

Protease degradomics: mass spectrometry discovery of protease substrates and the CLIP-CHIP, a dedicated DNA microarray of all human proteases and inhibitors

Christopher M. Overall^{1,2,*}, Eric M. Tam¹,
Reinhild Kappelhoff², Andrea Connor², Tom
Ewart³, Charlotte J. Morrison², Xose Puente⁴,
Carlos López-Otín⁴ and Arun Seth³

¹Department of Biochemistry and Molecular Biology,

²Department of Oral Biological and Medical Sciences,
University of British Columbia, Vancouver, British
Columbia V6T 1Z3, Canada

³Department of Molecular Pathology Sunnybrook and
Women's College Health Sciences Centre, Toronto,
Ontario M4N 3M5, Canada

⁴Facultad de Medicina Instituto Universitario de
Oncología Universidad de Oviedo, Campus El Cristo,
E-33006 Oviedo, Spain

*Corresponding author

e-mail: chris.overall@ubc.ca

Abstract

The biological role of most proteases *in vivo* is largely unknown. Therefore, to develop robust techniques to analyze the protease degradome in cells and tissues and to elucidate their substrate degradomes we have developed a dedicated and complete human protease and inhibitor microarray that we have called the CLIP-CHIP. Oligonucleotides (70-mers) for identifying all 715 human proteases, inactive homologs and inhibitors were spotted in triplicate onto glass slides with a dedicated subarray containing oligonucleotides for specific human breast carcinoma genes. Initial analyses revealed the elevated expression of a number of proteases in invasive ductal cell carcinoma including ADAMTS17, carboxypeptidases A5 and M, trypsin-gamma and matrilysin-2. Matrix metalloproteinases (MMPs) showed a restricted expression pattern in both normal and cancerous breast tissues with most expressed at low levels. However, of the several MMPs expressed in significant quantities, the carcinoma samples showed only slightly elevated amounts other than for MMP-28 which was strongly elevated. To discover new protease substrates we developed a novel yeast two-hybrid approach we term 'inactive catalytic domain capture' (ICDC). Here, an inactive mutant protease catalytic domain lacking the propeptide was used as a yeast two hybrid bait to screen a human fibroblast cDNA library for interactor proteins as a substrate trap. Wnt-induced signaling protein-2 (WISP-2) was identified by ICDC and was biochemically confirmed as a new MMP substrate. In another approach we used isotope-coded affinity tag (ICAT) labeling with tandem mass spectrometry to quantitate the levels of secreted or shed extracellular proteins in MDA-MB-231 breast carcinoma

cell cultures in the presence or absence of membrane type 1-MMP (MT1-MMP) overexpression. By this proteomic approach we identified and biochemically confirmed that IL-8, the serine protease inhibitor SLPI, the death receptor-6, pro-TNF- α and CTGF are novel substrates of MT1-MMP. The utility and quantitative nature of ICAT with MS/MS analysis as a new screen for protease substrate discovery based on detection of cleaved or shed substrate products should be readily adaptable to other classes of protease for assessing proteolytic function in a cellular context.

Keywords: metalloproteinase; MT1-MMP; oligonucleotide chip; proteinase; proteomics; transcriptome.

Introduction

The single most important task confronting protease biology today is understanding the biological roles of all individual proteases. *In vivo*, proteases generally do not function alone but in amplification cascades or regulatory circuits and in the presence of other proteins – whether substrates, binding proteins, inhibitors, other proteases, or proteins of the cellular milieu. In a binary manner, compartmentalization of proteases and substrates also profoundly modifies the biological activities of proteases. Thus, biochemical analysis of proteolytic activity *in vitro* is not sufficient to understand the *in vivo* activities and relevant substrates of proteases. So, although we may predict *in vivo* activities based on knowledge derived from current screens for substrates and can correlate protease expression with substrates by temporal and spatial localization through *in situ* hybridization, immunolocalization and *in situ* activity assays, traditional approaches lack the throughput, coverage and quantitation necessary to achieve a comprehensive understanding of proteolysis *in vivo*. To overcome these obstacles it is necessary to understand proteolysis on a system-wide scale. Hence, it is critical to know in a cell or tissue the expressed protease repertoire, termed the protease degradome (Lopez-Otin and Overall, 2002), all actual substrates of a protease – the substrate degradome – and whether they are cleaved or not in these complex biological milieus. Thus, in degradomics, proteomic and genomic approaches are applied to the study of proteases, their expression and activity, and the identification of protease substrates and inhibitors on a system-wide basis (Lopez-Otin and Overall, 2002).

One of the greatest technical challenges in confronting this issue is the identification of all protease substrates – potential and actual – given that there are more than 553 proteases present in the human genome (Puente et

al., 2003) and that many proteases cleave a number of proteins. Although peptide libraries (Deng et al., 2000; Turk et al., 2001) can determine consensus protease cleavage sequences, few of these approaches have led to the identification of significant numbers of new substrates after data base searches. The major reason for this is that substrates *in vivo* are generally not denatured proteins or peptides, but are folded proteins which limits protease susceptibility because of protein conformation and post-translational modification (Lopez-Otin and Overall, 2002). Critically, analysis of peptide sequences does not proffer information on exosites. Since exosites are substrate binding sites lying outside the active site cleft (Overall, 2001), the primary structure information in peptide libraries does not convey the more complex three-dimensional information nor the sequences of exosites that lie at a distance from the scissile bonds.

Cognisant of the limitations of peptide libraries we have previously developed a genetic technique to discover novel substrates by substrate trapping in the yeast two hybrid system. Recognizing that substrate binding to exosites outside the catalytic domain of proteases markedly improves catalytic efficiency (Overall, 2001), we used the hemopexin C domain of MMP-2 (gelatinase A) as an exosite bait for binding proteins that may be substrates (McQuibban et al., 2000). Termed 'exosite scanning' (Lopez-Otin and Overall, 2002; Overall et al., 2002), this was the first use of the yeast two hybrid system to discover protease substrates. However, not all proteases or substrates utilize exosites and a deficiency of yeast two hybrid screens is that protease catalytic domains cannot be used as bait since cleavage of a library protein in the yeast would not generate a transcriptional signal and so would not be detectable by this screen.

The best approach to identify substrates *in vivo* or in cells on a system-wide basis is to quantitatively identify the cleaved substrate degradome, which can then also be used as evidence of proteolytic activity *in vivo*. However, the labeling, detection and identification of neo N-termini generated after proteolysis amidst a vast background of primary and secondary amines is technically not yet possible. Moreover, the identification of cleaved substrates that may be present only transiently before further processing or clearance of unstable products, particularly for temporally rare or low abundant proteins, is very difficult by current procedures. Nonetheless this has been done by biochemical extraction or the use of neopeptide detection by antibodies specific for the new N- and C-termini of the cleaved substrates. Cleavage fragments of collagen have been extracted from tissue (Overall, 1987) and identified by neopeptide antibodies (Hughes et al., 1995; Billingham et al., 1997) directly showing the activity of the matrix metalloproteinase (MMP) collagenase *in vivo*. Other examples of the use of neopeptide antibodies to identify *in vivo* activities of MMPs and aggrecanases include those generated toward the proteoglycan aggrecan (Lemons et al., 2001) and the chemokine monocyte chemoattractant protein-3 (MCP-3) in arthritic synovial fluids (McQuibban et al., 2000). Although two-dimensional gel analysis followed by peptide fingerprinting or tandem mass spectrometry (MS/MS) allows for the identification of proteins from complex

biological samples, substrate identification is problematic as it is difficult to relate cleavage products with parent protein spots on 2D gels. Moreover, sensitivity still sets limits and so rare, but important, proteins that cannot be detected on gels will be missed by these procedures. By labeling proteins with stable isotope reagents, most notably isotope coded affinity tags, ICAT (Gygi et al., 1999), protein identification and comparative quantitation of protein levels by MS/MS can be made between experimental and control samples (see Figure 1). However, to date, there have been only two studies using proteomic approaches for protease substrate identification (Guo et al., 2002; Shen et al., 2003).

