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Updated Biological Roles for Matrix Metalloproteinases and New "Intracellular" Substrates Revealed by Degradomics[†]

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ABSTRACT: Shotgun proteomics techniques are conceptually unbiased, but data interpretation and follow-up experiments are often constrained by dogma, established beliefs that are accepted without question, that can dilute the power of proteomics and hinder scientific progress. Proteomics and degradomics, the characterization of all proteases, inhibitors, and protease substrates by genomic and proteomic techniques, have exponentially expanded the known substrate repertoire of the matrix metalloproteinases (MMPs), even to include intracellular proteins with newly recognized extracellular functions. Thus, the dogma that MMPs are dowdy degraders of extracellular matrix has been resolutely overturned, and the metamorphosis of MMPs into modulators of multiple signaling pathways has been facilitated. Here we review progress made in the field of degradomics and present a current view of the MMP degradome.

A TAIL OF MATRIX METALLOPROTEINASES

In the preproteomics era, often once a protein had been named and assigned function and location, a dogma became established that guided subsequent hypothesis-driven studies. Proteases that were homologous to tadpole tail (*1*) and mammalian "interstitial collagenase" and that could cleave extracellular matrix molecules were discovered, grouped into a family, and named systematically as the matrix metalloproteinases (MMPs)[1] (*2*, *3*). The search for MMP substrates was limited to extracellular matrix molecules (*4*), and these proteases were subgrouped on the basis of the types of matrix molecules that they could process, such as stromelysins and gelatinases (*3*).

As reflected by their name, the MMPs have for years been described as "collectively being able to degrade all components of the extracellular matrix", despite the fact that they have not all been tested against the ~140 protein components of extracellular matrix. Based upon this dogma, the hypothesis became that these proteases were responsible for matrix remodeling, basement membrane degradation (*5*), and, thus, cell migration (*6*), tumor metastasis (*7*), and neovascularization (*8*). Large MMP inhibitor programs were begun by more than 70 pharmaceutical and biotech companies, and phase III clinical trials were undertaken with broad-spectrum MMP inhibitors designed to block the extracellular matrix degradation properties of MMPs (*9*). However, these inhibitors had little therapeutic benefit, and encumbered with musculoskeletal side effects, the trials were halted (*10*−*12*). Initial reasons for the failure were attributed to trial design, patient selection, inappropriate interpretation of animal models, and inadequate dosing (*10*, *11*, *13*). We have proposed that this failure was also due to an incomplete picture of MMP biology resulting from inadequate knowledge of substrate repertoires and physiological pathways under MMP control, and that individual members of the MMP family have unique functions that render broad-spectrum inhibition undesirable and even advantageous for tumorigenesis and metastasis (*14*). So-called "anti-target" functions of MMPs were recognized, first for MMP-8 (*15*) and then for a clutch of MMPs, and now it appears that few MMPs can actually be ascribed soley detrimental roles in disease (*14*, *16*).

MMPS: FROM MATRIX TO MULTIFACETED

Later, the concept of MMPs releasing cryptic functions from matrix proteins emerged (*5*, *17*−*19*), for example, from within fibronectin (*17*, *20*) and laminin (*6*), and growth factors such as IGF from binding proteins (*19*, *21*). Sporadic reports began to appear for nonmatrix substrates of MMPs such as serpins (*22*, *23*), insulin-like growth factor binding proteins (*21*), galectin-3 (*24*), tachykinin peptides (*25*), IL1-β (*26*), tissue factor pathway

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[1]Abbreviations: 2D, two-dimensional; COFRADIC, combined fractional diagonal chromatography; CTGF, connective tissue growth factor; DIGE, difference in-gel electrophoresis; GAPDH, glyceraldehyde phosphate dehydrogenase; GO, gene ontology; HMGB1, high-mobility group box 1; hsp90α, heat shock protein 90-α; ICAT, isotope-coded affinity tags; ICDC, inactive catalytic domain capture; iTRAQ, isobaric tags for relative and absolute quantitation; LC, liquid chromatography; MMP, matrix metalloproteinase; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MCP-3, monocyte chemoattractant protein-3; PICS, proteomic identification of protease cleavage sites; PROTOMAP, protein topography and migration analysis platform; SDF-1, stromal-derived factor 1; SILAC, stable isotope labeling by amino acids in cell culture; TAILS, terminal amine isotopic labeling of substrates; TIMP, tissue inhibitor of metalloproteinases.

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inhibitor (*27*), and the FGF type 1 receptor (*28*). With mounting reports, the concept that MMPs could cleave proteins other than extracellular matrix proteins became more widely accepted (*29*). In fact, in the early 1990s, an expanded substrate repertoire was proposed for MMP-1, and an MMP-mediated link between extracellular matrix turnover and serpin function was suggested (*22*). This and a review by DeClerck et al. represent early references to what we now call the "protease web" (*14*). Relying upon hypothesis-driven studies and "let's look and see" type experiments, researchers found new substrates, but in a time-consuming haphazard manner. As more nonmatrix, and thus less predictable, substrates were discovered, it became clear that more robust and systematic methods for identifying MMP substrates and hence potential biological roles were required.

PROTEASE FUNCTION IS DEFINED BY SUBSTRATES

Identifying protease substrates amounts to far more than "stamp collecting", as those who groan "not another list of candidate substrates" should be aware, for not only does this allow researchers in other fields to progress, but protease substrates define the function(s) of the protease (*30*). For instance, if the extracellular matrix proteins collagen (*31*) and fibronectin (*20*) were the only known substrates of MMP-2, it could be concluded that the role of MMP-2 is to facilitate matrix remodeling (Figure 1A). However, if the chemokines stromal-derived factor 1 (SDF-1) (*32*) and monocyte chemoattractant protein-3 (MCP-3) (*33*) were the only known substrates of MMP-2, then it would likely be surmised that MMP-2 has a predominant anti-inflammatory role (Figure 1B). MMP-2 in fact can cleave a large number of substrates and so is multifunctional (Figure 1C). Only being aware of a portion of protease substrates is like having just the corner of a roadmap; it can set antiprotease drugs up for a crash right from the start. Roles of the most obvious substrates are not necessarily those of the greatest importance in vivo, and some of these proteases may be drug antitargets, i.e., beneficial to the host in disease, for instance the anti-inflammatory roles of MMP-2, making blanket inhibition an unwise choice (*14*, *34*).

