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Directed Evolution of Protease Beacons that Enable Sensitive Detection of Endogenous MT1-MMP Activity in Tumor Cell Lines

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SUMMARY

Directed evolution was applied to identify peptide substrates with enhanced hydrolysis rates by MT1-MMP suitable for protease beacon development. Screening of a random pentapeptide library, using two-color CLiPS, yielded several substrates identical to motifs in distinct collagens that shared the consensus sequence P-x-G↓L. To identify substrates with enhanced cleavage rates, a second-generation decapeptide library incorporating the consensus was screened under stringent conditions, which resulted in a MxPLG↓$^{M}/_{L}$M$^{G}/_{A}$R consensus motif. These substrates are hydrolyzed by human-MT1-MMP up to six times faster than reported peptide substrates and are stable in plasma. Finally, incubation of soluble protease beacons incorporating the optimized substrates, but not previous substrates, enabled direct detection of endogenous MT1-MMP activity of human-fibrosarcoma (HT-1080) cells. Extended substrate libraries coupled with CLiPS should be useful to generate more effective activity probes for a variety of proteolytic enzymes.

INTRODUCTION

The extracellular degradation of collagen is an essential step during developmental processes and in the remodeling and rapid growth of tissues. Collagen degradation is regulated by the expression, activation, and inhibition of several matrix metalloproteinases (MMPs). MMP family members including MMP-1, 2, 8, 9, 13, membrane type-1 MMP (MT1-MMP or MMP-14), and MT2-MMP are known to hydrolyze collagens (Aimes and Quigley, 1995; Haas et al., 1998; Knauper et al., 1997; Krane et al., 1996; Niyibizi et al., 1994; Ohuchi et al., 1997; Patterson et al., 2001). Although, most collagenases are secreted, MT1-MMP is membrane-anchored and has been found to mediate localized collagenolysis and invasive activity to both normal and malignant cells (Sabeh et al., 2009). MT1-MMP has been reported to cleave several types of collagen including types I, II, and III collagens (Ohuchi et al., 1997). Mice deficient in MT1-MMP exhibit inadequate collagen turnover and prominent defects that include dwarfism, arthritis, and connective tissue disease (Holmbeck K., 1999; Kenn et al., 2004). In addition, MT1-MMP deficient mice display loss of collagenolytic activity in skin fibroblast cells (Holmbeck K., 1999), and similar outcomes were observed in human fibroblasts and tumor cells when MT1-MMP expression was silenced by interfering RNA (Holmbeck, 1999; Lee et al., 2006; Sabeh et al., 2004). These observations support the assertion that MT1-MMP serves an important and nonredundant role in collagenolysis during a variety of physiological processes.

In addition to MT1-MMPs role in collagenolysis, several studies have reported that MT1-MMP is capable of hydrolyzing a variety of substrates including gelatin, β-casein, κ-elastin, and basement membrane or interstitial-associated proteins of the extracellular matrix (ECM) including fibronectin, vitronectin, laminin-1 and 5 (d'Ortho et al., 1997; Hornebeck and Maquart, 2003; Ohuchi et al., 1997; Pei and Weiss, 1996; Sato et al., 2005). Additionally, MT1-MMP contributes to shedding of cell adhesion molecules, such as CD44H which plays an important role in lymphocyte activation and homing, T cell activation, angiogenesis, and metastasis (Goodison et al., 1999; Kajita et al., 2001; Trochon et al., 1996). Also, several biologically relevant MT1-MMP substrates cleaved in cellular context were identified by quantitative proteomics methods using isotope-coded affinity tag labeling with tandem mass spectrometry, such as pentraxin 3, galectin-1, proTNFα, and Hsp90α (Butler et al., 2008; Tam et al., 2004). In addition to cleaving ECM proteins, MT1-MMP also has been found to activate MMP zymogens such as proMMP-2, proMMP-8 and proMMP-13 that are involved in tumor cell invasion and metastasis (Folgueras et al., 2004; Holopainen et al., 2003; Itoh and Seiki, 2004; Will et al., 1996). Elevated MT1-MMP activity has been detected in tumor and tumor stromal cells of many tissue types but particularly in brain, lung, gastric, and breast carcinoma tissues (Atkinson et al., 2007; Bisson et al., 2003; Lampert et al., 1998; Malhotra et al., 2002; Okada et al., 1995). Thus, MT1-MMP has been demonstrated to play an essential role in cell migration, invasion, cell morphology, metastasis, and angiogenesis in normal physiological and pathological events (Itoh and Seiki, 2004; Seiki, 2003; Stamenkovic, 2000; Zhou et al., 2000).

