some deformation. Further confounding the envelope analysis are the mutations that appear in the substrates themselves. Although not yet present in the majority of resistant strains, mutations near one or more of the *gag* cleavage sites allow more "extreme" mutations within the protease protein that would otherwise cripple viral replication. Such substrate mutations are likely to impact the shape of the substrate envelope. A refinement of the envelope analysis reported here will need to incorporate these additional factors.

Finally, while the approach outlined by King et al. is likely to enable the identification of new PIs to which HIV has more difficulty (or requires more genetic steps) in becoming resistant, a complete strategy for minimizing resistance also requires an understanding of conditions (e.g., regimen adherence, drug pharmacokinetics, and viral fitness) that define selective pressure in the host. Recent results indicate that pharmacokinetically boosted [6] regimens of existing PIs can erect a substantial barrier to resistance in vivo [6, 7], illustrating the fact that drug discovery is a multi-faceted effort and that all available tools are needed for defeating a plague such as HIV. The approach of King et al. is a welcome addition to the tool chest.

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Catching Proteases in Action with Microarrays

Proteases regulate many essential functions in biology, yet their precise roles are only beginning to be unraveled. In this issue, two related papers describe a novel method to dissect specific protease activities from complex mixtures [1, 2].

Proteolysis provides an irreversible means to activate or terminate signaling events in complex biological settings by destruction of proteins. With greater than 500 putative protease genes in the typical mammalian genome, understanding their precise roles in biology is daunting [3]. The generation of protease-deficient mice and small-molecule inhibitors has revealed essential functions for proteases in many areas of biology, ranging from cell cycle control [4] to antigen presentation [5] and extracellular matrix remodeling [6]. Functional redundancy within protease subfamilies and limited methods for identifying substrates in vivo complicate analysis. The majority of proteases are synthesized as zymogens and are activated only in specific subcellular compartments or upon stimulation. Furthermore, many proteases have endogenous inhibitors that attenuate their destructive capacity. As proteolysis is regulated posttranslationally, the evaluation of mRNA or polypeptide levels do not necessarily reflect their activity. To address the functional activity of proteases, two papers in this issue from Harris, et al. and Winssinger, et al. describe microarray technologies to identify proteases and their substrates from complex mixtures with peptide-nucleic acid (PNA)-encoded libraries of activity-based probes [1] and fluorogenic peptide substrates [2], respectively (Figure 1).

By and large, the characterization of protease substrates has been determined with purified proteins or peptide libraries in vitro, which then suggests potential substrates in vivo. The development of positional-scanning fluorogenic-substrate libraries has allowed the characterization of preferred amino acids at the site of proteolysis and has been used to determine the substrate specificities for several protease families in a high-throughput manner [7–9]. Using these positional-scanning substrate libraries, Harris, et al. evaluated the proteolytic activities in dust mite extracts that may be responsible for allergies [1]. By including class-specific protease inhibitors, they showed that the predominant proteolytic activities in dust mite extracts that cleaved P1 basic residues were attributed to cysteine proteases, whereas serine proteases were responsible for cleaving substrates with P1 proline residues. Although these positional-scanning peptide libraries revealed the overall proteolytic activities in dust mite extracts, the specific protease(s) responsible for these activities from complex mixtures remained to be identified.

In order to retrieve the cysteine-protease(s) from the dust mite extracts, Harris et al. turned to mechanism-based probes that irreversibly label active enzymes [1].