SUBSTRATE SCREENS

So how does one go about finding all the substrates of a protease, or reconcile substrates from cleavage and shedding events in cases in which the protease is unknown (*30*, *35*, *36*)? In around 2000, screening technologies began to emerge that were adapted for the discovery of new MMP substrates (*30*, *37*). Among these, techniques such as substrate phage display (*38*, *39*), proteomic identification of protease cleavage sites (PICS) (*40*), and combinatorial peptide libraries (*41*, *42*) can predict cleavage site consensus sequences which can aid bioinformatic identification of substrates, but they do not identify actual substrates and in fact indicate many proteins that are not bona fide substrates due to structure and localization considerations (*40*). Yeast two-hybrid screens using protease domains [an interactomics type of approach (*43*)], however, can reveal substrates (see Strategies for Degradomics), and the first use of this even detected very low abundance substrates such as chemokines (*33*). An expression cloning strategy that identifies gene products that interact with MMPs expressed in mammalian cultured cells, based upon their ability to compete with a measurable function of the MMPs, such as MMP-14-mediated activation of MMP-2 (*44*), has been successful in identifying several

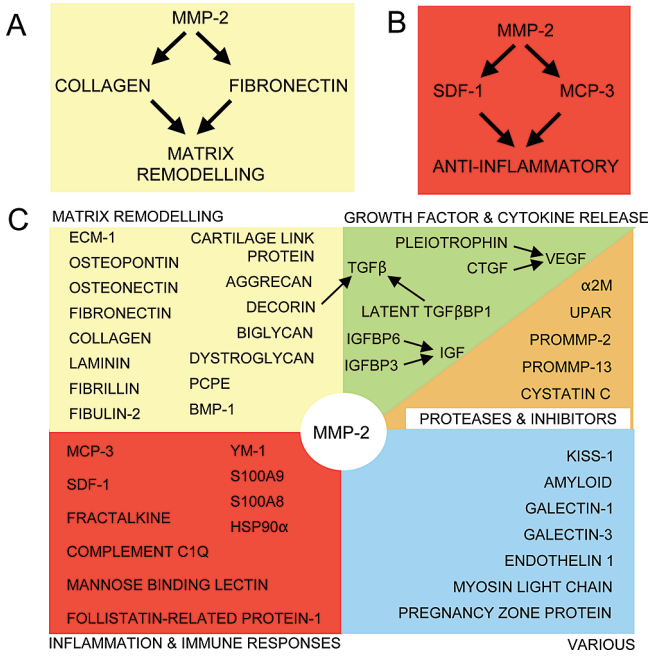

FIGURE 1: Substrates define protease function. Substrates such as collagen and fibronectin imply a role for MMP-2 in extracellular matrix remodeling (A), whereas the substrates stromal-derived factor-1 (SDF-1/CXCL12) and monocyte chemoattractant protein-3 (MCP-3/CCL7) advocate an anti-inflammatory role (B). In fact, MMP-2 can cleave a large number of substrates [MEROPS (http://merops.sanger.ac.uk/)] and therefore is multifunctional (C).

MMP substrates. These include KiSS-1 (*45*), lumican (*46*), and syndecan-1 (*47*).

Degradomics, the characterization of all proteases, inhibitors, and protease substrates present in an organism using genomic and proteomic techniques (*48*), can be divided into three main areas. Evaluation of the protease degradome, all the proteases, inhibitors, and their inactive homologues present, is approached using microarrays such as the CLIP-CHIP (*49*) and Hu/Mu ProtIn Chip (*50*) or qRT-PCR (*51*) to quantify protease and inhibitor gene expression, as well as profiling active proteases using activity-based probes (*52*, *53*). In this review, we will focus on the substrate degradome, all substrates of a protease, and the interactome, all proteins that interact with and are substrates or modify or localize the activity of proteases and inhibitors. It is not within the scope of this work to review mass spectrometry (MS) techniques; however, we will give a brief outline of the key points as applied to proteomics, and the reader is referred to pertinent reviews along the way. We will describe the key steps that are required for degradomics and how degradomic screens can be designed.

MASS SPECTROMETRY FOR PROTEIN IDENTIFICATION

The advent of MS approaches for protein identification has provided a breakthrough for high-throughput, unbiased proteomics (*54*, *55*), and coupled with strategies for quantification, MS is a powerful tool for degradomics.

To detect anything but the most abundant proteins in a sample and to simplify MS spectra so that they can be interpreted, a proteome must be fractionated prior to MS. There are two main separation strategies, two-dimensional (2D) gel electrophoresis and liquid chromatography (LC) (so-called "shotgun proteomics").

In 2D gel electrophoresis, proteome samples are separated first by isoelectric point (isoelectric focusing) and then by molecular mass (SDS−PAGE) (*56*, *57*). Protein spots of interest are excised and digested to peptides that are identified by MS (*58*). While this technique is affordable and can result in the identification of several thousand spots per gel, including protein variants and post-translational modifications, it is relatively low-throughput and reproducibility is an issue. Low-abundance proteins are difficult to detect without prefractionation or removal of high-abundance proteins, and proteins with extreme isoelectric points or molecular mass and hydrophobic proteins may be incompatible. The limited ability to analyze membrane proteins and the lack of resolution to separate proteins differing by only a few amino acids, which for MMP substrates such as chemokines can have profound effects on activity (*59*), are major impediments of 2D gel electrophoresis. However, 2D gel electrophoresis-based methods have been used successfully for MMP substrate discovery (*60*, *61*). Recently, "Protein Topography and Migration Analysis Platform" or PROTOMAP has combined one-dimensional (1D) SDS−PAGE with MS analysis and quantitation to directly map cleavage sites as well as identify substrates of caspases (*62*) and so has potential for the analysis of MMP function.

Gel-free proteomics approaches consist of multidimensional fractionation, commonly strong cation exchange and reverse phase C18 high-performance liquid chromatography (LC) (*63*, *64*). These techniques are high-throughput since they can be automated and run in-line with the mass spectrometer. There are advantages and disadvantages to both separation strategies (*65*, *66*), and a combination of both gives excellent proteome coverage (*67*, *68*).

Protein identification by MS is summarized below, and the reader is directed to reviews on the subject for more information (*54*, *69*, *70*). Proteins or simple mixtures of a few proteins (such as those excised from spots on a 2D gel, described below) can be identified by peptide mass mapping, where peptide masses are matched to theoretical peptide masses calculated for each protein in a database. This has largely been replaced by tandem mass spectrometry (MS/MS), where peptide spectra are used to search an in silico digested proteome sequence database. Peptides are further fragmented in the collision cell of the mass spectrometer to generate fragment ions of progressively lower mass to aid identification by fingerprinting. In some cases, de novo sequencing is possible, where the peptide sequence is iteratively decoded on the basis of its mass following the loss of each amino acid residue resulting from fragmentation at each peptide bond (*71*).

