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Activity Profile of Dust Mite Allergen Extract Using Substrate Libraries and Functional Proteomic Microarrays

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Summary

Enzymatic activity in the fecal droppings from the house dust mite has been postulated to contribute to the elicited allergic response. Screening dust mite extracts through 137,180 tetrapeptide fluorogenic substrates allowed for the characterization of proteolytic substrate specificity from the potential cysteine and serine proteases in the extract. The extract was further screened against a 4000 member peptide nucleic acid (PNA) encoded inhibitor library designed to target cysteine proteases using microarray detection. Affinity chromatography coupled with mass spectrometry identified Der p 1 as one of the proteases targeted by the PNA inhibitors in the dust mite lysate. A phenotypic readout of Der p 1 function in allergy progression was demonstrated by the inhibition of CD25 cleavage from T cells by dust mite extract that had been treated with the Der p 1 inhibitor identified from the PNA-encoded inhibitor library.

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Introduction

House dust mites are a major source of allergens and a major contributor to the rising incidence of allergic diseases such as bronchial asthma, perennial rhinitis, and atopic dermatitis [1–3]. At least seventeen allergic components have been identified in the two predominant dust mite species, *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* [4]. Some of these allergens have been characterized at the molecular level and many have been recognized as having enzymatic activity. For example, Der p 1 has been characterized as having cysteine protease activity, Der p 3 has trypsin-like activity, Der p 4 is homologous to amylase, Der p 6 has chymotrypsin activity, Der p 10 is homologous to tropomyosin, and Der f 15 is homologous to chitinase. Moreover, it has been hypothesized that proteolytic activity may be a common mechanism for other allergens, such as cockroach allergen Per a 1 [5], cat pelt allergen Fel d 1 [6], and allergens from rag weed [7]. Proteolytic activity in general has long been recognized to produce an allergic response when injected into human skin [8] and to disturb the normal function of the bronchial epithelium [9, 10]. More recently, the enzymatic activity present in fecal extracts has been shown to play an integral role in the allergic reaction elicited from these extracts [11–14]. While these studies implicate house dust mite protease activity in the development of allergic disease, the direct cellular mechanisms that the enzymatically active allergens use to promote the development of allergies are still not completely understood. Functional identification and characterization of the proteolytic activity and the protease(s) involved in allergen induction is needed to understand the mechanism by which dust mites cause allergic diseases.

Given that the enzymatic activity of the dust mite fecal extract may be essential for the full induction of the allergenic response, proteomic techniques that can distinguish between active and inactive proteins would be able to provide information on the active-state of the lysate. The use of positional scanning combinatorial libraries of peptide fluorogenic substrates has proven to be a versatile tool that provides insight into the functional characteristics of cysteine and serine proteases. To date, substrate libraries in this format have been used to characterize the substrate specificity of single proteases [15–17] or multicatalytic proteases such as the proteasome [18]. Here we demonstrate the use of the substrate libraries as a functional proteomic tool to identify overall class-specific protease activity in the dust mite allergen lysate.

Activity profiling with protease substrates of a complex mixture with potentially multiple proteases will yield an overall activity snapshot of the lysate. In some circumstances the activity profile can be sufficient to identify the protease(s) in the biological sample responsible for the activity. However, the substrate activity profiles of complex mixtures can be problematic because multiple proteases can act on the same substrate and be-

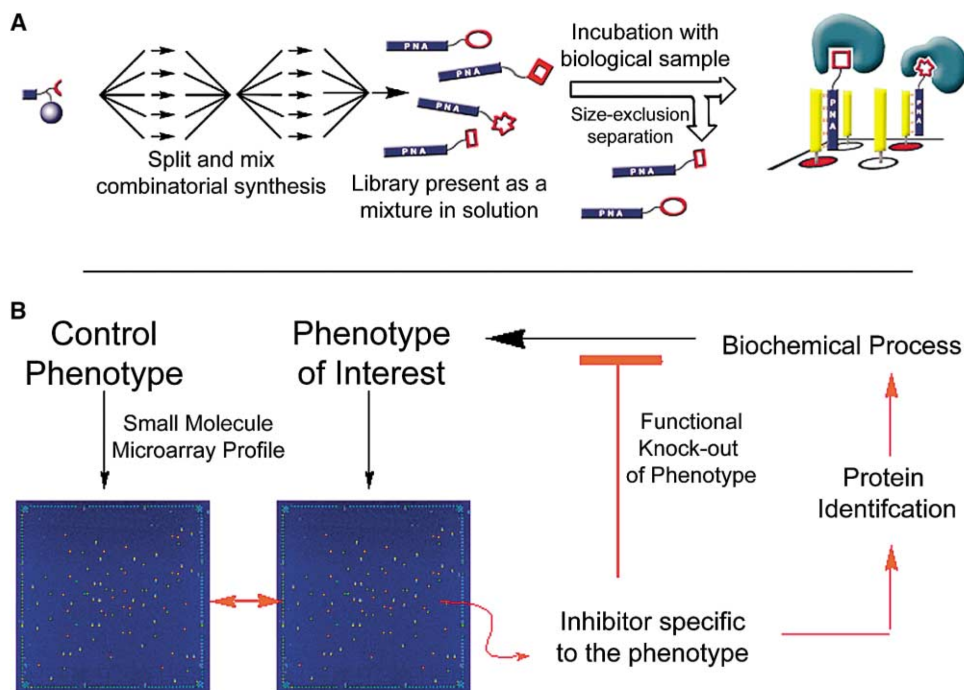

Figure 1. PNA-Encoded Libraries for Functional Profiling

(A) Synthesis and deconvolution of PNA-encoded library.

(B) Overview of functional profiling with substrate libraries and small molecule microarray.

cause a protease with a higher catalytic turnover can mask the activity of a less active protease. To address these issues, we recently reported a complementary technology of peptide nucleic acid (PNA)-encoded small molecule probes that has the ability to identify active proteins in a multiplexed and spatially addressable microarray format [19, 20]. The small molecule portion on the probe is designed to interact with and bind to proteins in a mechanism-dependent manner, therefore differentiating between active protein and protein that is present in a latent or inactive form. The PNA part of the probe functions to encode the synthetic history of the covalently attached small molecule and also allows for spatial deconvolution of the probe through hybridization on an oligonucleotide microarray (Figure 1A). Deconvolution of the PNA-encoded probes on oligonucleotide microarrays is an important feature of the method because multiple probes can be addressed at once in a miniaturized format-the format presented here has the potential of screening up to 400,000 probes in less than 300 μl. In our previous reports, we used the PNA-encoded small molecules in a diagnostic format through the design of specific inhibitor probes based on the peptide substrate specificity of the targeted proteases. However, the PNA-encoded probes can also be used to discover new proteolytic activities and does not require a priori knowledge of proteins in the sample. The encoding technique allows for the generation of chemical diversity in the small molecule portion of the probe through the facile use of split and mix synthesis. Using the PNA-encoded inhibitor probes allows for the analysis of organisms where the genomic or proteomic information is not known. Once the biological sample of interest is profiled against the PNA-encoded inhibitor probes, the probes can then be used as affinity labels in conjunction with mass spectrometry to identify the protease(s) responsible for the activity in the biological sample. In this way, the research effort can be focused on those protease(s) likely to function in the biological process being studied. Furthermore, the inhibitor identified from the PNA-encoded inhibitor profile can be used directly as a tool to investigate the function of the identified protease(s) in the biological process and validate the correlation between profile and phenotype (Figure 1B). Utilizing a 4000 member combinatorial PNA-encoded library targeting cysteine proteases, we describe the cysteine protease activity profile in the fecal extract from dust mites.

