitors can be developed, this technique may be useful in providing the functional equivalent of a conditional knockout of a given polypeptide if that species must be matured proteolytically. It should also be possible to extend this substrate-targeted inhibition approach to other types of protein-modifying events. Indeed, Nestler and co-workers have previously used epitope-targeted 'molecular forceps' to inhibit farnesylation of fusion proteins containing a peptide representing the C-terminus of Ras [7]. Other than the experiments reported here, this is the only other example of a synthetic substrate-targeted inhibitor of which we are aware.

While we are hopeful that the substrate-targeted inhibitor approach can eventually be developed into a useful general tool for the manipulation of protein-modification events, several important challenges must be met before this strategy achieves practical utility. Of course, peptides

such as LEPB are unlikely to be pharmaceutically useful reagents, but given impressive recent advances in methods to carry cell-impermeable molecules across membranes [22–26], they might be useful research tools. A more important current limitation is the modest affinities that the currently available epitope-binding peptides have for their targets. The LEPB molecule employed in this study exhibited an IC_{50} of approximately 70–150 μM under the conditions employed. The binding experiments shown in Fig. 5 indicate that this is most likely a reasonable reflection of its affinity for the target epitope under these conditions. Clearly, this is insufficient for LEPB to be employed in a practical sense, for example in a cell culture experiment, as an inhibitor of IL-1β maturation. Since only a few epitope targets have been examined to date, it is too early to say whether this affinity will prove to be typical of peptide–peptide complexes isolated using the Repressor reconstitution strategy or any other assay. But if this is the case, an important immediate goal will be to devise efficient, relatively high-throughput strategies by which to elaborate these molecules into high-affinity epitope binders without sacrificing synthetic expediency. For example, one straightforward approach would be to explore second-generation libraries comprised of peptides that resemble the initial hit. These could be screened by phage display [30] or some other in vitro technique that would allow the stringency of the binding conditions to be controlled. We are in the process of exploring several strategies to obtain high-affinity epitope-binding compounds.

It is worthwhile to comment here on why the apparent K_D of the LEPB–ICS complex seen here is significantly different than that reported previously (≈ 2 μM) [8]. While differences in conditions between the two experiments and the local context of the peptide play a role, we speculate that the nature of the assay used previously may have been even more of an issue. In that work, a GST 'pull-down' assay was employed in which various concentrations of GST–ICS were incubated with an extract containing overexpressed Repressor DBD-LEPB. The extent of complex formation was evaluated at each GST–ICS concentration by precipitation with glutathione-agarose beads followed by SDS–PAGE and Western blotting. GST is a homodimer [27] and thus displayed the ICS peptide as such. Furthermore, while the Repressor DBD is not a stable dimer, it does have some residual self-associating capability, particularly in extracts where it has been overexpressed. Thus, while the value reported for the K_D of the Repressor LEPB–GST–ICS complex is correct, it may not accurately reflect the true LEPB–ICS K_D due to avidity effects (i.e., the contribution of a 2:2 complex).

While this study and that of Nestler and colleagues [7] employed peptides as epitope-binding molecules, there is no reason to believe that appropriate non-peptidic molecules could not fulfill the same role. While it is unlikely that a truly small (< 500 Da) drug-like molecule could recognize an extended linear epitope, non-peptidic oligomers of some sort are more promising candidates. Such molecules may or may not exhibit enhanced cell permeability, but would at least be immune to proteases, an important feature if they are to be used as tools for cell culture or even in vivo experiments. Finally, it should be pointed out that single chain antibodies [28] or epitope-binding proteins based on alternative protein scaffolds [29] do have sufficient binding constants to be employed as substrate-specific manipulators of post-translational modifications. In cell culture experiments or even in transgenic animals, these molecules could be used to elicit the desired 'knockout' effect of a given event. However, such biological approaches are less desirable than the use of synthetic molecules since the latter can be applied at any time in the course of an experiment, making manipulation of the system far easier.

4. Significance

The feasibility of using epitope-binding compounds as substrate-targeted inhibitors of proteolysis has been demonstrated. While the peptide inhibitor in hand binds too weakly to be of practical utility, this novel result indicates a pathway for the future development of highly selective manipulators of proteolytic maturation of pro-proteins as well as other post-translational modifications of proteins.

5. Materials and methods

The purified ICE enzyme was kindly provided by Dr. Nancy Thornberry (Merck).

5.1. Peptide synthesis and labeling

All the peptides were synthesized using a Perkin-Elmer Synergy solid-phase peptide synthesizer using Fmoc chemistry. The peptides were cleaved from beads using trifluoroacetic acid. The desired peptides were purified by reverse-phase HPLC. Each peptide was characterized by electrospray mass spectrometry and provided the anticipated molecular ion.

Fluorescein-labeled LEPB was made by treating 25 mg of the beads on which the peptide was synthesized with excess piperidine for 30 min at room temperature. After removing most of the piperidine via syringe, 1 ml of carboxyfluorescein-*N*-hydroxysuccinimidyl ester in DMF (20 mg/ml) was added to the slurry and the resultant mixture was incubated at room temperature for 4 h. After this time, the beads were washed extensively with DMF and DMSO to remove excess starting material. The fluoresceinated peptide was cleaved from beads with TFA, purified by reverse-phase HPLC and characterized by mass spectrometry.