By comparison, transcriptome analysis of the expression patterns of all proteases and inhibitors is relatively easy and can be achieved today. Although DNA microarray technology and real time RT-PCR will be able to address this, no dedicated protease microarray chips have been developed that contain oligonucleotide or DNA probes for every human protease to date. Likewise, complete sets of RT-PCR probes for human proteases have yet to be developed. A similar status exists for protease inhibitors. But understanding the protease transcriptome is only part of the story – translation efficiency, secretion and trafficking, zymogen activation, autolysis, compartmentalization and regulation by inhibitors profoundly affects net activity. Hence, transcriptome analysis is of limited use in and by itself, but should be informative for addressing specific experimental questions. Profiling protease activities using active site probes reveals levels and the distribution of active proteases in tissues or cells statically or by real time *in vivo* imaging, but relatively few probes exist for cysteine (Greenbaum et al., 2002), serine (Liu et al., 1999) and threonine proteases (Nazif and Bogoy, 2001) and none have been reported for aspartate and metalloproteinases which rely upon a nucleophilic water molecule for catalysis and so are difficult to target irreversibly.

In this report (presented at the International Proteolysis Society 3rd Annual Meeting, Nagoya, Japan, November 2003) we describe how we have designed a dedicated 70-mer oligonucleotide microarray chip for all human proteases and inactive homologs, and protease inhibitors. We also review our other recently devised strategies of substrate discovery using new yeast two hybrid and proteomic approaches. Our aim was to develop a generally applicable high-throughput proteomic technique for protease substrate discovery on a system-wide basis that detects cleaved proteins and proteins proteolytically shed from the cell surface in complex biological samples conditioned by cells or tissues. In our first use of proteomics for substrate discovery (Tam et al., 2004) we utilized the power of ICAT labeling and MS/MS to identify differences in the levels of proteins that were degraded or processed in the pericellular matrix and medium, or shed from the cell surface of breast carcinoma cells transfected with a cell surface membrane type (MT) MMP (MMP-14, also termed MT1-MMP). MT1-MMP activates MMP-2 (gelatinase A; Sato et al., 1994; Morrison et al., 2001) and cleaves native type I collagen, fibronectin and other matrix proteins *in vitro* (Ohuchi et al., 1997; Tam et al., 2002) and so is important for normal growth and

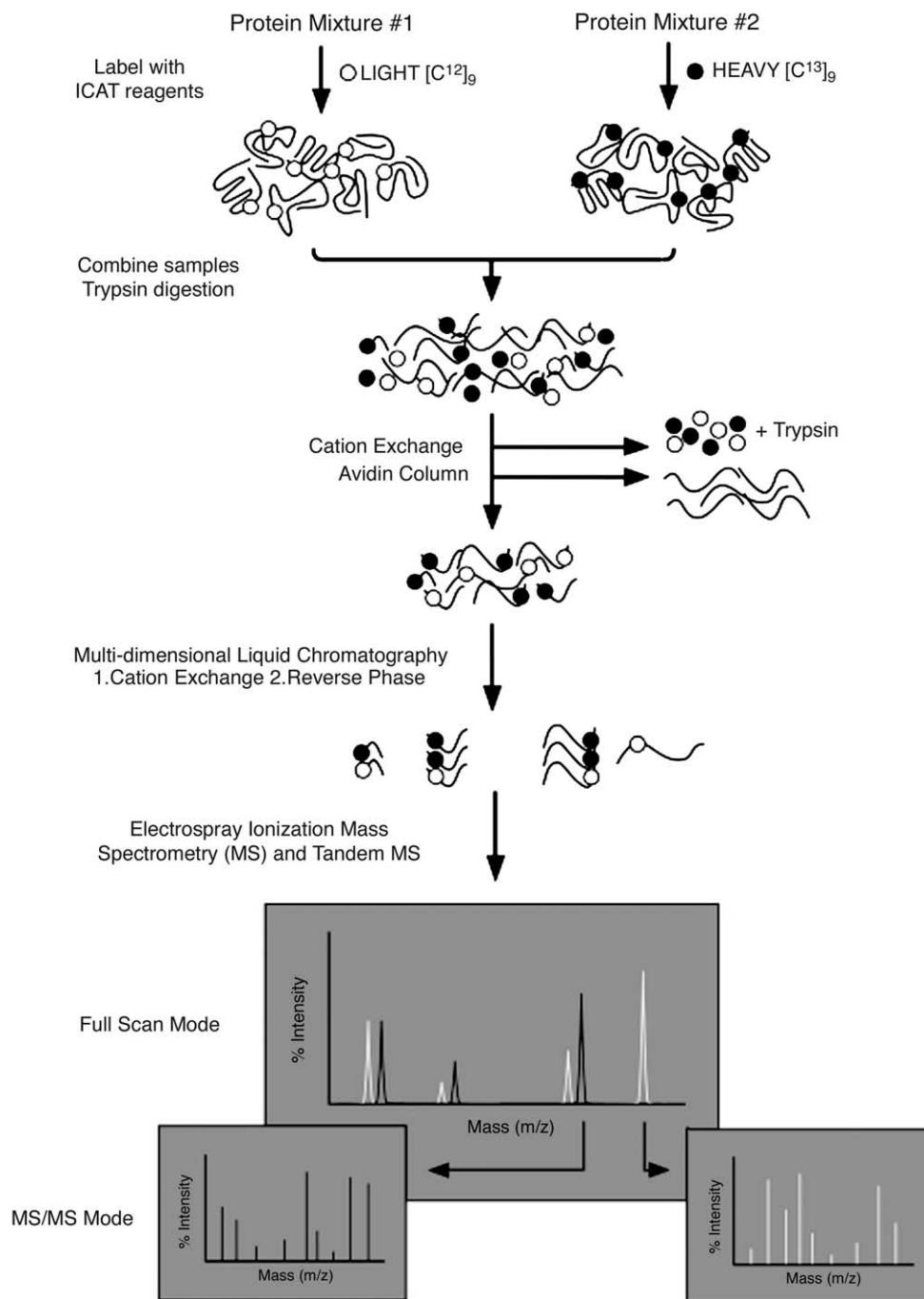


Figure 1 Schematic of sample preparation and analysis by multi-dimensional liquid chromatography ICAT tandem mass spectrometry.

development (Holmbeck et al., 1999) and in disease (Yamanaka et al., 2000; Egeblad and Werb, 2002; Seiki, 2003). MT1-MMP also processes the chemokines SDF-1 and MCP-3 (McQuibban et al., 2001, 2002), but the true *in vivo* substrates of MT1-MMP are largely unknown. For these reasons we studied MT1-MMP as a model cell surface protease to evaluate and validate this new proteomic technique.

Results

CLIP-CHIP

Hybridization analyses were performed using the human

CLIP-CHIP comparing protease and inhibitor expression from normal human mammary epithelium with invasive ductal carcinoma (Figure 2C). Table 1 presents a comparison of proteases and inhibitors comparing expression levels in the paired samples. Fluorescent signal strength >2000 is considered to show highly significant transcript expression. Representative data from one such pair highlighting the MMP section of the CLIP-CHIP is shown in Figure 2D. The normalized signal intensity, which corresponds to the amount of transcripts, was significantly elevated for MMP-2, 9, 15, 16, 17, 23, 24, 25 and 28 for both normal and cancer tissue. However, there was a trend of elevated MMP expression in the tumor samples. The other 14 MMPs (1, 3, 7, 8, 10, 11, 12, 13,

14, 19, 20, 21, 26, 27) were also expressed at low amounts.