QUANTITATIVE PROTEOMICS

Quantification strategies are valuable for degradomics as they allow differences in individual protein levels (including substrates) between samples to be discerned. This is particularly valuable for heirarchical substrate winnowing and the subtraction of background proteolysis in the sample. There are several techniques available (Table 1). The quantitative version of 2D gel electrophoresis, in which two differentially labeled samples are analyzed on a single gel, is called difference in-gel electrophoresis (DIGE) (*72*−*74*). For LC approaches, several labeling techniques in which relative amounts of each protein can be discerned by quantification of different isotopic labels in MS are available: isotope-coded affinity tags (ICAT) (*75*, *76*) and isobaric tags for relative and absolute quantitation (iTRAQ) (*77*) which have been used successfully for shotgun degradomics, stable isotope labeling by amino acids in cell culture (SILAC) (*78*, *79*), dimethylation (*80*), and ^{18}O labeling (Table 1). Some groups have employed label-free quantification methods to identify MMP substrates, but the quantification is less accurate than that from isotopic labeling methods (*81*, *82*). Many MS facilities run these techniques as services, so even laboratories without access to appropriate instrumentation can conduct degradomics (although both reagents and MS time are relatively expensive).

The most recent techniques, termed "N-terminomics", isolate proteolytically generated N-termini, allowing identification of the substrate and cleavage site in a single experiment, (reviewed in ref *83*). Such techniques include terminal amine isotopic labeling of substrates (TAILS), (*84*), combined fractional diagonal chromatography (COFRADIC) (*85*, *86*), acetylation of N-termini (*87*, *88*), and selective biotinylation of unblocked N-terminal α-amines chemically (*89*) and by subtiligase (*90*).

STRATEGIES FOR DEGRADOMICS

The most obvious type of degradomics screen is one comparing a proteome exposed to the protease of interest with an unexposed proteome (Figure 2A). This is typically performed in cell culture systems (*81*, *82*, *91*−*93*), and it can be applied to tissues [e.g., knockout vs wild-type mice (*94*)] or by exposing a proteome to recombinant protease in vitro (*60*, *84*). There will be changes in substrate levels with the protease compared with the control (Figure 2B,C), which can be quantified by stable isotope labeling techniques (Table 1) to give the ratio of protease-treated to control peptide and thus protein ratios. Via analysis of ratios, shedding or cleavage, and even the site of cleavage can be determined by peptide mapping (*92*). Complementary screens, for example, expression of protease versus vector control (*91*, *93*) and protease plus inhibitor versus vehicle control (*95*), that give opposite ratios (Figure 2B,C) can provide internal validation and increase confidence in substrate identification.

Several features of the MMPs can be exploited for degradomics in interactomic-type approaches. Mutation of the conserved active site glutamate residue (*96*), for example, glutamate 375 to alanine in MMP-2, renders the enzyme catalytically inactive (*97*). Since active site mutants are unable to cleave and thereby release substrates, substrates are trapped and this is exploited in inactive catalytic domain capture (ICDC) (*83*) (Figure 3A), which has been described for MMP-14 (*98*) and ClpXP protease (*99*). MMP domains ancillary to the catalytic domain, termed exosites (*100*, *101*), foster interactions that mediate complex formation (*102*, *103*), localization, and substrate presentation (*100*, *104*−*106*). Isolated exosites, for example, the collagen binding domain of the gelatinases or the hemopexin C domain (*107*), can be used to screen for interactors, commonly substrates, in exosite scanning (*108*) (Figure 3B). ICDC and exosite scanning can be incorporated into yeast two-hybrid or quantitative proteomic approaches. In a yeast two-hybrid screen, by exosite scanning using the hemopexin domain of MMP-2 against a human fibroblast cDNA library, the chemokine MCP-3 was discovered to be a bona fide substrate of MMP-2 (*33*, *108*). This led to the discovery that most chemokines are modified by MMPs (*109*), many of which bind the hemopexin C domain which inhibits their actions in biochemical assays with the recombinant domain. ICDC and exosite scanning with isotopic labeling can be operated as affinity capture screens using resin alone as a control

Table 1: Techniques That Have Been Used for Quantitative Degradomics

method	advantages	disadvantages	degradomics examples
DIGE (*72*)	removes intergel variation and facilitates identification of changes	low-throughput; incompatible with some proteins; difficult to detect low-abundance proteins	MMP-2 and MMP-9 (bronchoalveolar lavage fluid from wild-type vs *Mmp-2*/*9*$^{-/-}$ mice) (*94*)
ICAT (*75*, *132*)	absolute quantitation; biotin pullout of labeled cysteine residues reduces sample complexity	small number of peptides per protein, some proteins missed; only duplex; no discrimination between labeling of cellular and culture serum proteins	MMP-14 (conditioned medium, MDA-MB-231 cells transfected with MMP-14 or vector) (*91*); MMP-2 (conditioned medium, *Mmp-2*$^{-/-}$ fibroblasts transfected with MMP-2 or vector) (*93*); MMP-14 (conditioned medium and cell membrane, MDA-MB-231/MMP-14 treated with MMP inhibitor vs vehicle) (*95*)
iTRAQ (*133*)	labels N-terminus and lysine residues; high level of confidence due to many peptides per sample; up to eight samples	no discrimination between labeling of cellular and culture serum proteins	MMP-2 (conditioned medium, *Mmp-2*$^{-/-}$ fibroblasts transfected with MMP-2 or vector) (*92*)
SILAC (*79*, *134*)	metabolic incorporation of stable isotope-labeled amino acids in living cells; discrimination between cellular and culture serum proteins; early combination of samples reduces errors	labeling limited to cells in culture	γ-secretase (HeLa cells treated with γ-secretase inhibitor vs vehicle, cytosol and cell membrane) (*135*); Granzyme B (recombinant human and mouse granzyme B added to 3 SILAC-labelled JURKAT, K562 and YAC1 cell lysates) (*160*)
dimethylation (*80*)	(H)2 vs (D)2 formaldehyde; inexpensive; simple, fast reaction	labeling of N-termini and lysine residues; fractionation essential	MMP-2 (*Mmp-2*$^{-/-}$ mouse fibroblast secretome) (*84*)
$^{16}O/^{18}O$ differential labeling (*136*)	trypsin-catalyzed incorporation of 2O to the C-terminus of every cleaved peptide; simple reaction, no label removal required; $H_2{}^{18}O$ inexpensive; can be used with other proteases	some back-exchange (~5%) with ^{16}O unless trypsin is fully reduced and alkylated	Fas-stimulated Jurkat T-lymphocytes (apoptotic vs living) (*137*); HtrA2 (wild type vs inactive mutant) with apoptotic Jurkat T cell lysates (*138*)
label-free (peak height/ion count,[a] spectral counting[b]) (*139*, *140*)	no expensive labels required; no extra sample handling steps	samples analyzed separately; bias toward higher-abundance proteins; requires multiple data acquisitions for each sample; need to validate and normalize using spiked-in proteins; low dynamic range; reproducibilty may be an issue	MMP-9 (parent vs RNAi knockdown PC-3 ML cells) (*82*); MMP-9 (wild type vs active MMP-9 transfected RAW264.7) (*81*); caspases (apoptotic vs nonapoptotic Jurkat T cells) (*62*)

[a]Based on precursor ion intensities in the MS survey scan and quantification of peak areas of the peptide ions. [b]Based on the number of times a unique peptide is detected.