Results

Substrate Specificity Profiling of the Dust Mite Lysate Using Tetrapeptide Substrate Library

To assess the general proteolytic activity in the dust mite extract, kinetic screening was performed using a fluorogenic tetrapeptide substrate library in a positional scanning format (Figure 2A). Such libraries have been developed to determine the substrate specificity of serine, threonine, and cysteine proteases through the detection of substrate hydrolysis, as monitored by an increase in fluorescence over time. Each combination of two positions of the substrate is iteratively tested in six separate libraries to identify potential interdependence between the sites. The combined library information can then guide the identification of the tetrapeptide substrate specificity of the particular protease tested. The

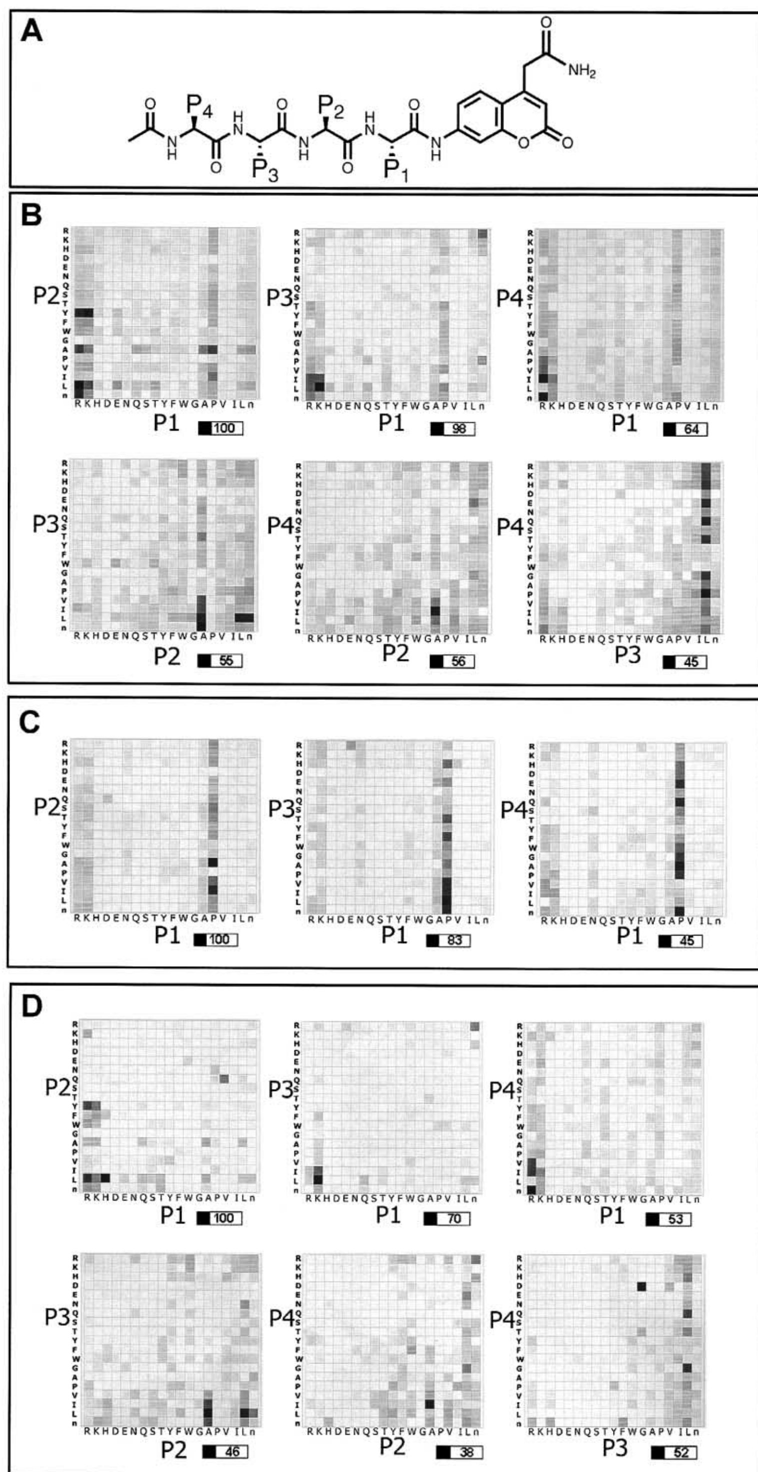

Figure 2. Substrate Specificity Profiles of Dust Mite Lysate in Positional Scanning Tetrapeptide Substrate Libraries

(A) General structure of library.

(B) Activity profile of dust mite lysate.

(C) Activity profile of dust mite lysate in the presence of cysteine protease inhibitor E64 (10 μM). No significant activity was observed in the P2 $\times$ P3, P2 $\times$ P4, and the P3 $\times$ P4, thus the plots are not shown.

(D) Activity profile of dust mite lysate in the presence of the serine protease inhibitor PMSF (1 mM). The shade of the square represents the rate of substrate cleavage (in relative fluorescence units) over time normalized across the libraries to 100 RFU/s. The number in the square at the bottom of each sublibrary represents the highest activity in the sublibrary as a percentage of the highest overall activity. The x and y axes represent the positions P1, P2, P3, and P4 amino acid that is held constant in the two-position fixed sublibrary represented. The two positions in the substrate that are not held constant contain an equimolar mixture of 19 amino acids (Cys is excluded and Met is replaced with Nle [indicated with "n"]) for a total of 361 substrates/well.

library presented in this manuscript consists of 19 amino acids at each of the four positions in the library, P1, P2, P3, and P4 for a total of 137,180 substrates screened. The natural amino acids methionine and cysteine were excluded as monomers in the library because of potential complications due to oxidation and disulfide bond formation. Norleucine, an isostere of methionine, is a nonnatural amino acid that was included in the library.

The resulting activity profiles from the dust mite extract show broad activity at all sites in the tetrapeptide sequence with a strong preference for P1 basic (arginine and lysine) and proline amino acids; P2 alanine, tyrosine, leucine, and norleucine amino acids; P3 aliphatic amino acids; and P4 aliphatic amino acids (Figure 2B). The activity observed in the lysate is presumably due to multiple proteases. In an attempt to identify and sepa-

rate the overall dust mite lysate specificity profile into functional classes, the dust mite lysate was incubated with the class-specific protease inhibitors, E-64 (L-trans-epoxysuccinyl-leucylamido (4-guanidino) butane) and PMSF (Phenylmethylsulfonyl fluoride), prior to screening through the library. The results indicate that the substrate activity of the dust mite lysate observed in the positional scanning library can be binned into two categories, papain-like cysteine protease(s) and serine protease(s). Incubation with E-64, a papain-like cysteine protease inhibitor, resulted in the attenuation of the P1-basic amino acid activity and the P2-alanine activity without modifying the activity of the P1-proline activity (Figure 2C). The P1-proline activity is likely due to a serine protease(s) not only because of its resistance to inhibition by E-64 but also because of the dominant dependence on the P1 position, a typical characteristic of serine proteases. Indeed, for the dust mite lysate treated with E-64, no activity was observed in libraries where P1 was not one of the fixed positions. The assignment of the P1-proline activity to a serine protease(s) was further validated by disappearance of the P1-proline activity upon incubation of the dust mite lysate with PMSF, a nonspecific serine protease inhibitor (Figure 2D).