Inactive catalytic domain capture

To discover protease substrates we have developed a new yeast two hybrid approach termed 'inactive catalytic domain capture' (ICDC) that utilizes as bait a mutated inactive catalytic domain as a substrate trap that binds but does not cleave substrates (Figure 3A; Lopez-Otin and Overall, 2002). We hypothesized that an inactive catalytic domain minus the zymogen propeptide would stably bind substrates but neither cleave nor release the protein and so would generate a transcriptionally active complex in the yeast nucleus. Screening of a human fibroblast cDNA library by the yeast two hybrid system using an inactive catalytic domain of MT1-MMP minus the propeptide as bait resulted in a number of positive clones identified – one of which was Wnt induced signaling protein-2 (WISP-2), a member of the CTGF family (Pennica et al., 1998). A recombinant protein, VTSP(Gly¹⁶⁴-Phe²⁵¹), representing the variable and thrombospondin modules of WISP-2 was cleaved by sMT1-MMP at two sites (Figure 3B). Both cleavages occurred within the variable domain, which connects the C-terminal half of WISP-2 to a N-terminal von Willebrand factor type-1C domain, and were identified as Gln¹⁷⁸-Gly¹⁷⁹ and Gly¹⁸⁴-Leu¹⁸⁵ by N-terminal sequencing and MALDI-TOF mass analyses (Figure 3B). To confirm these cleavages, full length WISP-2 was generated and incubated with sMT1-MMP and MMP-2. Cleaved proteins were analyzed by SDS-PAGE and Western blotting using a polyclonal antibody that recognizes a C-terminal epitope. As shown in Figure 3C, incubation of full length WISP-2 with sMT1-MMP resulted in the loss of the C-terminal epitope. In addition, MMP-2 was also found to cleave WISP-2 (Figure 3C).

ICAT and MS/MS analysis of extracellular proteins

The average relative abundance ratios for proteins found in the conditioned media were determined by ICAT for the experimental pairs of MDA-MB-231-transfected cells, MT1-MMP/vector and MT1-MMP/E240A, respectively. In our analysis we hypothesized that secreted proteins in the conditioned medium may be reduced in amount if degraded or processed by MT1-MMP and then subsequently cleared following reductions in stability. These proteins would have an ICAT ratio of labeled peptides from MT1-MMP:control < 1.0. In contrast, proteins may

increase in quantity in the conditioned medium if proteolytically shed from the cell surface or if they were released from the pericellular matrix. Such proteins would have an ICAT ratio of MT1-MMP:control > 1.0. Proteins with an average ICAT ratio in the 0.5–1.5 range were not considered to be significantly different and so only proteins outside this range were further analyzed. Analysis of the MT1-MMP/vector pair revealed that 47% (12% > 1.5; 35% < 0.5) of the 298 identified proteins showed altered levels upon MT1-MMP overexpression. In the MT1-MMP/E240A pair, 66% (5% > 1.5; 61% < 0.5) of the 173 proteins identified were so altered. Without inline MD-LC fractionation, the number of proteins identified was typically reduced to less than 100. A significant proportion of proteins identified were of intracellular origin – a reflection of the cell death that occurred in the 48-h serum-free media conditions necessary for optimizing proteolytic activity – and were not further analyzed. A selective list of potential MT1-MMP substrates was generated from the two data sets, which included protease inhibitors (SLPI and skin-derived antileukoproteinase), chemokines (IL-8, GRO- α , GRO- γ and macrophage migration inhibitory factor), cytokines (TNF- α and CTGF), cell receptors (death receptor-6 and neuropilin-1), latent transforming growth factor binding protein-4S, complement component-3, but very few extracellular matrix proteins (fibronectin and EGF-containing fibulin-like extracellular matrix protein-1). Shed MT1-MMP and MMP-1 (collagenase-1) were also elevated in the MT1-MMP transfectant cell medium, while TIMP-1 levels were unaltered.

Identification of new MT1-MMP substrates

IL-8, GRO- α and GRO- γ , members of the CXC family of chemokines, showed decreased levels upon MT1-MMP overexpression (ICAT ratio 0.34, 0.37 and 0.27, respectively). Incubation of IL-8 with MT1-MMP resulted in the removal of the first five residues as shown by MALDI-TOF and confirmed by Edman sequencing, which showed that the new N-terminus started at SAKELR (Figure 4A). By electrophoresis and MALDI-TOF analysis, no change in mass was detected for GRO- α and GRO- γ following sMT1-MMP incubation (not shown) indicating that an indirect effect of cell surface expression of MT1-MMP resulted in the decreased secretion of these two chemokines. Complement component-3, which displayed a similar ICAT ratio to GRO- γ , was also not susceptible to MT1-MMP proteolysis *in vitro* (not shown). In contrast, levels of the secretory leukocyte protease inhibitor, SLPI, were greatly increased upon MT1-MMP over-

Figure 2 The human protease CLIP-CHIP.

(A) Overview of the CLIP-CHIP microarray layout. The human microarray is in duplicate. In addition a dedicated subarray is included with 217 breast cancer related, prognosis and 192 control oligonucleotides (left side). (B) Proteases are arranged in their respective class. Thus, the microarray contains oligonucleotides for 21 aspartic proteases (Asp), 148 cysteine proteases (Cys), 184 metalloproteases (Metallo), 177 serine proteases (Ser), 28 threonine proteases (Thr) and 157 inhibitors (Inh), as well as 46 positive controls and 7 negative controls (light areas of the array). (C) Representative expression pattern of proteases and inhibitors in normal human breast tissue (only the cyanine 3 channel is shown). The white lines show the division of the five classes of proteases and inhibitors on the array. The color bar key reflects transcript expression levels; black, no expression, to white, high expression. (D) Normalized signal intensities of the 23 human MMP transcripts expressed in normal human breast tissue cells (normal, white) and invasive ductal breast carcinoma cells (cancer, black) from a third patient.

Table 1 Signal intensities of proteases and inhibitors highly expressed in normal human breast tissue cells (Normal 1, Normal 2) and invasive ductal breast carcinoma biopsies from two patients (IDC 1, IDC 2) analyzed on the CLIP-CHIP.

Class	Protease	Normal 1	IDC 1	Normal 2	IDC 2
Aspartic	Pepsin C	64 000	64 756	27 000	25 460
	Cathepsin D	65 000	65 002	27 500	29 120
Cysteine	Ubiquitin C-term. Hydrolase BAP1	20 077	22 235	0	5916
	Caspase-8	21 121	23 723	0	6300
	USP22	35 216	34 739	31 388	20 559
	USP40	36 904	32 863	18 424	8600
	Sentrin/SUMO protease-8	32 850	30 488	39 156	21 646
	Cezanne-2	0	22 236	0	21 060
Metallo	MMP28	9392	17 253	10 307	14 200
	ADAM30	12 098	0	38 877	21 658
	ADAMTS7	16 048	18 457	54 231	31 975
	ADAMTS17	10 095	19 326	32 566	28 476
	Carboxypeptidase A5	17 783	34 066	37 436	29 186
	Carboxypeptidase M	15 853	30 530	9540	18 800
	Mitochondrial processing protease	10 517	0	30 183	13 600
	Aminopeptidase-like 1	12 880	28 981	48 155	0
	Glu-carboxypeptidase-like 1	45 817	65 039	65 298	65 084
	Methionyl-aminopeptidase-like 1	13 914	22 734	32 079	19 003
	Serine	Kallikrein hK4	27 039	31 006	0
Tryptase γ		16 359	31 245	0	46 262
Matriptase-2		21 665	52 752	45 452	59 200
Pancreatic endopeptidase E (B)		25 081	34 107	0	48 282
Plasminogen		43 514	30 252	65 448	65 535
Threonine	Proteasome catalytic subunit 1i	10 302	0	3366	0
	Proteasome β 4 subunit	34 369	10 909	8355	23 147
	γ -Glutamyltransferase-1	65 440	32 568	42 873	65 535
	γ -Glutamyltransferase-2	0	6634	0	0
Inhibitors	Protease Inhibitor 9/CAP3	0	0	23 623	0
	Yukopin (SerpinB12)	23 387	20 377	62 902	24 169
	Antithrombin III	20 259	13 389	37 993	22 475
	Antileukoproteinase	18 396	18 696	31 798	21 702
	WAP four-disulfide core like 1	22 167	26 509	43 752	18 740
	Cystatin SA	0	0	23 451	40 947
	IGF binding protein-2	11 668	24 800	35 163	28 026
	Pregnancy zone protein	13 788	19 918	48 198	18 973
	Histatin 3	0	26 400	0	33 642

Normal1/IDC1 and Normal2/IDC2 sample pairs were hybridized together on the same slide. IDC1 was diagnosed as having estrogen receptor⁺, progesterone receptor⁻ mucinous carcinoma and invasive ductal carcinoma with <5% ductal carcinoma *in situ*; IDC2 was diagnosed as having estrogen receptor⁺, progesterone receptor⁺ invasive ductal carcinoma with ductal cell carcinoma *in situ* also present.

expression (ICAT ratio 4.95). We confirmed that SLPI was a novel substrate of MT1-MMP in *in vitro* assays. sMT1-MMP cleaved SLPI in a TIMP-2-inhibitable manner to generate a single product with an N-terminus of SGKSFKA that is identical to the intact inhibitor indicating that cleavage occurred near the C-terminus of SLPI (Figure 4B), where Leu72 is the S₁'-interacting inhibitory residue (Grutter et al., 1988). Potentially, cleavage in the C terminus not only inactivates SLPI but also releases SLPI bound to the cell surface or pericellular matrix to the medium.