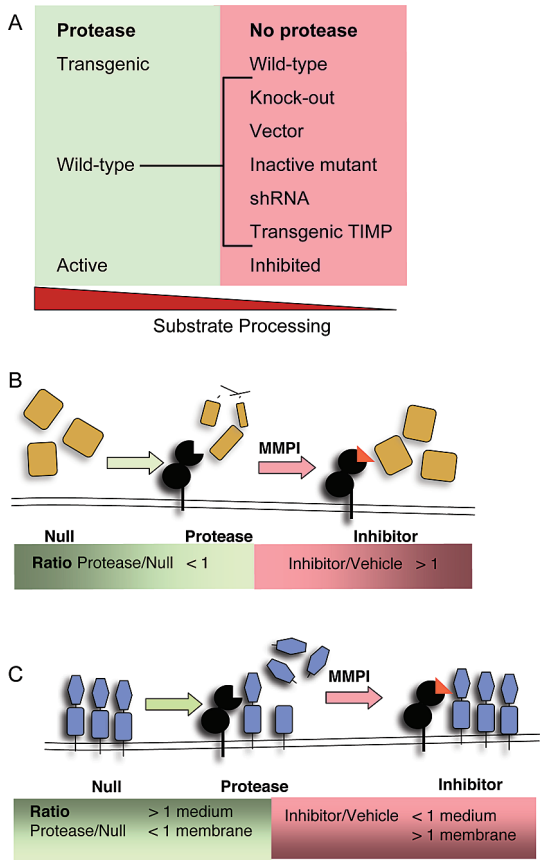

FIGURE 2: Degradomic screens. (A) Design of the degradomic screen. Comparisons that maximize the differences between the test sample and control in terms of substrate processing. (B) Soluble substrates. In the absence of protease (null), soluble substrates are present, but in the presence of protease, the substrate is cleaved and cleared. Using quantitative proteomics, the protease:null ratio of the substrate in the samples is <1. Comparing inhibited protease with vehicle-treated (active) protease, we find an inhibitor:vehicle ratio of >1. The latter screen can act as validation of the first. (C) Membrane proteins. In the case of membrane proteins, two components can be analyzed: the cell surface and the soluble phase. Proteolysis leads to a shift in location; shed ectodomains will be partitioned to the soluble phase and will decrease in number in the membrane, resulting in protease:null ratios of >1 for the culture medium and <1 for the membrane fraction. This is reversed by the addition of an inhibitor compared with vehicle-treated (active) protease.

for nonspecific binding (Figure 3) (reviewed in ref *83*) in a manner similar to chemical proteomics strategies that are being employed to screen for targets of kinase ATP mimetic inhibitor drugs (*110*, *111*).

There are relatively few reports ($\leq$12) of degradomic screens for MMP substrates to date. These are summarized in Table 2, along with degradomic screens for other mammalian proteases.

MMP SUBSTRATES REVEALED BY DEGRADOMICS

Although degradomics is in its infancy, these types of proteomic techniques are so powerful that the MMP degradome has already been vastly expanded and the dogma that MMPs are just matrix degraders has been resoundingly overturned, albeit 20 years after initial suggestions of an expanded substrate repertoire! In reality though, only a fraction of the candidate substrates that have been identified have been validated in vitro, let alone in vivo, partly because of their sheer number.

Selection of Candidate Substrates for Validation: Hierarchical Substrate Winnowing. How can a list of identified proteins be narrowed down to those that are biologically relevant, and which candidate substrates should be selected for validation? The first step is to set quantitative ratio cutoffs to reduce the false positive rate and to maximize the chance that the protein is an MMP substrate. This is sometimes done arbitrarily (e.g., ratios of <0.5 and >1.5), and there are statistical methods available (*112*); however, for MMPs, cutoffs can also be selected on the basis of the ratios obtained for known substrates detected in the screen (e.g., as in refs *92* and *95*) since so many MMP substrates are known and are typically detected in degradomic screens.

Of concern is whether we undermine the unbiased nature of proteomics by being dogmatic in how we select candidate substrates. Ultimately, selection of which candidates to validate from the "likely list" depends upon factors such as availability and bias. Some proteins are simply not available commercially, and those that are can be expensive. An invaluable source of candidate substrates for validation is gifts or collaborations from the researchers who study these proteins; this is a win−win situation as both parties gain valuable information. We again thank those researchers who generously supply proteins and constructs for validation of proteomic screens.

Apart from availability, protein selection may be influenced by familiarity. Many of the proteins on a proteomics hit list are likely to be unfamiliar, and this may lead to bias. For example, given the choice between a metastasis suppressor (KiSS-1) and a DNA binding protein [high-mobility group box 1 (HMGB1)] as a candidate substrate for an extracellular MMP, who would choose the nuclear protein? Yet HMGB1 is an MMP substrate (*95*) (G. S. Butler and C. M. Overall, unpublished observations) that can be extracellular (*113*) and is a drug target for sepsis (*114*).

There is software available to functionally annotate and categorize proteins, for example, Gene Ontology (GO) (http://www.geneontology.org/GO.database.shtml). This may allow selection of substrates on the basis of location; for extracellular proteinases such as the MMPs, one might select extracellular and cell membrane proteins since colocalization is a prerequisite for interaction and cleavage. Although it is powerful and useful software, GO relies upon database annotations that are not necessarily up to date, and even if they are, annotations are based on what is currently known.

INTRACELLULAR PROTEIN SUBSTRATES OUTSIDE OF THE CELL

Inhibitory assumptions that persist are that "intracellular" proteins found extracellularly are artifacts of cell death and lysis, that only proteins with a leader sequence exit the cell, and that intracellular proteins cannot function outside of the "reducing" environment of the cell. The beauty of proteomics is that it is conceptually unbiased; therefore, if a protein has a quantification ratio suggesting that it may be a substrate, should it be discarded because it is annotated as being intracellular, such as a glycolytic enzyme?