Synthesis of the 4000 Member PNA-Encoded Cysteine Protease-Directed Library

The library design was based on the premise that the active site cysteine of the protease would covalently and irreversibly bind to the peptide acrylate through Michael addition. Specificity of the acrylate probes for a protease can be achieved through the recognition of a particular amino acid sequence attached to the acrylate moiety (Figure 3). Encoding the amino acid sequence with peptide nucleic acids (PNA) allows for the use of split and mix chemistry [21] to generate large collections of compounds with a minimal number of synthetic steps (Figure 3). Encoding with the PNA sequence allows for the peptide acrylates to be screened in solution in small volumes with identification of the active compounds accomplished through spatial deconvolution on oligonucleotide microarrays after separation of the unbound probes from the protein-bound probes by size exclusion filtration. Visualization on the microarray was accomplished through the incorporation of a fluorescein molecule to each probe, allowing for fluorescence imaging.

Evaluation of the Library Synthesis and Normalization of the Probes

To normalize the intensity of the individual probes and to evaluate the theoretical model used to design the PNA sequences, a calibration curve was determined for each of the probes. Five different concentrations of probes were hybridized to the microarray and the intensity for each of the individual features was determined at the various concentrations. A plot of the fluorescence intensity versus concentration yielded a correlation factor for each probe and allowed for the evaluation of probes with problematic sequences—due either to problems of the chemical synthesis or of poor hybridization properties. The linearity of fluorescence intensity over the range tested was good for the majority of the probes with only 7 of the 4000 probes having a correlation coefficient of less than 0.8 and only 53 of the 4000 probes having a correlation coefficient of less than 0.9. Having thus determined the relative hybridization efficiencies of each individual probe in the library, the calculated slopes of the individual calibration curves were then used to correct for the variation in hybridization efficiencies in the subsequent experiments with the dust mite extract.

Profile of Dust Mite Lysate

The cysteine protease profile of the dust mite extract with the 4000 PNA encoded probes was obtained upon incubation of the lysate with the probe mixture and then separation of the unbound probes by spin filtration through a 30 kDa molecular weight cutoff filter. The retained sample containing the protein-bound probes was then hybridized to the oligonucleotide array and visualized by fluorescence imaging of the incorporated fluorescein molecule on the probe (Figure 4). The trends observed in the inhibitor profile of the dustmite lysate include activity for inhibitors with P1 lysine and norleucine, with minimal to little inhibitory activity seen for inhibitors with P1 aspartate or glutamine. The most prominent feature of the profile is for inhibitors that contain P2 alanine. The P3 and P4 positions of the inhibitor appear to be somewhat broad with the most striking feature being the absence of activity for inhibitors with P3 histidine, phenylalanine, or proline activity.

Capture of Dust Mite Proteins Interacting with Library Probes

A unique feature of the current approach is that the activity of a particular protease is measured on the microarray by the amount of protease trapped by the specific mechanism-based inhibitor. This allows for the quantitative assessment of the activity profile in the lysate as well as a mechanism to identify the captured protease. In the dust mite sample, the probe with the highest intensity on the microarray had the inhibitor sequence of Nle-Val-Ala-Lys (P4 to P1). This probe was resynthesized with a biotinylated linker to isolate and capture the interacting protein in the dust mite lysate (compound **12**, Figure 4). Incubation of the lysate with the biotinylated peptide inhibitor, compound **12**, was followed by capture with a monomeric streptavidin and elution with biotin. Sequencing of the captured protein(s) by mass spectrometry led to the identification of two major proteins, Der p 1 and Der p 10 (Figure 5A and Supplemental Data [available at http://www.chembiol.com/cgi/content/full/11/10/1361/DC1]); four unique peptides were matched to Der p 1 while two unique peptides were matched to Der p 10 (Figure 5A and Supplemental Data). Der p 1 is a 25 kDa protein that is homologous to the papain family of cysteine proteases. Der p 10 is a 33 kDa protein that is homologous to tropomyosin [22]. The major protein labeled by compound **12** in the dust mite lysate, as determined by SDS-PAGE separation and visualization of biotin-conjugated proteins, comigrates with purified Der p 1 (Figure 5B). Two lower molecular mass proteins, at approximately 15 and 12 kDa, were also labeled with compound **12**. It is currently not known if these lower molecular weight proteins are degradation products of Der p 1 or if they are additional proteins in the dust mite lysate that are also targeted by