Fibronectin, a known substrate of MT1-MMP, was also identified by ICAT and MS/MS to be in greater abundance in the medium upon MT1-MMP overexpression

(ICAT ratio 3.04) implying that MT1-MMP may shed fibronectin from the cell surface. Once again fibronectin shedding was specifically inhibited by TIMP-2, but not TIMP-1, confirming a requirement for MT1-MMP proteolysis (not shown). Although cleavage of fibronectin cannot be discounted as a shedding mechanism, cleaved fibronectin fragments were not detected using an antibody that recognizes the 15th type III module of fibronectin. Moreover, peptides ⁶⁷TYLGNALVCTCYGGSR⁸⁴ and ²¹⁹⁹LLCQCLGFGSGHFR²²¹⁴ found proximal to the N- and C-termini of the molecule, respectively, were identified by MS/MS and had similar ICAT ratios. Hence, the solubilized form of fibronectin was intact, indicating that cleavage of a fibronectin-binding cell surface molecule was

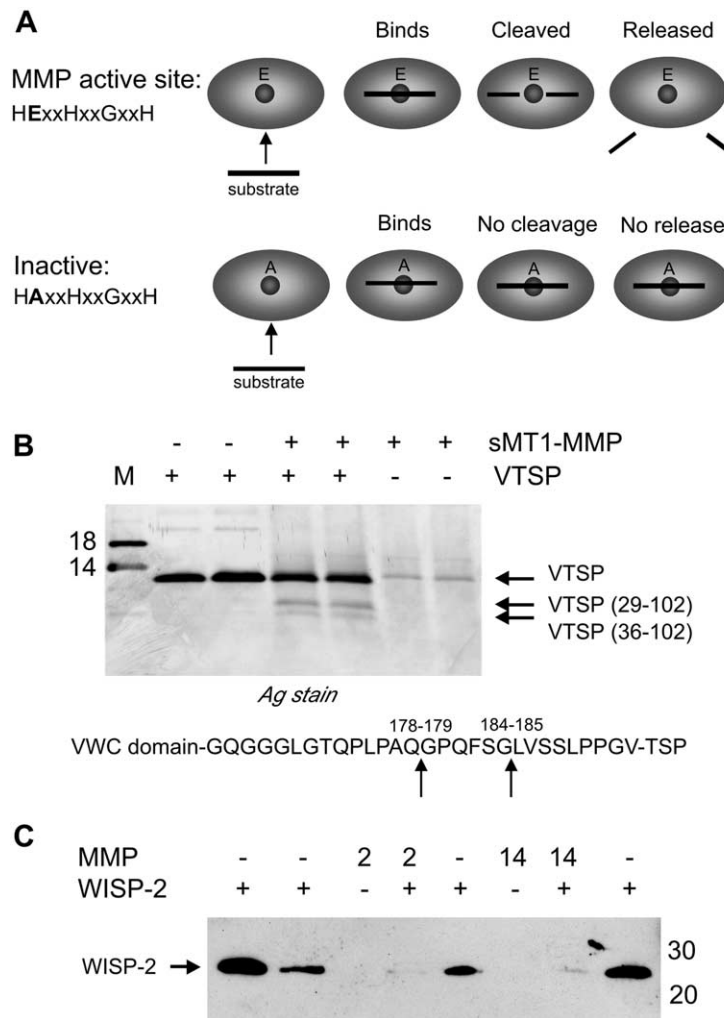


Figure 3 Inactive catalytic domain capture.

(A) Schematic of the ICDC principal using a MMP inactive catalytic domain as a yeast two hybrid bait. (B) SDS gel electrophoretic analysis of MT1-MMP cleavage of recombinant WISP-2 protein comprised of the variable and thrombospondin domains (VTSP) stained with silver nitrate after digestion and electrophoretic analysis. Sites of cleavage are indicated. (C) Western blot analysis of full length WISP-2 digestion by MMP-2 and MT1-MMP (MMP-14). Size of molecular mass markers are as labeled.

the mechanism responsible for fibronectin release and increase in medium levels that was revealed by the MS/MS analyses.

CTGF, a member of the CCN (CTGF/cysteine-rich 61/nephroblastoma overexpressed) family of extracellular matrix-associated signaling molecules (Brigstock, 1999) displayed an ICAT ratio of 1.74 and was cleaved by sMT1-MMP into 3 distinct lower molecular mass fragments (Figure 4C). N-terminal sequence analysis of the 17.5 and 16.2-kDa fragments revealed an identical N-terminus, YRLEDT, corresponding to cleavage at the Ala¹⁸¹-Tyr¹⁸² bond. The inflammatory cytokine, TNF- α , showed an ICAT ratio of 1.29, below our arbitrary cutoff. However, since pro-TNF- α can be shed by MMP-7 (Haro et al., 2000), we investigated the susceptibility of pro-TNF- α to MT1-MMP processing using a GST-pro-TNF- α fusion protein. sMT1-MMP cleavage of the fusion protein generated a product corresponding to the correct size of mature TNF- α (approximately 16 kDa; not shown). N-terminal sequencing revealed that the main site of cleavage was at the Ala⁷⁶-Val⁷⁷ bond, which generates the fully active TNF- α . Conditioned medium levels of death receptor-6, a member of the TNF receptor family with

unknown ligands (Pan et al., 1998), were increased upon MT1-MMP overexpression. A death receptor-6 fusion protein, DR6/Fc, consisting of a 350-residue ectodomain fused to the Fc region of IgG, was processed by sMT1-MMP (Figure 4D). Cleavage occurred within the ectodomain and not the Fc portion of the fusion protein as shown by first treating DR6/Fc with Factor Xa, to cleave a susceptible site between the ectodomain and the Fc region, prior to digestion with sMT1-MMP.

Discussion

The development of the CLIP-CHIP represents the first complete dedicated human protease and inhibitor DNA microarray chip reported to date. As such it will prove to be an extremely valuable and versatile tool for the genomic analysis of proteolytic function *in vivo*. The advantage of using a custom glass chip is that new protease or inhibitor oligonucleotides can be added in an agile manner at each chip printing for minimal cost. Thus, the massive parallel analysis of protease expression is not only possible now, but also readily adaptive with the

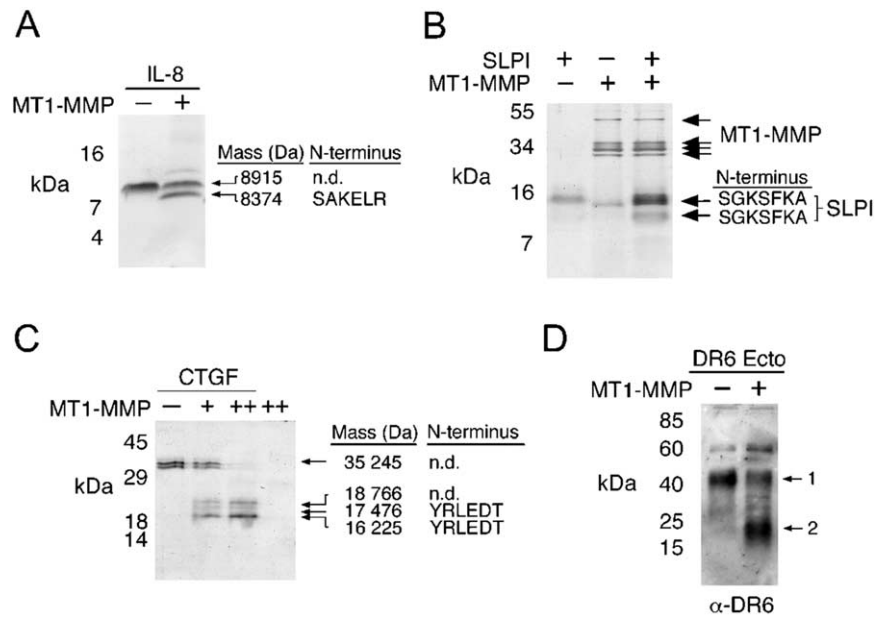


Figure 4 MT1-MMP processing of potential substrates identified from ICAT.