Certainly, stress and trauma can result in cell lysis and the release of intracellular proteins; here extracellular proteases such as MMPs may gain access to and cleave these inadvertent extracellular proteins as a clearance mechanism. This is important as the partitioning of many enzymes and their substrates is broken down and opportunistic cleavage of unintended

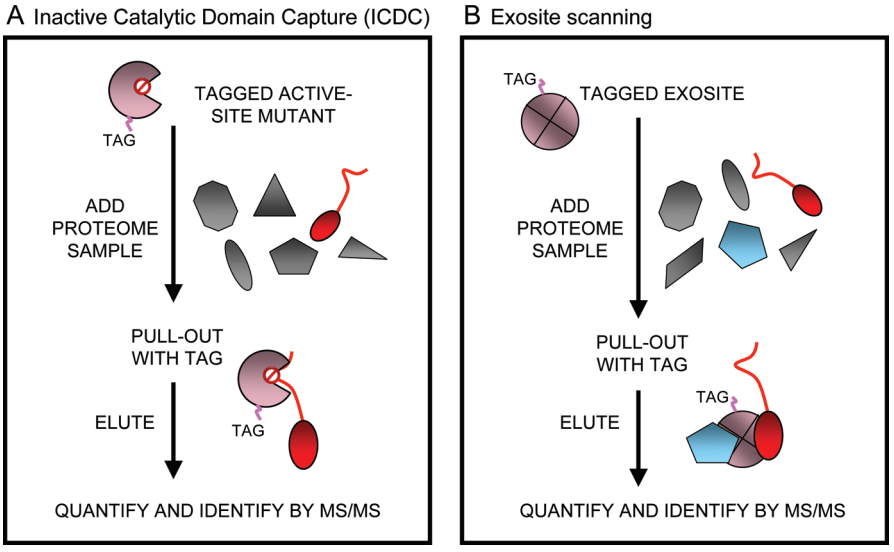

FIGURE 3: Interactomics-based approaches to degradomics. (A) Inactive catalytic domain capture. A catalytically inactive mutant binds substrates in a proteome sample but is unable to cleave and release them, resulting in capture. Bound proteins can be isolated by binding the tagged catalytically inactive domain to an affinity resin. (B) Exosite scanning. Recombinant exosites such as hemopexin C domain [or the fibronectin type II repeats of the gelatinases (not shown)] can be used to screen for interactors (often substrates) in a proteome. The tagged domains, e.g., polyhistidine or FLAG, can be isolated before or after exposure to the proteome using a tag reactive resin such as immobilized metal affinity chromatography or an anti-FLAG antibody column. Bound proteins can be identified by mass spectrometry. Nonspecifically bound proteins can be discounted by comparing eluted proteins to those eluted from resin alone using a quantitative labeling procedure, such as iTRAQ. Nonspecifically bound proteins will have a domain resin:resin ratio of ≈ 1. Specifically bound proteins will have a ratio of > 1.

substrates is potentially disastrous for cellular or tissue homeostasis. This is proposed for MMP-14 cleavage of the intracellular proteins, peptidyl prolyl cis−trans isomerase A, DJ-1, hsp90alpha, and γ-enolase, that we identified in degradomic screens (*92*, *95*), and more recently for MMP-9 cleavage of adenylyl cyclase-associated protein 1 (*115*). Although cell lysis does account for the presence of intracellular proteins in culture media, many but not all intracellular proteins are repeatedly detected in secretomes (Table 2 in ref *34*) and thus may be selectively secreted or translocated. Nonclassical secretion pathways are relatively poorly investigated, yet multiple signal sequence-independent secretion pathways (exosomes, microparticles, secretory lysosomes, and membrane translocation) have been reported (*116*−*120*). It is likely that many proteins are present in multiple locations (*34*, *121*), depending upon various conditions or signals in vivo, and that these are identified by proteomics, whereas they were neither sought nor found by traditional protein biochemical methods.

With regard to the difference in environment inside and outside of the cell, there may be specific microenvironments that mimic intracellular conditions, or it may be that proteins are multitasking; i.e., they perform one function inside of the cell and a completely different one outside (*34*, *121*). For example, glucose-6-phosphate isomerase is a well-established intracellular metabolic enzyme mediating the interconversion of glucose 6-phosphate to fructose 6-phosphate. On the surface of sperm, this same protein mediates sperm agglutination (sperm surface antigen-36) (*122*) and when secreted by tumor cells (autocrine motility factor) or T-cells (neuroleukin) acts as a cytokine stimulating cell migration and metastasis (*123*) or as a neurotropic mediator through CXXC chemokine activity (*124*−*126*), respectively. Novel extracellular roles of other seemingly irrelevant proteins may be highly relevant in the context of MMP processing and regulation, as we have shown for chemokines (*109*). For example, secreted in response to inflammatory stimuli, the DNA-binding protein HMGB1 and the chaperone peptidyl prolyl cis−trans isomerase A are major mediators of inflammation, the latter being a potent immune cell chemoattractant (*127*, *128*), and the molecular chaperone, hsp90alpha, is implicated in cancer invasion (*129*). Therefore, whereas such proteins are labeled intracellular, many of these are also bona fide extracellular proteins, have important extracellular biological roles, and are substrates modified by MMP activity. This nascent field promises to be very exciting with much new biology to be learned. On the other hand, some of the newly identified MMP substrates are intracellular and arise from reports of MMPs translocating into the cell and exerting both intracellular proteolytic and novel nonproteolytic activities (e.g., transcription factor activity) on bona fide intracellular substrates (reviewed in refs *34* and *130*). These are included in Table 1 of the Supporting Information which summarizes a current view of the classes of proteins in the MMP degradome, and the processes that MMPs modulate (although classification is both subjective and dependent upon what is currently known).