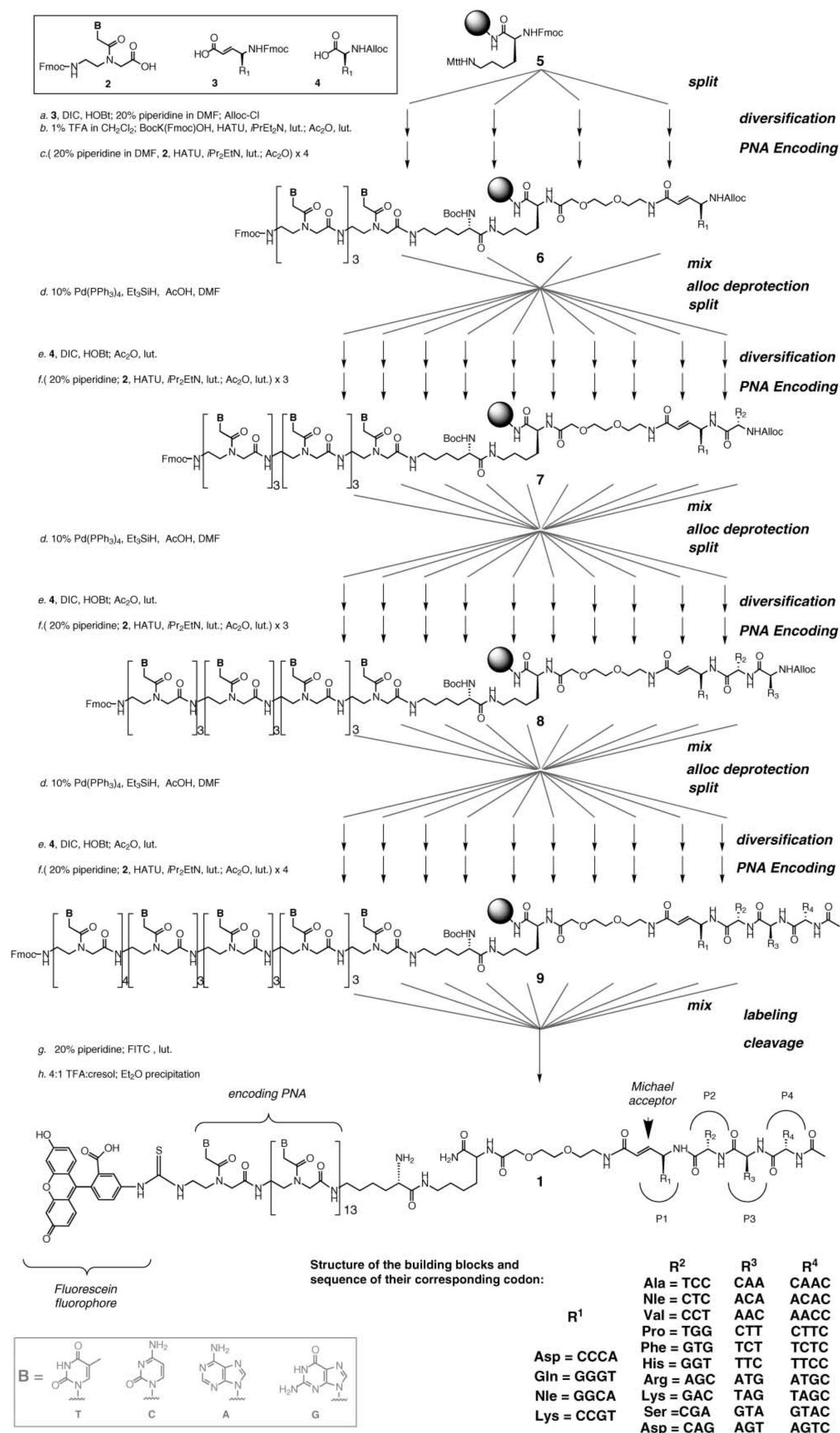

Figure 3. General Synthesis and Structure of the 4000-Compound PNA-Encoded Acrylamide Library 1

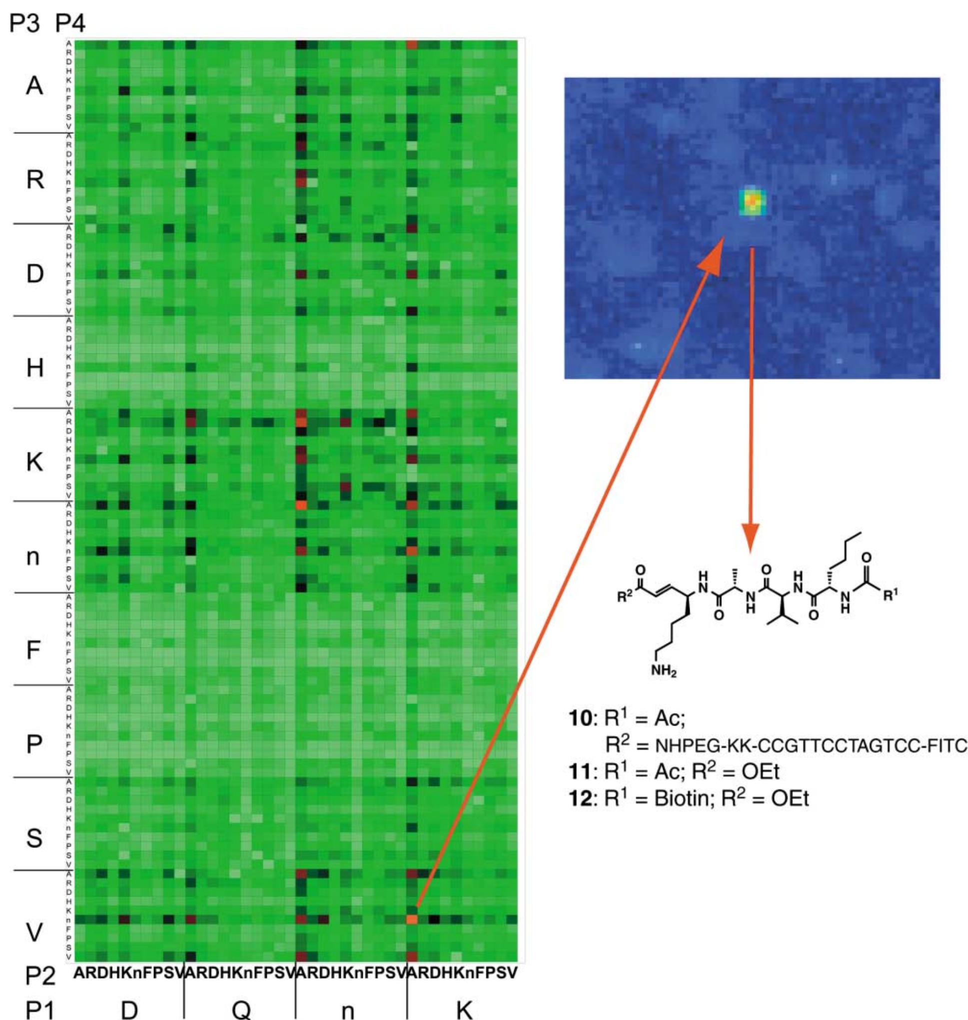

Figure 4. Profile of the Dust Mite Extract with the 4000-Compound PNA-Encoded Acrylamide Library
Green represents the lowest intensity on the microarray and red represents the highest intensity on the microarray.

the inhibitor. Compound **11** and its biotinylated analog, compound **12**, were determined to be potent irreversible inhibitors of Der p 1 with apparent second order inhibition rate constants of $(8.5 \pm 0.3) \times 10^3$ M^{-1} s^{-1} and $(9.1 \pm 0.4) \times 10^3$ M^{-1} s^{-1}, respectively. These data indicate that the probes identified from the library target Der p 1 in the dust mite lysate.

Substrate Specificity of Der p 1

To assess the substrate specificity of Der p 1, the protease was run through the tetrapeptide fluorogenic substrate library in a positional scanning format. The results from the library show that the major substrate specificity determinant for Der p 1 is in the P2 position for the alanine amino acid. The preference for P2-alanine is not only clearly observed from the specificity profile, but also by the fact that higher substrate hydrolysis activities are observed in the libraries where P2 is held as a constant amino acid, 100% of the highest activity for P1×P2, 52% for P2×P3, and 77% for P2×P4 versus 20% for P1×P3, 16% for P1×P4, and 8% for P3×P4 (Figure 6). Der p 1 shows only a slight preference for basic amino acids in P1 and P3 and shows a P4 preference for aliphatic amino acids such as isoleucine, proline, valine, leucine, and norleucine.