5.2. Construction of the GST–ICS expression vector (pGST–ICS)

Oligonucleotides encoding the peptide sequence NEAYVHD-

GPVRSLNCIIHRD (amino acids present in the ICS are underlined) were inserted into pGEXcs digested with *Nco*I and *Bam*HI. After transformation of *E. coli* and purification of the plasmid by standard methods, the construct was verified by DNA sequencing.

5.3. Construction of the expression vectors for the **Matched** and **Mismatched** proteins (pMATCH and pMISMATCH)

Two oligos: 5′-CCGGGGGAACGAAGCATACGTACACGACGGACCCGTAAGAAGCCTAAACGTA and 5′-CCGGTACGTTTAGGCTTCTTACTGGTCCGTCGTGTACGTATGCTTCGTTCCC were annealed and ligated into the vector pGFPuv (Clontech) that had been digested with restriction enzymes *Xma*I and *Age*I. The products were verified by DNA sequencing. A pair of PCR primers was used to amplify the DNA fragment encoding both the ICS peptide and the GFP protein. The PCR product was digested with *Eco*RI and *Nco*RI restriction enzymes, gel-purified and ligated into vector pGEXcs digested with *Eco*RI and *Nco*RI to provide pMATCHED. The construct was verified by DNA sequencing.

To make pMISMATCHED, two oligos: 5′-TCGAGGGAGTCGTCTGGTTCAAAGACTCAGTCGGCGTCTCAGGGAACGC and 5′-CATGGCGTTCCCTGAGACGCCGACTGAGTCTTTGAACCAGACGACTCCC were annealed and ligated into the vector pGEXGGH digested with *Xho*I and *Nco*I. This vector was subsequently digested with *Nco*I and *Sma*I. A PCR product containing sequences encoding the MBP was then cut with the same enzymes and inserted into the *Nco*I/*Sma*I-cut vector to provide pMISMATCHED. This construct was verified by DNA sequencing.

5.4. Purification of GST fusion proteins

The pGST–ICS, pMATCHED or pMISMATCHED plasmids were transformed into BL21 (DE3) competent cells and plated on a LB plate supplemented with ampicillin, and grown at 37°C overnight. A single colony was inoculated into 2 ml of LB medium supplemented with ampicillin. The 2-ml culture was inoculated into 50 ml of LB medium supplemented with ampicillin and grown to saturation. The 50-ml culture was inoculated into 1 l of LB medium with ampicillin and grown to an OD_{600} of 0.5. The culture was induced with 0.5 mM IPTG and grown at 37°C for 3 h. The cells were harvested by centrifugation at 3500 rpm for 15 min. The pellet was resuspended in 25 ml of PBS buffer solution supplemented with protease inhibitor tablets (Boehringer Mannheim). The suspension was frozen with liquid nitrogen and stored at −70°C overnight. The suspension was thawed at room temperature and subjected to sonication. The resulting mixture was centrifuged at 12 000 rpm for 45 min. The supernatant was added to 1 ml of glutathione-fused beads and tumbled at 4°C for 2 h. The beads were centrifuged at 3000 rpm for 5 min, then washed three times with PBS solution supplemented with 500 mM NaCl. The beads were equilibrated with PBS and bound GST fusion proteins were released by the addition of 10 mM of reduced glutathione in TE buffer. The eluted sample was dialyzed against PBS at 4°C to remove the reduced glutathione.

5.5. K_D measurement by fluorescence polarization experiment

A series of 200-μl solutions was set up with HEPES buffer (10 mM, pH 7.5), 10% sucrose, 2 μg of Melittin fluoresceinated LEPB (20 nM) and GST–ICS protein (0, 10 μM, 25 μM, 50 μM, 100 μM and 200 μM). The solutions were allowed to come to equilibrium by incubating at room temperature for 20 min. The samples were then placed into the cavity of a fluorescence spectrometer equipped to measure anisotropy (PanVera Beacon 2000). The polarization of the emitted light was recorded. Three identical experiments were conducted and the values were averaged.

5.6. Proteolysis inhibition assay

10 μM of **Matched** protein, 10 μM of **Mismatched** protein, 10% sucrose and HEPES buffer (10 mM, pH 7.5) were mixed with 0–1000 μM LEPB (Fig. 1) or 0–1000 μM 19-mer control peptide (Fig. 2) in a total volume of 30 μl. The mixture was left at 4°C for 30 min, then allowed to warm up to room temperature for 5 min. To the reaction mixture was added ICE (540 Units). After a 2-min incubation at 25°C, the reactions were stopped with 4× SDS-containing dye solution. The reaction mixtures were then analyzed by SDS–PAGE (Fig. 1). The time course experiment employed the same general protocol, but employed 250 μM of LEPB or the control 19-mer. After quenching at the appropriate times with denaturing gel-loading buffer, the reaction mixtures were analyzed by SDS–PAGE and staining with Coomassie blue. The data shown in Fig. 4 were obtained by densitometric scanning of the stained gels.

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