PROTEASE WEB

Proteases do not tend to act alone: many protease substrates are zymogens and inhibitors, the cleavage of which can modulate the net proteolytic potential of a system. Thus, the protease web impinges upon diverse biological processes (Figure 4). Rather than depicting the entire known degradome of humans (~560 proteases, > 150 inhibitors, and their substrates and interactors), Figure 4 depicts interconnections of just two MMPs (MMP-2 and MMP-14) and one of the inhibitor substrates of MMP-2 (cystatin C), and even then, one can see how complicated the map becomes. MMPs are nodes in the web that can modulate the activity of other proteases, and thus, perturbations in the web can be propagated. Hence, it is often impossible to determine the direct role of a protease in knockout mice, where some proteases have multiple protease and inhibitor substrates. All that can be said from such knockout or transgenic mice is that the phenotype

Table 2: Mammalian Protease Substrates Revealed by Degradomics[a]

screen design	techniques	validated substrates	possible effect of processing
		Matrix Metalloproteinases	
		Secreted	
MMP-2 hemopexin domain vs ConA-treated fibroblast cDNA library (*33*)	yeast two-hybrid, exosite scanning	monocyte chemoattractant protein 3	generation of receptor antagonist, downregulated inflammation
MMP-2 vs vector in *Mmp-2*$^{-/-}$ fibroblasts (conditioned medium) (*93*)	ICAT	heparin affinity regulatory peptide/pleiotrophin	abrogation of mitogenic and migration enhancing activity, VEGF release and stimulation of angiogenesis
		connective tissue growth factor	reduced level of protein expression and extracellular matrix formation, VEGF release and stimulation of angiogenesis
		follistatin-like related protein-1	inhibition of proinflammatory activity, accelerated cell growth, cytokine release
		IGFBP-6	release of IGFII
		cystatin C	reduced level of inhibition of cathepsins B, H, and L
Δpropeptide MMP-2 vs Δpropeptide E375A-MMP-2 in *Mmp-2*$^{-/-}$ mouse embryonic fibroblasts (conditioned medium) (*92*)	iTRAQ	osteopontin	effects on receptor interactions affecting bone turnover, wound healing, inflammation, and immune responses
		galectin-1	modulation of inflammation, angiogenesis, and immune responses
		fractalkine (CX_3CL1)	shedding of N-terminal chemokine domain from cell surface
		procollagen C-proteinase enhancer	release of C-terminal netrin domain from ECM, altered procollagen processing
MMP-2 incubated with secretome from mouse *Mmp-2*$^{-/-}$ fibroblasts (*84*)	N-terminomics, dimethylation, TAILS	bone morphogenetic protein-1	modulation of procollagen maturation
		fibulin-2	modulation of extracellular matrix
		extracellular matrix protein-1	modulation of extracellular matrix
		biglycan	release of bone morphogenetic protein-4 from extracellular matrix
wild type vs *Mmp-2*/*9*$^{-/-}$ mice asthma model (bronchoalveolar lavage fluid) (*94*)	2D DIGE	Ym1	loss of chemoattractant activity
		S100A8	altered chemotactic activity, loss of proinflammatory activity
		S100A9	loss of proinflammatory activity
MMP-9 vs shRNA MMP-9 knockdown or MMPI, in metastatic prostate cancer cells (conditioned medium) (*82*)	label-free quantitation	leukemia inhibitory factor	modulation of cytokine activity
		protease nexin-1	alleviation of thrombin, plasminogen activator, or plasmin inhibitory activity
MMP-9 (wild type vs active MMP-9 transfected RAW264.7) (*81*)	label-free quantitation	amyloid protein precursor integrin $\beta 2$	ectodomain shedding, reduced β-amyloid peptides, modulation of cell adhesion
MMP-11 vs E276A MMP-11 incubated with MCF-7 secretome (*84*)	N-terminomics, TAILS	α-1 antitrypsin	loss of serine proteinase inhibitory activity
		Cell Surface	
plasma with or without the MMP-14 catalytic domain (*60*)	nonquantitative 2D gels	plasma gelsolin	polymerization of extracellular actin → pathology
		apolipoprotein A-I	accumulation of cholesterol, atherosclerosis

Table 2. Continued

screen design	techniques	validated substrates	possible effect of processing
		apolipoprotein E	accumulation of cholesterol, atherosclerosis
		apolipoprotein C-II (validated in ref *141*)	decreased lipoprotein lipase activity
vector vs MMP-14 in MDA-MB-231 cells (conditioned medium) (*91*)	ICAT	connective tissue growth factor	release of VEGF and stimulation of angiogenesis
		interleukin-8	increased chemokine activity
		secretory leukocyte protease inhibitor	removal of inhibitory activity
		fibronectin	release from cell sites
		death receptor 6	ectodomain shedding
		pro tumor necrosis factor α	maturation of tumor necrosis factor α
MMPI vs vehicle in MMP-14-transfected MDA-MB-231 cells (conditioned medium and cell membranes) (*95*)	ICAT	gamma enolase	degradation and clearance
		peptidyl cis−trans isomerase A	modulation of proinflammatory activity
		DJ-1	pathogenesis of Parkinson's disease
		heat shock protein 90α	regulation of cell invasion
		galectin-1	effects on cell adhesion, migration, and proliferation
		pentraxin 3	regulation of innate immunity and inflammation
		progranulin	effects on inflammation, repair, and tumorigenesis
		Cyr61	modulation of proliferation, chemotaxis, angiogenesis, and cell adhesion
		follistatin-related protein 3	release of activin, myostatin, or bone morphogenetic proteins
		Niemann-Pick C2	effects on cholesterol binding
		iduronate-2-sulfatase	effects on glycosaminoglycan metabolism
		thrombospondin-1	modulation of binding interactions
		dickkopf-1	increased Wnt activity, effects on joint remodeling
		Other Proteases	
		Secreted	
HtrA1 serine protease PDZ domain vs mouse embryonic and adult brain cDNA libraries (*142*)	yeast two-hybrid, exosite scanning	type III procollagen α1 C-propeptide	collagen quality control, regulation of C-propeptide bioactivity?
		GM130	
		Cell Surface	
ADAM-17-transfected vs MMPI-treated A431 cells (glycoproteins extracted from conditioned medium) (*143*)	2D DIGE	desmoglein 2-activated leukocyte cell adhesion molecule	loss of cell adhesion
ADAM-17 vs vector in *Adam-17*$^{-/-}$ DRM cells (glycoproteins extracted from conditioned medium) (*144*)	1D PAGE, *N*-ethyliodoacetamide (d_0 or d_5) labeling	–	–
meprin zymogen vs active form in MDCK (epithelial) cells (conditioned medium) (*145*)	nonquantitative 2D gels	–	–