Inhibition of Dust Mite Protease Cleavage of CD25

One major advantage of the PNA-encoded small molecule libraries is that the inhibitor identified from the screen can be used to directly interrogate the activity of the identified protein in the biological process being studied. Dust mite lysate and Der p 1-dependent cleavage of the IL-2 receptor α chain (CD25) was used to establish the ability of the small molecule inhibitor (**11**, Figure 4) identified from the PNA-encoded microarray screen to reverse the dust mite allergy phenotype [23, 24]. The results indicate that upon incubation of PBMCs with dust mite extract or isolated Der p 1, there is a decrease in the surface expression of CD25 by $CD4^+$ T cells as compared to cells that are treated with inactive dust mite extract or inactive Der p 1. The decrease in surface expression of CD25 has previously been shown to be correlated with increased levels of soluble CD25, indicating direct proteolytic cleavage by dust mite prote-

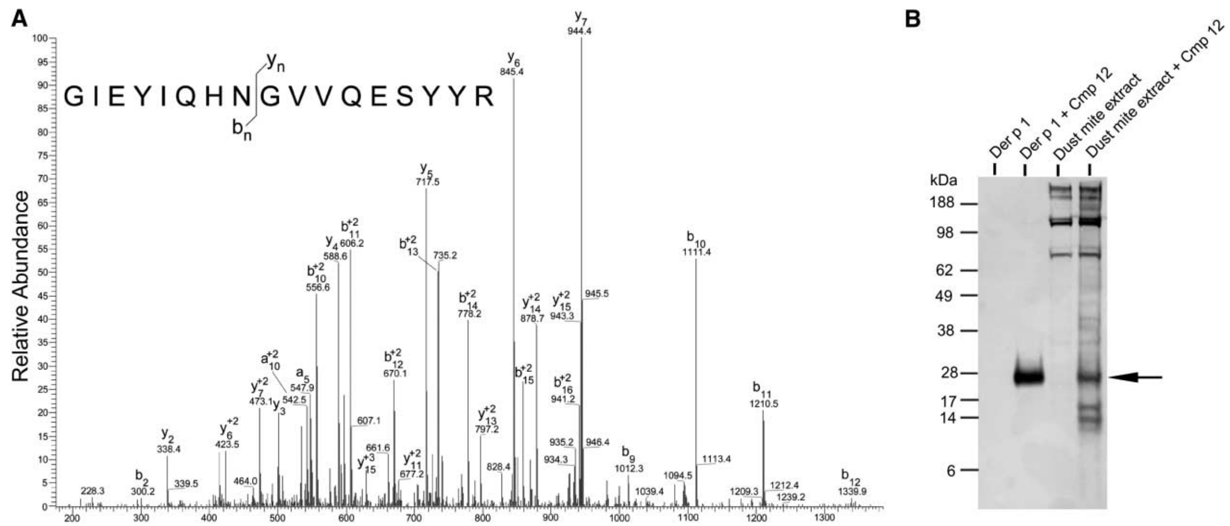

Figure 5. Characterization of a Protein Associated with a Microarray Inhibitor

(A) MS/MS spectrum of R.GIEYIQHNGVVQESYYR.Y peptide identified from affinity capture from dust mite extract by compound **12**. (Additional spectra are presented in Supplemental Data available online at http://www.chembiol.com/cgi/content/full/11/10/1361/DC1.)

(B) Detection of dust mite proteins reactive to compound **12**. Dust mite proteins were incubated with compound **12** and then separated on SDS-PAGE and blotted with streptavidin-peroxidase (see methods).

ases and not other modes of downregulation [23]. Pretreating the dust mite lysate or Der p 1 with compound **11** resulted in a dose-dependent protection of CD25 on the surface of $CD4^+$ T cells (Figure 7).

Discussion

A goal of proteomic research is to characterize proteins on a genome-wide scale to better understand the role of specific proteins in both normal and disease processes. The therapeutic uses of this information include the identification of biomarkers to monitor disease progression, identification of proteins to target for therapeutic intervention, and to further define the mechanism of action of proteins. Because of the potentially high payoff that this information provides, much effort has been devoted to technologies that investigate cellular events on a genome-wide scale. At the transcription level,

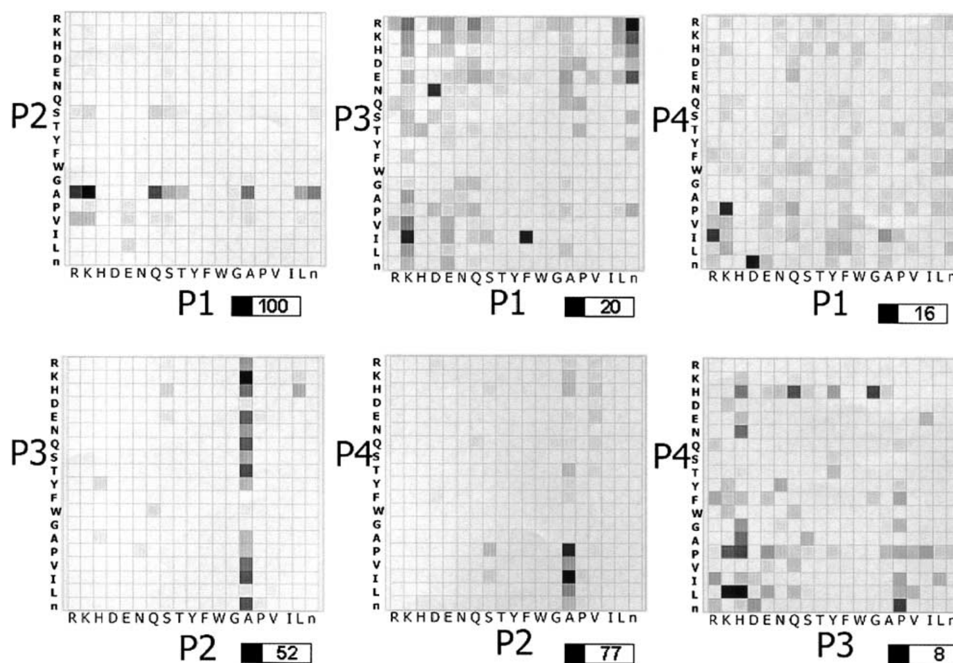

Figure 6. Substrate Specificity Profile of Purified Der p 1

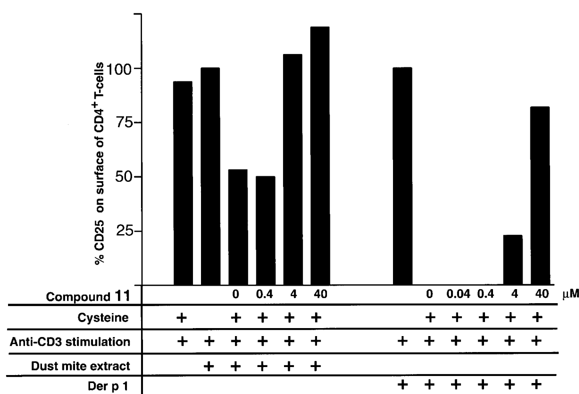

Figure 7. Inhibition of Dust Mite Allergen- and Der p 1-Mediated CD25 Cleavage on anti-CD3 Stimulated PBMCs by an Inhibitor Identified from the 4000-Member PNA-Encoded Library, Ac-nVAK-acrylate (Compound **11**)

The y axis represents the percentage of $CD4^+$ T cells expressing CD25 after treatment with active dust mite lysate or active Der p 1 versus inactive dust mite lysate or inactive Der p 1 (see Experimental Procedures).

mRNA expression profiling has proven useful in rapidly addressing the changes in gene expression and in the identification of potential therapeutic targets [25]. At the translational level, chromatography in conjunction with mass spectrometry has been successful in analyzing the content of proteins in a given sample [26, 27]. However, protein function is not always regulated at the transcription and translation level, but rather it is regulated at the posttranscriptional and posttranslational level through modifications such as splicing, phosphorylation, acetylation, specific localization, cofactor requirement, endogenous inhibitors, and protein truncation, just to name a few [28–30]. With the variety of posttranscriptional and posttranslational modifications, the functional proteome may far exceed the unique genome sequences by 10- to 100-fold. Multiple functional proteomic approaches have started to emerge to address the challenge of assigning functional information to proteins and protein classes [30–33]. This study introduces the combined use of two functional proteomic technologies, positional scanning fluorogenic substrate libraries and PNA-encoded small molecule libraries, to functionally identify and characterize proteases involved in dust mite-induced allergic hypersensitivity. Both techniques show that the proteolytic proteome of the dust mite lysate consists of multiple activities from both cysteine and serine protease classes.