(A, B) IL-8 and SLPI were incubated with sMT1-MMP for 18 h at 37°C. Samples were then separated on 15% Tris-tricine gels under non-reducing conditions. Proteins were visualized by Coomassie blue or silver nitrate staining. (A) The mass and sequence of cleaved and uncleaved IL-8 was determined using MALDI-TOF MS and N-terminal Edman sequencing. (B) SLPI cleavage was inhibited by TIMP-2. N-terminal sequences of cleaved and uncleaved SLPI are shown. (C) CTGF was treated with sMT1-MMP (+,++) for 18 h at 37°C and analyzed by SDS-PAGE (15%) and MALDI-TOF MS. The MT1-MMP cleavage site was determined by N-terminal sequencing of the cleaved CTGF fragments. (D) The ectodomain of DR6 was incubated with sMT1-MMP (18 h, 37°C). Samples were electrophoresed by SDS-PAGE (15%) and Western blotted using α-DR6 antibody. Uncleaved (1) and MT1-MMP cleaved (2) DR6 are indicated.

inclusion of any newly identified proteases and inhibitors possible. Moreover, having a dedicated subarray containing oligonucleotides relevant to the experimental question being addressed further focuses the experiment without distraction from analyses of many thousands of genes that is possible in larger arrays. Work is currently in progress to design and validate oligonucleotides for the complete murine protease degradome. We will replace one human array with the complete murine protease and inhibitor array in order to examine protease contributions from human xenograft cells and tissues in murine mouse models by hybridization on the same chip. Until then laser dissection microcapture will be necessary to analyze the stromal versus tumor source of proteases in human breast carcinoma.

The preliminary results to date indicate a restricted pattern of MMP expression in human breast cancer tissues. Even though 21 of the 23 human MMPs appear to be more highly expressed in samples of breast cancer tissue, most were only expressed at a low level as evident by low signal intensities. The nine MMPs (MMP-2, 9, 15, 16, 17, 23, 24, 25, 28) that were present in higher amounts were generally expressed in the cancer tissues at higher levels. Thus in general, our data confirm a general but restricted up-regulation of MMPs in cancer. However, since whole biopsies were analyzed the stromal versus tumor cell source of proteases is not revealed by these analyses.

In substrate discovery, one approach alone will not be able to provide complete coverage of the protease substrate landscape. Genetic and proteomic approaches are complementary with each having their distinct advantages and disadvantages. Here we have expanded our yeast

two hybrid approaches by developing a new screen to complement exosite scanning (McQuibban et al., 2000). Termed ICDC, we used the inactive catalytic domain of MT1-MMP to trap substrates in a yeast two hybrid screen. As proof of principle of this novel approach WISP-2, a potential oncogene (Pennica et al., 1998) was identified as an interactor that we confirmed was a novel substrate of MT1-MMP. Although the function of WISP-2 is unknown, based on homology with the structurally homologous CTGF, cleavage in the variable domain is likely to be an inactivating cleavage (Brigstock, 2003) – in addition to leading to release of the protein from the cell membrane or pericellular matrix. Definitive analysis of the effect that MMP proteolysis has on this signaling molecule awaits further characterization that is currently underway in our laboratory. Thus, exosite scanning and ICDC yeast two hybrid screens have the potential to identify novel protease substrates present at low levels in tissues or cells that are more readily detectable following genetic amplification and that may be missed by other approaches. Notably, we used protein domains that are normally disulfide-containing as yeast two hybrid baits. Together with the many ‘hits’ found of proteins that also contain disulfide crosslinkages when normally expressed in the extracellular environment, these novel approaches confirm our previous validation of the yeast two hybrid system for screening of interactions involving extracellular disulfide-containing protein domains that appear to adopt a functionally equivalent biological fold at the lower temperatures used for yeast growth compared to mammalian body temperatures.

Despite the utility of yeast two hybrid approaches to search for substrates, in particular for temporally rare or

otherwise low abundant proteins, more rapid techniques of substrate discovery on a system-wide basis are needed with the capability of identifying and quantitating substrates directly from cells, tissues and body fluids. Proteomic approaches for the identification of protease substrates have only slowly been adopted due, in part, to the difficulty in assigning cleavage products to MS peaks or to relating cleavage products with parent protein spots on 2D gels. In comparison to gel-based analyses, mass spectrometric-based approaches provide more extensive and rapid proteome coverage. By using ICAT to label protein in control and proteolyzed samples that were then identified by MD-LC MS/MS, we have developed a new, rapid degradomic screen for protease substrate discovery. In MDA-MB-231 cells, we identified several proteins that were biochemically confirmed as novel substrates of MT1-MMP thereby validating the utility of this approach for substrate degradomics.

IL-8, a potent neutrophil chemoattractant, belongs to the CXC chemokine family which contain the essential ELR motif near the N-terminus (Belperio et al., 2000). MT1-MMP was found to cleave IL-8 at the N-terminus to remove the first five residues to generate IL-8 (6–77), which is more potent than the untruncated form (Van Damme et al., 1989). This cleavage is selective as MT1-MMP did not cleave the ELR-positive CXC chemokines GRO- α and GRO- γ , whereas MMP-9 is known to cleave both IL-8 and GRO- α (Van den Steen et al., 2000). Therefore, the reduction in the levels of GRO- α and GRO- γ appears to be an indirect response to the overexpression of MT1-MMP. The serine protease inhibitor, SLPI, was cleaved by MT1-MMP to generate a single fragment with an identical N-terminus as the full-length molecule. This indicates that MT1-MMP processing occurred proximal to the inhibitory C-terminal region (Grutter et al., 1988). Previously, it was shown that SLPI was resistant to MMP-8 proteolysis (Henry et al., 2002). Thus, our studies reveal a novel MMP substrate in which the activity of a serine protease inhibitor is predicted to be abrogated upon MT1-MMP proteolysis. Potentially, elevated MMP expression may lead to elevated serine protease activity. This is now under active investigation in our laboratory.

Overexpression of MT1-MMP activity also led to shedding of intact fibronectin from the MDA-MB-231 cell surface rather than being degraded despite fibronectin being a known substrate of MT1-MMP (Ohuchi et al., 1997; Tam et al., 2004). This not only shows that proteomic data should be confirmed by biochemical and cell based investigations but highlights the importance of confirming biochemical data with *in vivo* based investigations. The mode of fibronectin release is not clear. It has been reported that MT1-MMP degrades transglutaminase, a fibronectin co-receptor, and reduces the adhesion and migration of cells on fibronectin (Belkin et al., 2001). MT1-MMP also cleaved the ectodomain of death receptor-6, a new member of the TNF receptor family that regulates apoptosis through its cytoplasmic death domain and activation of the TRADD/FADD/caspase-8 pathway (Pan et al., 1998). Death receptor 6 is an orphan receptor since specific ligands have not been identified. Ligands that bind death receptors belong to the TNF family of cytokines, of which TNF- α is the most

characterized. ADAM-17 is the enzyme thought to be primarily responsible for the generation of mature TNF- α from a membrane bound precursor (Black et al., 1997; Moss et al., 1997). However, MMP-7 can also process TNF- α (Haro et al., 2000). Here, we show that MT1-MMP cleaves pro-TNF- α at the Ala⁷⁶-Val⁷⁷ bond to generate mature, fully active TNF- α . Thus, MT1-MMP may also be involved in generating proinflammatory signals that are executed by TNF- α under certain circumstances on the cell surface.