Table 2. Continued

screen design	techniques	validated substrates	possible effect of processing
		Extracellular Matrix	
ADAMTS1 thrombospondin repeat I/Cys-rich region vs human placenta cDNA library (*146*)	yeast two-hybrid, exosite scanning	tissue factor pathway inhibitor-2	altered binding properties and location
ADAMTS1 vs parent 293T cells (conditioned medium) (*147*)	2D DIGE	−	−
		Intracellular	
caspases: apoptotic vs living Fas-stimulated human Jurkat T-lymphocytes (lysates) (*137*)	N-terminomics, $^{16}O/^{18}O$ labeling, COFRADIC	−	−
caspase 1 (or 3) vs buffer-soaked gel of THP-1 cells or blood monocytes lysates (*148*)	diagonal gel electrophoresis	poly(ADP-ribose) polymerase GAPDH triosephosphate isomerase α-enolase aldolase pyruvate kinase	disruption of glycolysis
caspase 3-treated vs untreated MCF-7 cell lysate proteins (*149*)	2D DIGE	vinculin	regulation of cytoskeleton
granzyme A vs Ser → Ala inactive mutant incubated with Jurkat proteins (cell lysates) (*150*)	2D DIGE	heterogeneous ribonuclear protein K	disrupt pre-mRNA binding and translational silencing of genes responsible for cell death
granzyme B (human & mouse) vs JURKAT, K562 and YAC1 cell lysates (*160*)	SILAC, N-terminomics, COFRADIC	poly(ADP-ribose) polymerase caspase-3	cell death cell death
granzyme B (S183A inactive mutant) vs human peripheral blood lymphocyte cDNA library (*151*)	yeast two-hybrid, ICDC	filamin	caspase-independent cell death
granzyme B vs vehicle incubated with YAC-1 mouse lymphoma proteins (cell lysates) (*152*)	2D DIGE	heat shock protein 70/90 organizing protein (Hop) procapase 3	loss of scaffold for stress response proteins, activation of apoptosis
granzyme K vs Ser → Ala inactive mutant incubated with Jurkat proteins (cell lysates) (*150*)	2D DIGE	heterogeneous ribonuclear protein K	disrupt pre-mRNA binding and translational silencing of genes responsible for cell death
granzyme M vs Ser → Ala inactive mutant incubated with HeLa proteins (cell lysates) (*153*)	nonquantitative 2D gels	β-tubulin ezrin	disrupt microtubule network, cell death disrupt actin−plasma membrane linkage of cytoskeleton and cell signaling → cell death
cathepsin P (rodent) vs buffer incubated with rat choriocarcinoma proteins (cell lysates) (*154*)	2D DIGE	α-tubulin Gp96 calreticulin (vasostatin)	disrupt microtubule network, cell death release from the cell (ER) for antitumor adjuvant activity modulation of intracellular ER chaperone activity, modulation of extracellular activities, angiogenesis, proliferation, adhesion of endothelial cells, trophoblast maturation

Table 2. Continued

screen design	techniques	validated substrates	possible effect of processing
calpain-μ large subunit 5-EF-hand domain vs brain cDNA library (*155*)	yeast two-hybrid, exosite scanning	heterogeneous ribonuclear protein K	effects on gene expression
		heterogeneous ribonuclear protein R	
calpain 3 (C129S inactive mutant) vs mouse skeletal muscle cDNA library (*156*)	yeast two-hybrid, ICDC	aldolase A	localization at sarcomere, regulation of calcium release (substrate in vitro but may not be processed in vivo)
calpain 3 transgenic vs wild-type mice (muscle homogenates) (*157*)	2D DIGE	myosin light chain 1	sarcomere remodeling
HtrA2/Omi vs HtrA2 S306A inactive mutant incubated with cell lysates from apoptotic Jurkat T cells (*138*)	N-terminomics, $^{18}O/^{16}O$ labeling, COFRADIC	actin	cytoskeletal disruprtion
		α-tubulin	
		β-tubulin	
		vimentin	
		elongation factor 1-α	abrogation of translation
		eIF-4G1	
		TIF-1β	effects on gene expression
		HADH2	effects on amyloid Aβ binding
		KIAA1967	apoptosis
		KIAA0251	
ubiquitin-specific protease 10 N-terminal residues 1−300 vs mouse cortical collecting duct cell cDNA library (*158*)	yeast two-hybrid	sorting nexin 3	stabilization by deubiquitinylation
ubiquitin-specific protease 10 vs mouse embryo and hu lymphocyte cDNA libraries (*159*)	yeast two-hybrid	ras-GTPase activating protein	interactor (rather than a substrate) that inhibits deubiquitinylation
selective γ-secretase inhibitor vs vehicle in HeLa cells (cytosolic and cell membrane fractions) (*135*)	SILAC	dystroglycan	regulation of extracellular matrix−cytoskeleton interaction, role in muscular dystrophy
		delta/notch-like EGF-related receptor	regulation of notch signaling
		desmoglein-2	regulation of intercellular adhesion, modulation of apoptosis
		natriuretic peptide receptor-C	signaling role
		plexin domain-containing protein-2	?
		vasorin	regulation of TGF-β signaling

[a]Since the field is relatively new and there are few MMP degradomics reports, degradomics screens for other proteases are included to give an idea of what kinds of screens are possible. Where substrates are reported, only those validated by secondary means are shown (excludes validation of ratios by Western blotting of samples used in the screens and validations using peptides rather than proteins). Abbreviations: MMPI, MMP inhibitor; IGFBP, insulin-like growth factor binding protein; eIF-4G1, eukaryotic translation initiation factor 4γ1; HADH2, L-3-hydroxyacyl-coenzyme A dehydrogenase; VEGF, vascular endothelial growth factor; ER, endoplasmic reticulum; TGF, transforming growth factor.