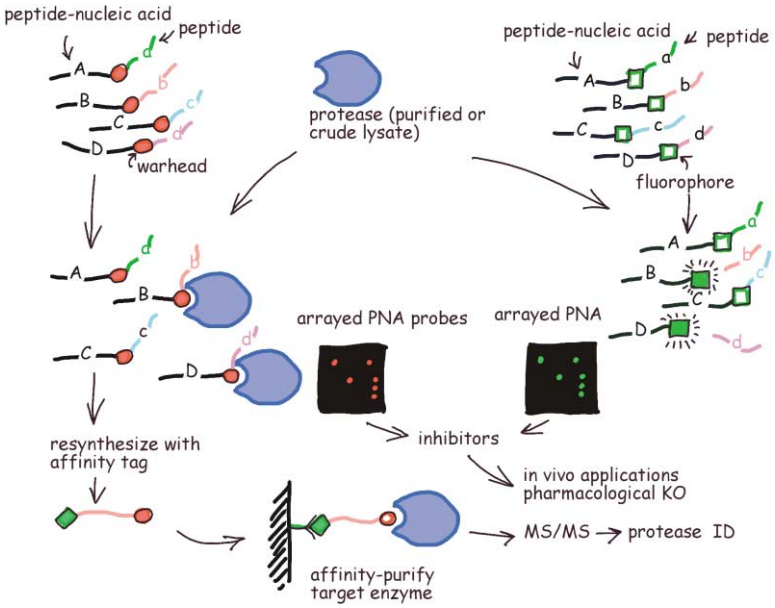

Figure 1. Characterization of Protease Activities from Complex Mixtures with PNA-Encoded Libraries of Activity-Based Probes and Fluorogenic Substrates

After the reaction of purified protease(s) or cell lysates with PNA-encoded libraries, the identity of an activity-based probe or a peptide substrate is revealed upon hybridization with oligonucleotide microarrays. The PNA-encoded libraries enable irreversible inhibitors and peptide substrates to be rapidly identified, which facilitates the development of small molecule inhibitors for in vivo applications.

Activity-based probes appended with visualization or affinity tags (I^{125}, fluorescent dyes, azides, biotin, or haptens) have been developed for several enzyme families and are powerful tools to measure functional enzymes in complex mixtures [10]. Thus, dust mite extracts were reacted with a library of PNA-encoded peptide acrylate inhibitors to selectively label cysteine-proteases in solution. After removal of unreacted PNA-encoded peptide acrylates, PNA-labeled cysteine-proteases were hybridized onto DNA-microarrays to identify reactive peptide acrylates, an elegant approach that was previously established by the same authors [11]. The most potent PNA-encoded inhibitor was then synthesized as a biotinylated derivative and used to retrieve the targeted protease(s) after enrichment with streptavidin beads. Mass spectrometry and SDS-PAGE analysis showed that Der p 1 and Der p 10 are the cysteine-proteases labeled by the most potent peptide acrylate inhibitor, with Der p 1 being the major target. It has been proposed that cleavage of CD25 (interleukin-2 receptor α chain) on regulatory T cells by Der p 1 induces allergies. Indeed, treatment of dust mite extracts with the Der p 1 inhibitor blocked cleavage of CD25 in a dose-dependent manner. This activity-based profiling approach, in combination with PNA-encoded libraries, illustrates how specific proteases from complex mixtures could be identified for a phenotype of interest and provides a powerful method for functional proteomic studies.

In the accompanying paper, Winssinger, et al. demonstrate that the PNA-encoding strategy can also be applied to fluorogenic peptide libraries [2]. The mixture of fluorogenic peptides in positional-scanning libraries precludes the identification of a precise peptide substrate for the protease of interest and only suggests the preferred amino acids at each position of the peptide substrate. To resolve this issue, fluorogenic peptide microarrays were printed on glass slides for spatial separation of individual substrates [12]. While these peptide microarrays have good reactivity and enable high-throughput analysis of protease substrate specificity, the immobilization of substrates on a solid support can affect the interaction of substrates with enzymes. By using PNA-encoded libraries, Winssinger, et al. allowed the hydrolysis of fluorogenic peptides to occur in solution, which upon hybridization with oligonucleotide arrays, reveals the identity of the cleaved peptides [2]. Importantly, Winssinger, et al. demonstrate that the attachment of PNA to fluorogenic peptides does not significantly interfere with substrate hydrolysis when compared with unmodified fluorogenic peptides. From a practical point of view, the fluorescent emission of rhodamine is relatively insensitive to changes in pH and enables the microarray analysis to be performed with standard instrumentation. Experiments with individual PNA-encoded rhodamine-peptide substrates and purified proteases established good specificity and sensitivity ($-$100 pmol) for the method. Moreover, these PNA-encoded fluorogenic peptide libraries can also be applied to complex mixtures of proteases from cell lysates or extracts, which reveal differences in protease profiles when comparing nonapoptotic and apoptotic cells as well as clinical blood samples.