CTGF was also identified by ICAT as a potential MT1-MMP substrate which we then showed was cleaved by MT1-MMP at the Ala¹⁸¹-Tyr¹⁸² bond, a position found within the linker region between the von Willebrand factor type-1C domain and the thrombospondin-1 domains of CTGF (Brigstock, 1999). This site is proximal to the Met¹⁹⁴-Ile¹⁹⁵ bond which is cleaved by soluble MMPs 1, 3, 7 and 13 (Hashimoto et al., 2002). *In vivo*, CTGF is unstable and is converted into low molecular weight forms (10–20 kDa) by unidentified cell surface proteases (Ball et al., 1998), a result which is consistent with our data demonstrating that MT1-MMP can process CTGF.

Protein quantification and identification by ICAT and MS/MS is a powerful technique that we have adapted for the proteomic investigation of protein shedding and degradation and protease substrate discovery on a system-wide scale. However, differences in protein abundance as measured by ICAT must be interpreted with caution since changes in protein expression induced by the transfected gene are also detected – an important consideration for interpreting other studies using transfected cells. Approaches such as this are also providing us with a critical stepping stone to the development of new proteomic approaches for the more difficult task of directly detecting cleaved proteins in complex biochemical samples or living cells and tissues by targeting, labeling and identifying the neo N-termini of cleaved products against the vast background of N-termini of full length proteins and secondary amino groups of lysine residues. This technique we term ‘CLIP-TAILS’ (Terminal Affinity Isotope Labeling of Substrates) is currently in development in our laboratory for direct mass spectrometric identification and quantitation of substrate cleavage in complex samples by specific neo N-terminal tagging, affinity purification, quantitation and identification of cleaved protein substrates and the scissile bond in the same experiment.

Proteases regulate many aspects of protein function including activity, localization, shedding, exposure of cryptic binding sites and release of neoproteins thereby achieving precise control over many cellular processes. The CLIP-CHIP will extend our knowledge of the expression patterns of proteases on a system-wide basis. Together with elucidating the substrate degradome of a protease this should facilitate our understanding of the role of proteases *in vivo*. By establishing the physiological function of a protease, its role in pathology can be more readily ascertained which is a necessary prerequisite for drug target identification. Traditionally, the MMPs were viewed as enzymes of catabolism being primarily involved in the degradation and turnover of the extracellular matrix. However, MMPs are now known to process a diverse range of extracellular proteins other than those

of the matrix (Egeblad and Werb, 2002). The novel substrates identified here reveal MT1-MMP to be an important cell signaling protease and further supports our view that MMPs are critical processing proteases rather than just nonspecific degraders of matrix. The effect of cleaving CTGF, TNF α and other growth factors would reduce extracellular matrix synthesis and formation. Thus MMPs exert potent control on the extracellular matrix by acting in concert to desassemble matrix by reducing synthesis to augment any matrix degradative role MMPs may perform.

Materials and methods

Protease and inhibitor oligonucleotides

Using public and private databases we recently annotated and compiled the complete list of human proteases and inhibitors from mouse and man (Puente et al., 2003). In humans a total of 552 genes encode proteases, 72 more than annotated in MEROPS v6.2. In addition, we have compiled a list of 157 human protease inhibitors and more recently identified six new proteases in the database. Using this information we designed a oligonucleotide microarray for every known human protease and inhibitor that we have called the CLIP-CHIP. Oligonucleotides (571), synthesized as unique 70-mers, for each protease, inactive homolog and inhibitor were obtained from the Human Genome Oligo Set v2.0 (Qiagen, Mississauga, ON, Canada). Oligonucleotides for genes not represented in the Human Oligo Set (144) were synthesized after extensive BLAST analyses to identify unique sequences. Each oligonucleotide utilized a 5'-C6 amino linker for attachment to an epoxy silane glass matrix. Forty six positive control oligonucleotides (representing house keeping genes) and 7 negative controls (synthetic oligonucleotides with no cross reactivity to any known human sequence) from the Human Genome Oligo Set 2.0 were included in the microarray. Additional controls were spotted in a subarray containing 192 positive, 80 negative and 16 spiking oligonucleotides (Ambion, Austin, TX, USA) for normalization.

CLIP-CHIP microarray

Oligonucleotides (75 pmol) were dissolved in 15 μ l printing buffer (3 \times SSC, 0.1% SDS) and were spotted onto a glass surface coated with epoxy silane (SpotArray, Perkin Elmer, Boston, MA, USA). Each oligonucleotide was spotted in triplicate and the array was printed in duplicate onto the glass slide. The CLIP-CHIP is designed with oligonucleotides for proteases arranged logically into families and divided into the five protease classes and inhibitors (Figure 2A, B). A dedicated subarray is included in the design of the CLIP-CHIP for gene expression analysis of targeted genes of interest according to experimental needs. Thus, v1.2 of the CLIP-CHIP includes oligonucleotides specific for 217 breast carcinoma-associated genes of interest and 192 control oligonucleotides for normalization. Before use, any unreacted groups from the epoxy silane surface of the glass slides were blocked with 100 mM ethanolamine, pH 8 for 2 h.

mRNA sample preparation, hybridization and analysis

Total RNA was prepared from frozen biopsy sections of patients with invasive ductal carcinoma in the Sunnybrook and Woman's College Health Science Centre Breast Carcinoma Collection, with signed patient consent. Normal human breast tissue total RNA was isolated as a mixture from breast tissue including

gland, fat and fibrillar cells. mRNA was isolated from total RNA preparations by extraction with Trizol (Invitrogen, Burlington, ON, Canada) using DynaBeads (Dyna, Oslo, Norway). cDNA (500 ng) from normal and tumor tissues was generated by reverse transcription (LabelStar, Qiagen, Mississauga, ON, Canada) substituting amino-allyl dUTP (Sigma, Oakville, ON, Canada) for dTTP. In a following reaction NHS-ester cyanine 3 or cyanine 5 (Amersham, Piscataway, NJ, USA) were coupled to the amino-allyl-dUTP. After purification equal amounts of cyanine 3- and cyanine 5-coupled cDNA were hybridized to the CLIP-CHIP in hybridization buffer containing 5 \times SSC and 0.3% SDS for 16 h at room temperature in the dark. Following hybridization the slides were washed in 2 \times SSC, 0.2% SDS for 15 min and then in 0.1 \times SSC for 15 min at 42°C. The microarray slides were dried by centrifugation (5 min, 500 g) and read in an Affymetrix 428 (MWG, High Point, NC, USA) microarray laser scanner using 532 nm for the cyanine 3 channel and 635 nm for the cyanine 5 channel. Gains were set individually for each channel. The tagged image files of both channels were used for normalization and analysis using Jaguar 2.0 software (MWG, High Point, NC, USA). The signals from the cyanine 3 and cyanine 5 channels were normalized using the signals of the control oligonucleotides in the subarray for calculation of the normalization factor. Data for each gene analyzed were calculated as the mean of the triplicate spots for each of the duplicate arrays per slide (n=6 total).

Yeast two hybrid

Saccharomyces cerevisiae strain HF7c containing HIS3 and LacZ reporter genes were transformed with plasmids vectors pGBT9 and pGAD424 (BD Biosciences Clontech, Palo Alto, CA, USA) containing the cDNA sequences for the fusion proteins with Gal4 DNA binding domain and the Gal4 activation domain (McQuibban et al., 2000). To these Gal4 protein domains were tethered the proteolytically inactive (Glu240Ala) human catalytic domain of MT1-MMP (Tyr111-Gly284) lacking the propeptide and terminating before the linker and hemopexin C domain and transmembrane tether. A cDNA library was fused to the Gal4 activation domain as described previously (McQuibban et al., 2000). The efficacy of the fusion protein constructs was demonstrated by automated sequencing of both cDNA strands. Yeast cells were plated on appropriate selective medium and tested for expression of reporter genes. β -galactosidase activity resulting from up-regulation of the LacZ reporter gene was quantitated by a colorimetric assay as detailed in the Matchmaker Two-Hybrid System product protocol (BD Biosciences Clontech, Palo Alto, CA, USA).