We have previously described the use of positional scanning substrate libraries in monitoring the substrate specificity profile of single cysteine and serine proteases or the multicatalytic proteasome, a threonine protease [16–18]. The information from the libraries has proven useful in the design of substrates and inhibitors to target specific enzymes. In the current approach, positional scanning tetrapeptide substrate libraries were used to monitor overall cysteine-, serine-, and threonine-proteolytic activity in the physiologically relevant context of dust mite allergens. Catalytic activity against multiple substrates was observed in the lysate. Incubation of the lysate with two class-specific protease inhibitors showed that the activity observed in the substrate library could be broken down into papain-like cysteine protease(s) and serine protease(s).

To address the issue of targeting specific proteases in the dust mite lysate, a library of PNA-encoded affinity probes was designed and synthesized. These probes consist of a reactive acrylamide group for covalent attachment to the enzyme, a bifunctional linker allowing for the cosynthesis of the specificity element, and a PNA sequence. The specificity element in the presented work consists of a tetrapeptide sequence. The reactive acrylamide group is targeted to react specifically with the nucleophilic cysteine in the active site of cysteine proteases. The PNA portion of the molecule not only encodes the specificity element, but also allows for deconvolution on the highly miniaturized format of oligonucleotide arrays. We have previously demonstrated the use of these types of probes in a diagnostic format with designed specificity elements targeting specific cysteine proteases [19, 20]. The advantage of using the PNA probes in a library format is that several thousand probes can be used to interrogate a biological sample in a minimal volume. A specific profile for the dust mite lysate was demonstrated using a library of 4000 PNA encoded probes. The major profile observed on the microarray was for probes with nonacidic P1 amino acids, a strong preference for alanine in the P2 position, and aliphatic amino acids in P4.

The important advantage of the PNA-encoded technology presented here is that inhibitors identified in the profile can be used to directly identify and validate their target. The targeted proteins are covalently linked to the small molecule and the protein can be directly isolated from the lysate and identified using mass spectrometry. Two proteins, Der p 1 and Der p 10, were identified upon incubation of the dust mite lysate proteins with a biotinylated version of the probe showing the highest intensity, P4 to P1 sequence of Nle-Val-Ala-Lys. Der p 1 was identified as the major protein captured by the probe. Der p 10 was also identified but with overall lower peptide coverage as determined by mass spectrometry than Der p 1. Der p 1 is a cysteine protease homologous to papain and its capture is in agreement with the mechanism-based inhibitor design of the probes. Der p 10 is also a dust mite protein but shows homology to tropomyosin rather than to a cysteine protease. The capture of Der p 10 by the probe is not completely understood based on the design of the probes. Further investigation of Der p 10 is needed to determine if its interaction with the probe simply arises from nonspecific binding to the probe or if it represents specific binding to a nonannotated function or site of the protein.

Further characterization of purified Der p 1 in the 137,180 member positional scanning substrate libraries revealed that the enzyme shows a strong preference for P2 alanine and P4 aliphatic amino acids—a result consistent to the profile observed in the 4000 member PNA-encoded library. The difference in substrate profile between the dust mite extract and the purified Der p 1 reveal that additional cysteine protease activity is found in the lysate that cannot be attributed to purified Der p 1. In particular, Der p 1 does not demonstrate P2

leucine, norleucine, or tyrosine activities that are prominent in the dust mite extract. The alternative activity may be due to additional proteins or may indicate a change in Der p 1 substrate specificity due to its interaction with other proteins or cofactors in the lysate. The 4000 member PNA library did not include P2 leucine or tyrosine, and thus the identity of the protease responsible for this activity could not be determined using inhibitor probe capture. Identification of additional proteases in the lysate using the presented method may require expansion of the PNA-inhibitor library sets to include alternative scaffolds and sequences.

The activity of Der p 1 appears to be a major factor in the initiation and propagation of an immune response. Previous studies have demonstrated a selective enhancement in total IgE levels in mice that have been immunized with active Der p 1 compared to mice that have been immunized with inactive Der p 1 [34, 35]. The mechanism of this selective enhancement has been demonstrated through the cleavage of several key proteins including the α subunit of the IL-2 receptor, CD25 [23, 24]. Cleavage of the IL-2 receptor would decrease the autocrine growth factor effect from IL-2 on the Th1 subset of T cells and bias the immune response toward the Th2 subset, thus enhancing allergic hypersensitivity. Reversal of CD25 cleavage by dust mite lysate was observed upon treatment of PBMCs by dust mite lysate pretreated with the small molecule inhibitor identified from the PNA-encoded inhibitor screen.

Significance

The functional profiling tools described in this manuscript allow for the detection and dissection of protease activities in physiologically relevant biological samples. The technologies yield a direct readout of the functional state of the biological sample and provide tools to directly interrogate the sample of interest. Using these tools we have shown that several proteolytic activities exist in dust mite lysates and that these activities are important in enhancing the allergenic effect of the sample. Whether proteolytic activity is a fundamental property of allergens to elicit an immune response is still debatable. Use of the technologies and tools presented here should help address this issue by allowing for the demonstration, identification, and characterization of proteolytic activities from multiple diverse airborne allergens.

Experimental Procedures

Preparation and Screening of the Tetrapeptide Substrate Library

Tetrapeptide coumarin substrate libraries (Figure 2A) were synthesized using methods described previously [17, 18, 36]. Commercial dust mite extract reagents were obtained from Hollister-Stier Laboratories, LLC (Spokane, WA) and used without further purification. The preparation of Der p 1 is described below and was used at 20 nM in PBS buffer supplemented with 5 mM DTT. Dust mite lysate was diluted 1000-fold in activity buffer consisting of PBS buffer supplemented with 5 mM DTT with or without a 10 min preincubation with the class specific inhibitors, E-64 at 10 μM or PMSF at 1 mM. The preincubated lysate was then added to the substrate library and the rate of fluorescence increase over time was monitored for 1 hr at 37°C with a λ_{ex} 380 nm and λ_{em} 450 nm in a Molecular Devices Gemini XS microtiter plate reader. The approximate concentration of the substrates in the library was 0.25 μM/substrate/well at 361 substrate/well (Cys was excluded and Met was replaced by the isosteric nonnatural amino acid norleucine, Nle, n).