It is clear that many biological processes are regulated posttranslationally, and proteolysis is a prime example. Therefore, new approaches that can detect how such activities are modulated in a high-throughput fashion are essential for functional proteomics. The development of PNA-encoded libraries provides a powerful method for profiling the activity of proteases and recovering them from complex mixtures [1, 2]. The ability to perform reactions with PNA-encoded libraries in solution is key, which avoids artifacts with immobilized inhibitors/substrates, on the likely assumption that PNA tags themselves do not interfere with enzyme activity. In addition, the use of microarray technology enables the miniaturization of protease assays for high-throughput analysis and will be particularly useful for clinical samples when limited amounts of material are available. The informa-

tion encoded in the PNA-encoded inhibitor/substrate enables the design of small-molecule inhibitors, which can then serve as tools for cellular assays and as a further basis for drug design. Indeed, the same group of researchers has developed PNA-encoded small-molecule libraries for irreversible protease inhibitors [13]. The use of PNA-encoded libraries should significantly reduce the steps required to identify the relevant protease(s) and their substrate(s) for a phenotype of interest. It will be exciting to see if these strategies can also be extended to small molecules that bind proteases reversibly as well as to other enzyme families. Finally, many changes in protein function cannot be detected in cellular lysates, and therefore future experiments will require the development of cell-permeable probes to monitor changes in vivo.

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A Suppression Strategy for Antibiotic Discovery

High-throughput phenotype screening and target identification have been combined in an effort to isolate antimicrobial, small-molecule therapeutics [1]. This approach, developed by Brown and colleagues and reported in this issue, is a major technological advance for antimicrobial drug discovery.

The discovery and development of novel antimicrobials via target-based approaches has historically been plagued by difficulties associated with optimizing small molecule leads out of biochemical screens while preserving or improving upon antimicrobial activity. This is due in large part because the factors governing small-molecule permeability and substrate selection criteria for efflux pumps in bacterial cells are poorly understood phenomena. However, high-throughput, phenotype-based screening methods offer a new promising strategy for identifying compounds from high-throughput screens that elicit a specific biological response. Unlike target-based screening of biochemical activities, phenotype-based screening selects for compound candidates that can penetrate cells, remain relatively unaffected by efflux pumps, and function properly in vivo. Thus, many of the former problematic issues affecting target-based screening are circumvented.

For example, by using a phenotype-based screen, Mitchison, Schreiber, and colleagues [2] identified an inhibitor of mitosis in mammalian cells with monopolar spindles, out of a library of 16,320 compounds. The inhibitor discovered, monastrol, attacks the motility of the mitotic kinesin Eg5, preventing normal spindle bipolarity and thereby validating it as a potential anticancer drug. At the time of this study, the only other previously known inhibitors of kinesin were cell impermeable. This work clearly demonstrates the advantages of employing phenotype screens in finding compounds that have novel activities within a biological system.

However, there is a slight problem. Although phenotype screens allow the rapid and selective identification of compounds that elicit a specific biological response, the mode of action of active compounds cannot be effectively and clearly deduced given the inherent complexity resulting from the large number of possible targets whose function is altered by the presence of the biological modifier. The success rate of finding a specific mechanism of action hinges on the stringency afforded by the phenotype screen as well as the level of knowledge of the possible targets impacted by the small-molecule effector. In the aforementioned example, Mitchison and Schreiber's search for a target was facilitated by the fact that the small molecule caused a mitotic