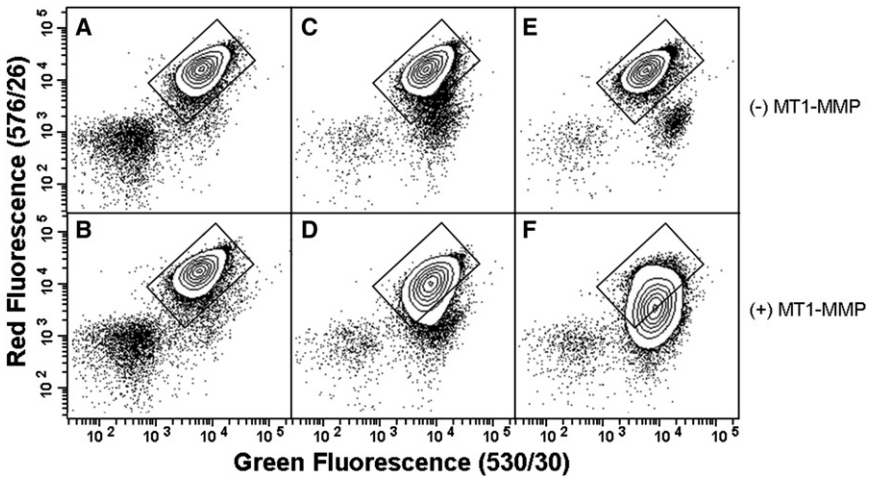

Figure 1. Use of FACS to Screen Peptide Substrate Libraries (CLiPS) for MT1-MMP Substrates

Flow cytometric analysis of cell populations representing the 5 mer random library prior to screening without MT1-MMP (A) or with MT1-MMP (B), the final sorted populations from the 5 mer random library without (C) or with MT1-MMP (D), and the 10 mer focused library without (E) or with (F) MT1-MMP. See also Table S1.

Given MT1-MMP's diverse functions, inhibitors and probes have been developed to inhibit or measure MT1-MMP activity in vivo (Devy et al., 2009; Fujita et al., 2003). For example, a FRET-based biosensor has been shown to enable detection and localization of MT1-MMP activity on the membrane of HeLa cells that are cotransfected with both the membrane-anchored MT1-MMP probe and MT1-MMP (Ouyang et al., 2008). However, the MT1-MMP cleavable substrate used in the probe was hydrolyzed at a similar rate by MT2-MMP and MT3-MMP, and the method required a high concentration of MT1-MMP (~60 nM) for cleavage. In another strategy, a MT1-MMP-activated single photon emission computed tomography (SPECT) imaging probe was constructed by fusing a radionuclide to an inhibited cell penetrating peptide (CPP) to selectively target MT1-MMP expressing cells. However, the probe was also activated in cells that do not express MT1-MMP, indicating nonspecific cleavage of the MT1-MMP substrate by other MMPs (Watkins et al., 2009). Thus the specific detection of MT1-MMP activity in cell culture and in vivo will require substrates with improved activity and selectivity.

The substrate specificity of MT1-MMP has been investigated in several previous studies. Using the substrate phage display method, the consensus substrate sequence for MT1-MMP was found to be $Px^{G}/_{P}L$ at the P3-P1′ sites (Ohkubo et al., 1999). Most of the substrates identified using this method were also cleaved by MMP-2 and MMP-9. Another study using the substrate phage methodology suggested that the specificity for MT1-MMP can be conferred by arginine at the P4 position and the absence of proline at the P3 position through mutational analysis (Kridel et al., 2002). However, phage library selections did not reveal a clear consensus for MT1-MMP selective substrates (Kridel et al., 2002). In addition, these studies did not characterize the activity and selectivity of other collagenases toward the MT1-MMP substrates. Consequently, we sought to identify MT1-MMP substrates that exhibit rapid hydrolysis rates using two color cellular libraries of peptide substrates (CLiPS) (Boulware and Daugherty, 2006; Boulware et al., 2010). Using CLiPS methodology, substrates are displayed on the surface of bacterial cells, allowing quantitative measurement of the extent of substrate cleavage using flow cytometry, an advantage over substrate phage. The use of CLiPS and substrate extension provided a highly effective directed evolution strategy, wherein selection stringency could be tuned using FACS. We applied this method to identify enhanced substrates suitable for direct MT1-MMP activity measurements in cells.