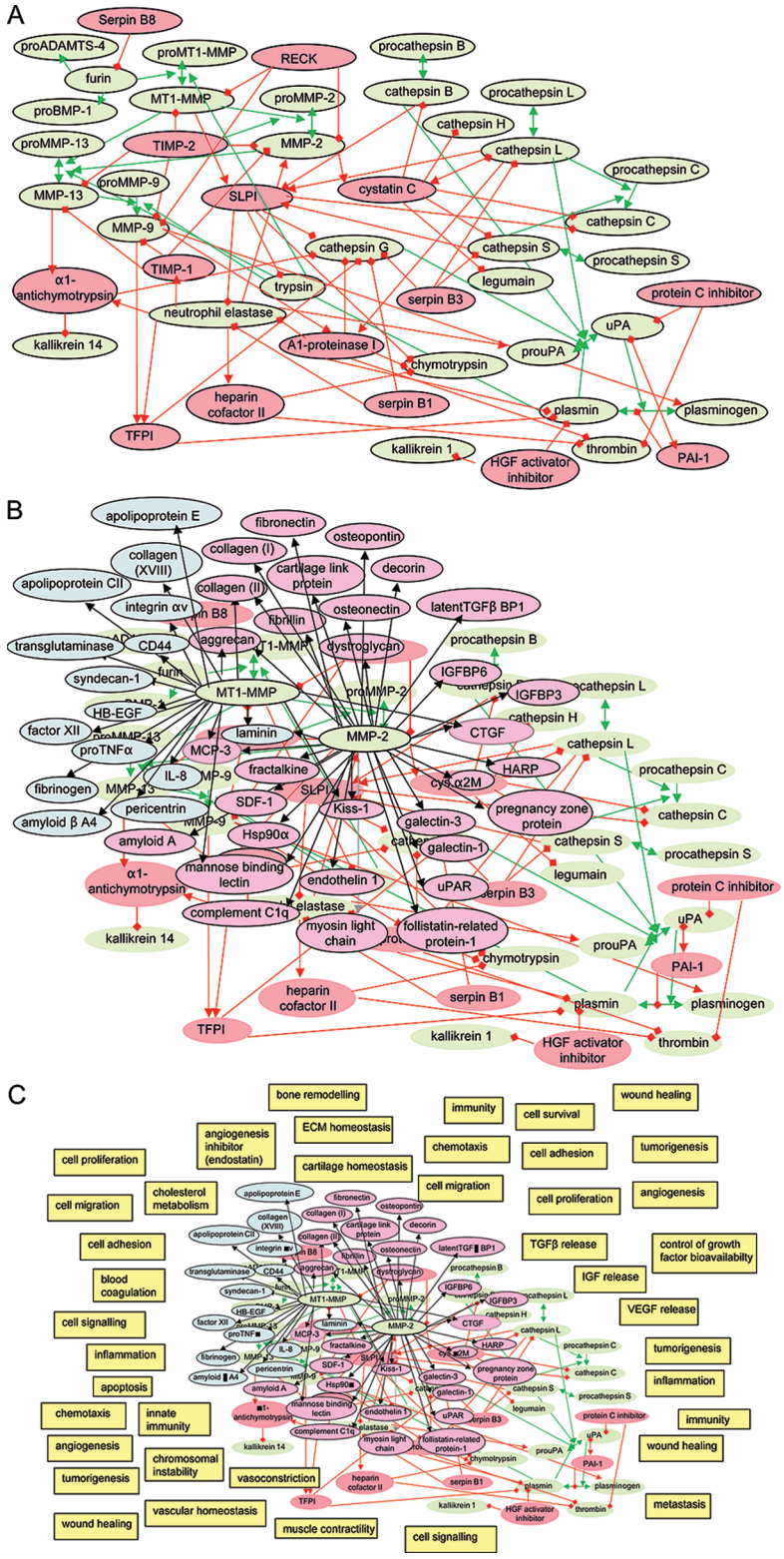

FIGURE 4: Protease web. (A) Excerpt from the protease web. Protease and inhibitor substrates of MMP-2 and MMP-14 (which activates MMP-2), and cystatin C (which is inactivated by MMP-2) are mapped. Red arrows indicate an inactivating cleavage, and a red diamond indicates an inhibitory interaction. A green arrow indicates an activating cleavage. Proteases are colored pale green and inhibitors pale red. (B) Intersection of the protease web with the proteome. The protein substrates of MMP-2 and MMP-14 are overlaid on panel A in pink and blue, respectively. (C) Processes modulated by the protease web. Biological pathways and processes that are mediated by these substrates of MMP-2 and MMP-14, and that are therefore potentially regulated by these two proteases, are added to panel B. Abbreviations: ADAMTS, disintegrin and metalloproteinase with a thrombospondin type 1 motif; β2M, β2-macroglobulin; CTGF, connective tissue growth factor; HARP, heparin affin regulatory peptide; HB-EGF, heparin-binding epidermal growth factor-like growth factor; HGF, hepatocyte growth factor; IGFBP, insulin-like growth factor binding protein; IL-8, interleukin-8 (CXCL8); MCP-3, monocyte chemoattractant protein-3 (CCL7); MMP, matrix metalloproteinase; MMP-14, membrane type 1 MMP (MT1-MMP); PAI-1, plasminogen activator inhibitor-1; RECK, reversion-inducing cysteine-rich protein with kazal motifs; SDF-1, stromal-derived factor-1 (CXCL12); SLPI, secretory leukocyte protease inhibitor; TFPI, tissue factor pathway inhibitor; TGFβBP1, transforming growth factor β-binding protein 1; TNF, tumor necrosis factor; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor.

is the response of a complex system to the loss or gain of a protease gene that was initiated by the knocked out or overexpressed protease. It is in fact often extremely difficult to ascribe one activity of a cleaved substrate directly to expression levels of the genetically modified protease. Nonetheless, animal models are invaluable for target validation, and for elucidation of the role of each protease, biochemical and pathway validations are required. In view of this interconnectivity and interdependence of components of not only the protease web but also the proteome, it is crucial to fully elucidate the protease web and how it modulates the proteome. Because of the connectivity of the protease web, strategies for modifying the activity of one protease will likely affect others as well as their substrates and their functions. This has been shown using an MMP inhibitor to treat MDA-MB-231 cells, where many proteases and inhibitors of different classes were affected (see Table 2 of ref *83*), as well as a multitude of other proteins (*95*). It has been suggested that mapping these unintended effects allows correction using combination strategies to increase the efficiency and avoid side effects of therapies (*131*).

THE FUTURE

MMPs modulate the activities of a wide range of extracellular and intracellular proteins and thus regulate cell proliferation, adhesion, migration, growth factor bioavailability, chemotaxis, and signaling. These roles can be affected by extracellular matrix protein cleavage, but more importantly by proteolytically modifying the signaling environment of the cell. This can be facilitated by the release of soluble growth factors and cytokines from masking proteins such as insulin-like growth factor binding proteins and pleotrophin, representing a significant emerging class of MMP substrates. Exciting new evidence also indicates many new unexplored roles of MMPs following the cleavage of intracellular proteins located outside of the cell for legitimate and important functions. Thus, MMP activity is crucial for processes such as angiogenesis, tumorigenesis, metastasis, immunity, inflammation, wound healing, and vasodilation. Now there is the potential for a combination of therapeutic interventions directed at many different facets of cancer and inflammation in addition to the therapies for such pathologies that were originally envisioned for MMPs, but this must be done with care given the many new key roles for MMPs. While this makes drug target validation difficult for MMPs, it also reveals the exciting place that MMPs hold at the heart of many physiological and pathological processes.

SUPPORTING INFORMATION AVAILABLE

A semicomprehensive view of proteins that are processed by MMPs (Table 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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