Preparation of the 4000 Member PNA-Encoded Library Targeted toward Cysteine Proteases

All peptide synthesis reagents and resins were purchased from Novabiochem, PNA monomers as well as HATU were purchased from Applied Biosystem, and other chemicals were purchased from Aldrich. The general procedure for PNA oligomerization was Fmoc deprotection using 20% piperidine in DMF for 2.5 min followed by a coupling using PNA monomer (4.0 equivalents [eq]), diisopropyl ethylamine (4.0 eq), HATU (3.5 eq), and 2,6-lutidine (6.0 eq) (preincubated for 2 min) twice for 1 hr. The general procedure for Alloc deprotection was treatment of the resin with a solution of palladium tetrakis (0.5 eq) and acetic acid (10 eq) in dichloromethane followed by the addition of a triethylsilane solution in DMF and agitation for 30 min. The resin was then briefly washed with a 1% solution of Hünig's base in DMF to freebase the polymer-bound amine. The amino acid couplings were carried out using DIC (4.0 eq), HOBt (4.0 eq) and monomer (4.0 eq) in DMF (preincubated for 5 min) for 2 hr. All reactions were carried out at room temperature (23°C) and all couplings were capped with acetic anhydride (5.0 eq) and 2,6-lutidine (5.0 eq) in DMF for 5 min. The full synthetic details and characterization of the library synthesis have been recently described elsewhere [37]. The library synthesis is summarized in Figure 3. Briefly, Rink amide resin loaded with orthogonally protected lysine (**5**) was selectively deprotected at the α terminus, derivatized with a polyethyleneglycol spacer (Applied Biosystem) and split into four pools which were each coupled to an acrylic acid (**3**). The orthogonal Mtt group from the lysine linker was removed and a lysine residue was added followed by the first codon of each respective pool, thus affording the encoded pools of resin **6**. The four pools were mixed, the Alloc group was removed, and the resin was redistributed into 10 pools which were each coupled to a unique amino acid followed by encoding with their respective codon via a reiteration of PNA couplings to obtain 10 pools of resin **7**. Two more cycles of mixing, Alloc deprotection, splitting, amino acid coupling, and PNA encoding afforded resin **9**, which was mixed, labeled at the PNA N terminus with fluorescein isothiocyanate (4.0 eq) and 2,6-lutidine in DMF for 5 hr, and cleaved from the resin with TFA:*m*cresol (4:1). The crude cleavage product was precipitated in diethyl ether and pelleted by centrifugation (9800 $\times$ g). The pellet was washed several times with Et_2O then dissolved in a 1:1 mixture of AcCN:H_2O (1:1, 20 ml) and lyophilized to obtain a yellow powder.

Design and Calibration of PNA Encoding Sequences

Each tetrapeptide was encoded to a PNA 14-mer using four codon sets (Figure 3). The sequences of the codons were chosen to balance two primary design goals. First, the codons should be sufficiently different so that every PNA sequence hybridizes specifically to its complement on the DNA microarrays. This was addressed by choosing codons that differed by at least two bases. Second, the codons should be similar enough that all the sequences have similar melting temperatures. This was addressed by predicting the melting temperature (T_m) of sequences in the library using the method developed by Griffin and Smith [38]. According to this method, the formula for the melting temperature (°C) of a 14-mer is described in equation (1) where H(seq) and G(seq) are functions of the sequence.

$$T_m = -273.15 + (298 \cdot H(seq))/(H(seq) - G(seq) - 3.14) \quad (1)$$

The most significant factor in the sequence-dependence of the T_m is the G/C content. As a result, the most salient feature of the resulting codon sets is that every codon in a given set has the same G/C content. A solution of $4\times10\times10\times10$ codon sequences was selected and used to encode the amino acids in the inhibitor portion of the probes.

To correct for differences in intensities of the hybridized PNA probes due to differences in the melting temperatures of each probe, a calibration curve was determined for each of the probes. The calibration curves were generated using the customized 12 μm/feature oligonucleotide array from Affymetrix that contained 4000 DNA sequences that were complementary to the 4000 PNA se-

quences in the cysteine protease inhibitor library. Five concentrations of probes corresponding to 10–500 relative fluorescence units were applied directly to the oligonucleotide microarray in PBS and allowed to incubate for 4 hr before removal of the nonhybridized probes from the microarray. The microarray was then visualized and the fluorescence intensities of the individual features were quantitated. The slopes for each of the features were then used as a correction factor in the experiment.

Incubation of Dust Mite Lysate with 4000-PNA-Encoded Probes

The dust mite lysate was diluted 1:100 in 500 μl of PBS buffer supplemented with 5 mM DTT and 5% DMSO. The 4000-probe mixture was added to the dust mite sample as 10 μl of an approximately 10 mM stock concentration of the probes in DMSO, yielding an overall approximate probe concentration of 200 μM and an individual probe concentration of 50 nM. The sample was allowed to incubate at room temperature for three hours. The sample was then loaded on an ultrafree 30 kDa molecular weight cutoff filter (Millipore) and washed with 1× PBS buffer (5 × 500 μl). The volume of the sample retained in the 30 kDa filter was then adjusted to 200 μl with PBS and fluorescein-conjugated DNA control probes were added to the sample. The sample mixture was then added to a customized 3 μm/feature oligonucleotide array from Affymetrix that contained 4000 DNA probes that were complementary to the 4000 PNA probes in the cysteine protease inhibitor library. The array was visualized after 16 hr incubation.

Preparation of Acylated and Biotinylated nVAK-Acrylate

The peptides **11** and **12** were prepared by solid phase Fmoc synthesis using DIC/HOBt mediated coupling; DBU induced Fmoc deprotection with a 2-chlorotrityl resin. Thus a solution of Fmoc-Lys(Boc) acrylic acid (2.0 eq) and diisopropyl ethylamine (2.0 eq) was added to 2-chlorotrityl resin (1.0 eq, Novabiochem) swelled in dichloromethane (10 ml/g) and the reaction mixture was agitated at ambient temperature for 3 hr. Subsequent Fmoc deprotections were carried out with DBU (10 eq) in dichloromethane (10 ml/g) for 4 min at ambient temperature. The following peptide couplings were performed using standard Fmoc amino acids (4.0 eq) preactivated with DIC (4.0 eq)/HOBt (4.0 eq) in DMF (10 ml/g) and added to the resin. The couplings were carried out at ambient temperature for 3 hr. The final amino acids were capped with acetic anhydride (5.0 eq)/ lutidine (5.0 eq) in DMF (10 ml/g) or NHS-biotin (2.0 eq, Novabiochem)/ lutidine (5.0 eq) in DMF (10 ml/g), leading to compounds **2** and **3**, respectively. The compounds were released from the resin using a 1% solution of TFA in dichloromethane for 30 min, then diluted with toluene and concentrated in vacuo. The compounds were redissolved in a 5% ethanol solution in dichloromethane and WSC (2.0 eq, Novabiochem) was added. After 3 hr, the reactions were washed with 0.1 M aqueous HCl solution, dried over $MgSO_4$ and concentrated in vacuo. The compounds were resuspended in a minimum of dichloromethane and TFA was added to reach a 50% concentration. After 30 min, the solutions were precipitated over diethyl ether and the desired compounds were pelleted by centrifugation (15,000 × g) to obtain compounds **11** (calculated for $C_{26}H_{47}N_5O_6$ $(MNa)^+$: 548.68, found: 548.69) and **12** (calculated for $C_{34}H_{59}N_7O_7S$ $(MNa)^+$: 732.94, found: 732.93).