Cell culture

Human MDA-MB-231 breast carcinoma cells were stably transfected with FLAG-tagged human MT1-MMP as described previously (Tam et al., 2002). For controls, cells transfected with a catalytically inactive MT1-MMP mutant (E240A) that retained the propeptide (to avoid potential dominant negative effects by binding substrate and masking cleavage sites) or pCR3.1 vector alone under G418 selection were used. To collect cleaved or degraded extracellular and pericellular matrix proteins and shed ectodomains from the cell membrane, conditioned media from cells grown in serum-free DMEM containing ascorbic acid (50 μ g/ml) for correct collagen synthesis and formation, were harvested after 48 h. The conditioned medium was treated with protease inhibitors (6.7 μ M EDTA, 1 mM PMSF, 10 μ M leupeptin, 1 μ M pepstatin A) to prevent further proteolysis, clarified by centrifugation (30 min, 1000 g) and filtration (0.22 μ m) before concentration by ultrafiltration.

ICAT labeling, MD-LC and MS/MS

Proteins (100 µg) from MT1-MMP, E240A and vector-transfected cell media were paired (MT1-MMP/vector or MT1-MMP/E240A) and labeled with either isotopically heavy [¹³C]₉ or light [¹²C]₉ cleavable ICAT™ reagents (Applied Biosystems, Foster City, CA, USA) for 2 h at 37°C (Figure 1). Samples were combined, trypsin digested (18 h, 37°C) and fractionated on a cation-exchange column. Labeled peptides were then purified over an avidin-affinity column and the eluted peptides were fractionated by multi-dimensional (MD) liquid chromatography (LC) before electrospray ionization and tandem mass spectrometry. MD-LC was performed on a BioX-SCX column with the peptides step eluted by ammonium acetate, pH 4.0. These peptides were subsequently loaded onto an inline 75 µm I.D.×15 cm PepMap nano separation column and fractionated using an acetonitrile gradient (0 to 64%). MS analysis of the separated peptides was performed using a QStar Pulsar™ I mass spectrometer (MDS-Sciex, Concord, ON, Canada) after nano-spray ionization (BC Genome Centre, Victoria, BC, Canada). MS analysis was used to quantify peptide abundance ratios and the peak pairs representing peptides from the two cell sources that were different in abundance were then subjected to MS/MS fragmentation. Using MASCOT, proteins were identified from peptide sequences queried against the human NCBI protein database. Labeling and analysis was performed three times.

Protease, substrates and assays

To confirm the proteolytic susceptibility of potential substrates identified from the yeast two hybrid and tandem mass spectrometry analyses *in vitro* protease assays were performed. Soluble (s) human MT1-MMP lacking the transmembrane and cytoplasmic tail was expressed and purified as described (Tam et al., 2002). sMT1-MMP was incubated with synthetic human interleukin (IL)-8, growth related oncogene (GRO)-α and GRO-γ (Clark-Lewis et al., 1997), or recombinant human secretory leukocyte protease inhibitor (SLPI), human death receptor-6 fused to the Fc region of IgG and human connective tissue growth factor (CTGF), kindly provided by Dr. D. Brigstock (Columbus, OH, USA). Human pro-TNF-α fused to GST was expressed and purified. The variable and thrombospondin modules of human Wnt induced signaling protein-2 (WISP-2) (Gly¹⁶⁴-Phe²⁵¹) were expressed in *E. coli* and purified by immobilized Ni-chelating resin. Full length WISP-2 was expressed in CHO cells according to published procedures (Morrison et al., 2001). Protease assays were performed in 50 mM Tris-HCl, 200 mM NaCl, 5 mM CaCl₂, 3.8 mM NaN₃, 0.05% Brij, pH 7.4 for 18 h at 37°C and the incubation products analyzed by MALDI-TOF or ESI-TOF MS. Proteins were also visualized by Coomassie R-250 staining or Western blotting after Tris-tricine gel electrophoresis.

Acknowledgments

This work was supported by grants from the National Cancer Institute of Canada and the Canadian Institutes of Health Research. E.M.T. is supported by a CIHR Strategic Training Fellow Award in the Cell Signals in Mucosal Inflammation and Pain program and a Canadian Arthritis Network Trainee Award. C.M.O. is supported by a Canada Research Chair in Metalloproteinase Biology.

References

- Ball, D.K., Surveyor, G.A., Diehl, J.R., Steffen, C.L., Uzumcu, M., Miranda, M.A. and Brigstock, D.R. (1998). Characterization of 16- to 20-kilodalton (kDa) connective tissue growth factors (CTGFs) and demonstration of proteolytic activity for 38-kDa CTGF in pig uterine luminal flushings. *Biol. Reprod.* 59, 828–835.
- Belkin, A.M., Akimov, S.S., Zaritskaya, L.S., Ratnikov, B.I., Der-yugina, E.I. and Strongin, A.Y. (2001). Matrix-dependent proteolysis of surface transglutaminase by membrane-type metalloproteinase regulates cancer cell adhesion and locomotion. *J. Biol. Chem.* 276, 18415–18422.
- Belperio, J.A., Keane, M.P., Arenberg, D.A., Addison, C.L., Ehlert, J.E., Burdick, M.D. and Strieter, R.M. (2000). CXC chemokines in angiogenesis. *J. Leukoc. Biol.* 68, 1–8.
- Billinghurst, R.C., Ionescu, M., Reiner, A., Bourne, R., Rorabeck, C., Mitchell, P., Diekmann, O., Tschesche, H., Chen, J., Van Wart, H. and Poole, A.R. (1997). Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. *J. Clin. Invest.* 99, 1534–1545.
- Black, R.A., Rauch, C.T., Kozlosky, C.J., Peschon, J.J., Slack, J.L., Wolfson, M.F., Castner, B.J., Stocking, K.L., Reddy, P., Srinivasan, S. et al. (1997). A metalloproteinase disintegrin that releases tumour-necrosis factor-α from cells. *Nature* 385, 729–733.
- Brigstock, D.R. (1999). The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family. *Endocr. Rev.* 20, 189–206.
- Brigstock, D.R. (2003). The CCN family: a new stimulus package. *J. Endocrinol.* 178, 169–175.
- Clark-Lewis, I., Vo, L., Owen, P. and Anderson, J. (1997). Chemical synthesis, purification, and folding of C-X-C and C-C chemokines. *Methods Enzymol.* 287, 233–250.
- Deng, S.J., Bickett, D.M., Mitchell, J.L., Lambert, M.H., Blackburn, R.K., Carter, H.L., 3rd, Neugebauer, J., Pahel, G., Weiner, M.P. and Moss, M.L. (2000). Substrate specificity of human collagenase 3 assessed using a phage-displayed peptide library. *J. Biol. Chem.* 275, 31422–31427.
- Egeblad, M. and Werb, Z. (2002). New functions for the matrix metalloproteinases in cancer progression. *Nat. Rev. Cancer* 2, 161–174.
- Greenbaum, D., Baruch, A., Hayrapetian, L., Darula, Z., Burlingame, A., Medzhradszky, K.F. and Bogoy, M. (2002). Chemical approaches for functionally probing the proteome. *Mol. Cell Proteomics* 1, 60–68.
- Grutter, M.G., Fendrich, G., Huber, R. and Bode, W. (1988). The 2.5 Å X-ray crystal structure of the acid-stable proteinase inhibitor from human mucous secretions analysed in its complex with bovine alpha-chymotrypsin. *EMBO J.* 7, 345–351.
- Guo, L., Eisenman, J.R., Mahimkar, R.M., Peschon, J.J., Paxton, R.J., Black, R.A. and Johnson, R.S. (2002). A proteomic approach for the identification of cell-surface proteins shed by metalloproteinases. *Mol. Cell Proteomics* 1, 30–36.
- Gygi, S.P., Corthals, G.L., Zhang, Y., Rochon, Y. and Aebersold, R. (2000). Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. *Proc. Natl. Acad. Sci. USA* 97, 9390–9395.
- Gygi, S.P., Rist, B., Gerber, S.A., Turecek, F., Gelb, M.H. and Aebersold, R. (1999). Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* 17, 994–999.
- Haro, H., Crawford, H.C., Fingleton, B., Shinomiya, K., Spengler, D.M. and Matrisian, L.M. (2000). Matrix metalloproteinase 1-dependent release of tumor necrosis factor-α in a model of hermiated disc resorption. *J. Clin. Invest.* 105, 143–150.
- Hashimoto, G., Inoki, I., Fujii, Y., Aoki, T., Ikeda, E. and Okada, Y. (2002). Matrix metalloproteinases cleave connective tissue growth factor and reactivate angiogenic activity of vascular endothelial growth factor 165. *J. Biol. Chem.* 277, 36288–36295.

- Henry, M.T., McMahon, K., Costello, C., Fitzgerald, M.X. and O'Connor, C.M. (2002). Secretory leukocyte proteinase inhibitor and elafin are resistant to degradation by MMP-8. *Exp. Lung Res.* **28**, 85–97.
- Holmbeck, K., Bianco, P., Caterina, J., Yamada, S., Kromer, M., Kuznetsov, S.A., Mankani, M., Robey, P.G., Poole, A.R., Pidoux, I. et al. (1999). MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. *Cell* **99**, 81–92.
- Hughes, C.E., Caterson, B., Fosang, A.J., Roughley, P.J. and Mort, J.S. (1995). Monoclonal antibodies that specifically recognize neoepitope sequences generated by 'aggrecanase' and matrix metalloproteinase cleavage of aggrecan: application to catabolism *in situ* and *in vitro*. *Biochem. J.* **305**, 799–804.
- Lemons, M.L., Sandy, J.D., Anderson, D.K. and Howland, D.R. (2001). Intact aggrecan and fragments generated by both aggrecanase and metalloproteinase-like activities are present in the developing and adult rat spinal cord and their relative abundance is altered by injury. *J. Neurosci.* **21**, 4772–4781.
- Liu, Y., Patricelli, M.P. and Cravatt, B.F. (1999). Activity-based protein profiling: the serine hydrolases. *Proc. Natl. Acad. Sci. USA* **96**, 14694–14699.
- Lopez-Otin, C. and Overall, C.M. (2002). Protease degradomics: a new challenge for proteomics. *Nat. Rev. Mol. Cell Biol.* **3**, 509–519.
- McQuibban, G.A., Butler, G.S., Gong, J.H., Bendall, L., Power, C., Clark-Lewis, I. and Overall, C.M. (2001). Matrix metalloproteinase activity inactivates the CXC chemokine stromal cell-derived factor-1. *J. Biol. Chem.* **276**, 43503–43508.
- McQuibban, G.A., Gong, J.H., Tam, E.M., McCulloch, C.A., Clark-Lewis, I. and Overall, C.M. (2000). Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. *Science* **289**, 1202–1206.
- McQuibban, G.A., Gong, J.H., Wong, J.P., Wallace, J.L., Clark-Lewis, I. and Overall, C.M. (2002). Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties *in vivo*. *Blood* **100**, 1160–1167.
- Morrison, C.J., Butler, G.S., Bigg, H.F., Roberts, C.R., Soloway, P.D. and Overall, C.M. (2001). Cellular activation of MMP-2 (gelatinase A) by MT2-MMP occurs via a TIMP-2-independent pathway. *J. Biol. Chem.* **276**, 47402–47410.
- Moss, M.L., Jin, S.L., Milla, M.E., Bickett, D.M., Burkhart, W., Carter, H.L., Chen, W.J., Clay, W.C., Didsbury, J.R., Hassler, D. et al. (1997). Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor- α . *Nature* **385**, 733–736.
- Nazif, T. and Bogoyo, M. (2001). Global analysis of proteasomal substrate specificity using positional-scanning libraries of covalent inhibitors. *Proc. Natl. Acad. Sci. USA* **98**, 2967–2972.
- Ohuchi, E., Imai, K., Fujii, Y., Sato, H., Seiki, M. and Okada, Y. (1997). Membrane type 1 matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. *J. Biol. Chem.* **272**, 2446–2451.
- Overall, C.M. (2001). Matrix metalloproteinase substrate binding domains, modules and exosites. Overview and experimental strategies. *Methods Mol. Biol.* **151**, 79–120.
- Overall, C.M., McQuibban, G.A. and Clark-Lewis, I. (2002). Discovery of chemokine substrates for matrix metalloproteinases by exosite scanning: a new tool for degradomics. *Biol. Chem.* **383**, 1059–1066.
- Overall, C.M., Wiebkin, O.W. and Thonard, J.C. (1987). Demonstration of tissue collagenase activity *in vivo* and its relationship to inflammation severity in human gingiva. *J. Periodont. Res.* **22**, 81–88.
- Pan, G., Bauer, J.H., Haridas, V., Wang, S., Liu, D., Yu, G., Vincenz, C., Aggarwal, B.B., Ni, J. and Dixit, V.M. (1998). Identification and functional characterization of DR6, a novel death domain-containing TNF receptor. *FEBS Lett.* **431**, 351–356.
- Pennica, D., Swanson, T.A., Welsh, J.W., Roy, M.A., Lawrence, D.A., Lee, J., Brush, J., Taneyhill, L.A., Deuel, B., Lew, M. et al. (1998). WISP genes are members of the connective tissue growth factor family that are up-regulated in wnt-1-transformed cells and aberrantly expressed in human colon tumors. *Proc. Natl. Acad. Sci. USA* **95**, 14717–14722.
- Puente, X.S., Sanchez, L.M., Overall, C.M. and Lopez-Otin, C. (2003). Human and mouse proteases: a comparative genomic approach. *Nat. Rev. Genet.* **4**, 544–558.
- Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E. and Seiki, M. (1994). A matrix metalloproteinase expressed on the surface of invasive tumour cells. *Nature* **370**, 61–65.
- Seiki, M. (2003). Membrane-type 1 matrix metalloproteinase: a key enzyme for tumor invasion. *Cancer Lett.* **194**, 1–11.
- Shen, M., Guo, L., Wallace, A., Fitzner, J., Eisenman, J., Jacobson, E. and Johnson, R.S. (2003). Isolation and isotope labeling of cysteine- and methionine-containing tryptic peptides: application to the study of cell surface proteolysis. *Mol. Cell Proteomics* **2**, 315–324.
- Tam, E.M., Morrison, C.J., Wu, Y.I., Stack, M.S. and Overall, C.M. (2004). Membrane protease functional proteomics: isotope coded affinity tag/tandem mass spectrometry identification of novel MT1-MMP substrates. *Proc. Natl. Acad. Sci. USA* **101**, 6917–6922.
- Tam, E.M., Wu, Y.I., Butler, G.S., Stack, M.S. and Overall, C.M. (2002). Collagen binding properties of the membrane type-1 matrix metalloproteinase (MT1-MMP) hemopexin C domain. The ectodomain of the 44-kDa autocatalytic product of MT1-MMP inhibits cell invasion by disrupting native type I collagen cleavage. *J. Biol. Chem.* **277**, 39005–39014.
- Turk, B.E., Huang, L.L., Piro, E.T. and Cantley, L.C. (2001). Determination of protease cleavage site motifs using mixture-based oriented peptide libraries. *Nat. Biotechnol.* **19**, 661–667.
- Van Damme, J., Decock, B., Conings, R., Lenaerts, J.P., Opdenakker, G. and Billiau, A. (1989). The chemotactic activity for granulocytes produced by virally infected fibroblasts is identical to monocyte-derived interleukin 8. *Eur. J. Immunol.* **19**, 1189–1194.
- Van den Steen, P.E., Proost, P., Wuyts, A., Van Damme, J. and Opdenakker, G. (2000). Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO- α and leaves RANTES and MCP-2 intact. *Blood* **96**, 2673–2681.
- Yamanaka, H., Makino, K., Takizawa, M., Nakamura, H., Fujimoto, N., Moriya, H., Nemori, R., Sato, H., Seiki, M. and Okada, Y. (2000). Expression and tissue localization of membrane-types 1, 2, and 3 matrix metalloproteinases in rheumatoid synovium. *Lab. Invest.* **80**, 677–687.