Capture of Protein Functionally Interacting with the Biotinylated Compound 12

Compound **12** at a concentration of 10 μM was allowed to incubate for 1 hr at room temperature with 2000 μl of 1:100 dilution of dust mite extract in PBS supplemented with 5 mM DTT. Ultralink immobilized monomeric avidin resin (Pierce) was then added to the sample and incubated at room temperature for 1 hr. The resin was then washed with 10× resin volume of PBS and captured proteins were eluted with 5 mM biotin. The samples were then prepared for mass spectrometry using standard protocols.

Inhibition Kinetics of Der p 1 with Compounds 11 and 12

The apparent second order rate constant, $k_{obs}/[I]$, for the irreversible inhibition of Der p 1 by compounds **11** and **12** (Figure 4) was determined using previously described methods [39, 40]. Briefly, progress curves were obtained using 10 nM Der p 1 and 50 μM of the fluorescent substrate Ac-Pro-Lys-Ala-Lys-acc (K_m = 202 μM) in the presence of multiple concentrations of compounds **11** and **12** that ranged from 50–20,000 nM. The observed inactivation rate constant (k_{obs}) was calculated by direct fit of the data to equation (2) where ν_0 is the initial velocity, ν_s is the steady state velocity, and t is time.

$$[\text{fluorescence}] = \nu_s t + (\nu_0 - \nu_s)[1 - \exp(-k_{obs}t)]/k_{obs} \quad (2)$$

Labeling of Dust Mite Extracts and Der p 1 with Compound 12

Detection of the proteins labeled in the dust mite lysate was accomplished using previously described methods [41, 42]. Dust mite extract and Der p 1 were incubated with 10 mM DTT and 10 μM compound **12** in PBS buffer for one hour at room temperature. Reactions were quenched with sample buffer and resolved by reducing SDS-PAGE on a 4%–12% Bis-tris gel. Gels were then transferred to PVDF, blocked, and probed with 1:10,000 dilution of streptavidin-peroxidase (Calbiochem). Western blots were then developed with ECL+ and resulting signals were recorded with a Storm 860 scanner (Amersham).

Identification of Protein(s) Functionally Interacting with Probe by Mass Spectrometry

Captured protein in water (0.01 to 0.4 mg/ml) was diluted with an equal volume of 8 M urea, 25 mM Tris HCl. Samples were reduced (DTT, 10 mM for 45 min) and alkylated (iodoacetamide, 20 mM for 15 min in the dark) prior to digestion with trypsin (1 μl of 0.1 mg/ml trypsin for 4 hr at 37°C). Samples were analyzed by LC-MS/MS using a ThermoFinnigan LCQ Deca XP Plus mass spectrometer modified with a home-built nanospray source configured for online desalting as previously described [43]. Samples were loaded onto a 100 μm internal diameter precolumn packed with 2 cm of Monitor 5 μm, C18 (Column Engineering) and desalted for 10 min at 5 μl/min with 0.1 M HOAc. After desalting, the precolumn was placed inline with an integrated microcolumn-ESI emitter (360 μm OD × 75 μm internal diameter, 5 μm tip opening) packed with 10 cm of the same packing material, and the ACN concentration was increased from 0% to 50% over 30 min. The flow from the HPLC pump was split prior to the precolumn to achieve 250 nl/min. The mass spectrometer continuously acquired one full scan spectrum followed by tandem-MS spectra of the three most abundant ions from the full scan spectrum. Tandems-MS spectra were correlated to peptide sequences in the NCBI nonredundant database (ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nr.gz) using SEQUEST; no cleavage enzyme was specified during the database search. The resulting peptide assignments were further restricted to trypsin-specific peptides and matches were manually verified. Confirmed peptide sequences were searched against the nonredundant database using pBLAST to validate a unique protein identity.

Der p 1 Preparation

Der p 1 was isolated from house dust mite fecal pellets (Indoor Biotechnologies, Cardiff, UK) using a multistep procedure, involving immunoaffinity chromatography on immobilized monoclonal anti-Der p 1 antibody (clone 4C1, Indoor Biotechnologies, Cardiff, UK), removal of contaminating serine proteases on immobilized soybean trypsin inhibitor (Sigma, Poole, UK), and finally FPLC to remove low molecular weight contaminants [34]. The purity of the preparation was confirmed by N-terminal sequencing, SDS-PAGE analysis, and by demonstrating that enzymatic activity is completely dependent on preactivation with cysteine and is totally blocked by the class-specific inhibitor E-64 [34]. Protein concentration was determined using a BCA microtiter plate assay and confirmed spectrophotometrically using the empirical absorption coefficient value for Der p 1 of $E^{1\%}$ (280 nm) = 16.4. The catalytic activity of Der p 1 was ascertained in a continuous rate (kinetic) assay using the fluorogenic peptide substrate Boc-Gln-Ala-Arg-AMC [44].

CD25 Cleavage Assay in the Presence of Identified Inhibitor

Heparinized whole blood from healthy nonatopic volunteers (obtained with prior consent and Ethical Committee approval) was used for separation of PBMC on a histopaque density gradient (HISTOPAQE-1077, Sigma). Cells (2×10^5/250 μl) were suspended in serum-free AIM-V medium, supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Gibco Life Technologies Limited, Paisley, UK), and 5 mM HEPES (Sigma), and cultured in flat bottom 96-well plates (Corning Costar Corporation, New York, New York) at 37°C in a humidified atmosphere of 5% CO_2. Mouse anti-human CD3 antibody was purified on a protein G column from the

supernatant of a hybridoma cell line (OKT3, ECACC, Porton Down, UK). Lymphocytes were stimulated with immobilized anti-CD3 antibody (2 μg/ml) to induce surface expression of CD25 (IL-2 receptor α chain). Six hours later, different concentrations of cysteine protease inhibitor (**11**, Figure 4) were added, followed by purified Der p 1 (5 μg/ml) or whole dust mite extract (25 μg/ml) (both activated with 5 mM cysteine), and the cultures were continued for a further 18 hr.

Cells were harvested, washed twice with PBS, 0.1% BSA, and azide (PBA) and then stained for lymphocyte surface markers for 20 min at 4°C in the dark. The cells were then washed twice with PBA and fixed in 0.5% formaldehyde in isotonic azide-free solution. The $CD3^{+}CD4^{+}$ T lymphocytes were then analyzed by flow cytometry for CD25 expression [23, 24]. Flow cytometry was performed using a Coulter Altra analyzer (Beckman Coulter, Highwycombe, UK) and 50,000 events were collected for each sample. Dead cells and monocytes were excluded by forward and side scatter characteristics. The quadrant markers for the bivariate dotplots were set based on the appropriate isotype controls. The percentage of cells expressing CD25 after treatment with proteolytically active Der p 1 or dust mite extract with cells that have been treated with proteolytically inactive Der p 1 or dust mite extract were calculated for the different concentrations of compound 11 and are represented in Figure 7.

Supplemental Data

Supplemental Data for this article is available online at http://www.chembiol.com/cgi/content/full/11/10/1361/DC1/.

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