RESULTS

Identification and Directed Evolution of MT1-MMP Substrates Using CLiPS

To identify optimal five residue cleavage motifs for MT1-MMP, a cellular library of peptide substrates (CLiPS) consisting of more than 10^7 unique random pentamer substrates displayed on the surface of *Escherichia coli* (Boulware and Daugherty, 2006) was screened using fluorescence activated cell sorting (FACS). A two-step screening process was applied to enrich library members that display substrates efficiently cleaved by the catalytic domain of MT1-MMP. First, cells exhibiting green and red (green+ red+) fluorescence were sorted after labeling with a red fluorescent probe, streptavidin R-phycoerythin (SA-PE), that recognizes the uncleaved N terminus and a green fluorescent probe (*Alaj*GFP-Mona SH3) that binds to the scaffold's C terminus irrespective of cleavage. In the second step, library members with cleaved substrates (green+ red-) were sorted after incubation with MT1-MMP (80 nM for 2 hr) and labeling with red and green probes. The process was repeated to enrich clones displaying rapidly cleaved substrates (Figure 1). Individual identified substrates exhibited a clear preference for proline at the P3 position, glycine at the P1 position, and a medium-sized hydrophobic residue (L, M, I) at the P1′ position; the resulting consensus substrate sequence was P-x-G-(L/M/I). Additionally, the P2′ position exhibited a modest preference for arginine, methionine, or leucine. The pentapeptide substrates identified by CLiPS exhibited a high degree of identity to motifs in known substrates including several collagen types. In fact, the most rapidly cleaved substrate PQGLL is identical to a known pentapeptide motif in type-1 collagen.

In an effort to identify substrates with improved hydrolysis rates and identify residue preferences further from the scissile bond, a secondary library of the form xxxPx(G/P)(L/M/I)xxx was constructed. Screening the extended substrate library under more stringent conditions (60 nM MT1-MMP for 1 hr) enriched a population with substantially improved cleavage

Table 1. Amino Acid Sequences and Second-Order Rate Constants for the Substrates Resulting from Sorting the 5-mer Random Library (5-peptide), and the 10-mer Focused Library (10.peptide) for MT1-MMP

Substrate	P6	P5	P4	P3	P2	P1	P1′	P2′	P3′	P4′	P5′	P6′	P7′	k_{cat}/K_M (μM^{-1} Min^{-1})
5.1			s	**P**	Q	**G**	**L**	**L**	q					0.15 ± 0.01
5.2			s	**P**	Q	**G**	**L**	R	q					0.15 ± 0.02
5.3			s	**P**	M	**P**	**L**	**M**	**A**					0.12 ± 0.01
5.4			s	**P**	I	**G**	**L**	R	q					0.12 ± 0.02
5.5			s	**P**	Q	**G**	**M**	**L**	q					0.11 ± 0.01
5.6			s	**P**	R	**G**	**M**	**L**	q					0.11 ± 0.01
5.7			s	**P**	S	**G**	**L**	R	q					0.07 ± 0.01
5.8			s	**P**	R	**G**	**I**	**M**	q					0.06 ± 0.01
5.9			s	**P**	K	**G**	**L**	**L**	q					0.05 ± 0.01
5.10			s	W	G	**G**	**L**	R	q					0.02 ± 0.01
10.1	S	**L**	A	**P**	**L**	**G**	**L**	Q	R	**R**				0.56 ± 0.02
10.2	W	**M**	K	**P**	Q	**G**	**M**	R	L	M				0.54 ± 0.02
10.3	G	**M**	L	**P**	**L**	**G**	**M**	R	**G**	S				0.40 ± 0.02
10.4	L	Q	L	**P**	S	**G**	**L**	**M**	**G**	**R**				0.36 ± 0.04
10.5	W	R	A	**P**	**L**	A	**L**	**M**	S	**R**				0.34 ± 0.03
10.6	G	**M**	V	**P**	V	**G**	**L**	**M**	**A**	G				0.34 ± 0.04
10.7	V	G	E	**P**	R	**G**	**M**	**L**	**A**	G	S	L	K	0.32 ± 0.01
10.8	L	R	V	**P**	**L**	**G**	**L**	**M**	**G**	**K**				0.32 ± 0.03
10.9	G	**M**	R	**P**	**L**	**G**	**I**	A	**G**	**R**				0.16 ± 0.02

The second-order rate constants were determined using flow cytometry. The consensus motif from the 5-mer library was P-x-(G)-(L/M/I), and for the 10-mer focused library the consensus motif was (M/L)-x-P-L-G-(L/M)-(L/M)-(G/A)-(R/K).

kinetics when compared to the primary random library (Figure 1). Lower concentrations of MT1-MMP (20 and 30 nM) and shorter incubation times (15 and 25 min) were attempted but did not enrich improved substrates. Individual clones randomly selected from the final sort exhibited a consensus of ($^{M}/_{L}$)xPLG(L/M)($^{L}/_{M}$)($^{G}/_{A}$)($^{R}/_{K}$) (Table 1). Lysine and arginine were observed at the P4′ position in about half of the substrates. The substrates identified using CLiPS were searched against the human proteome using the ScanProsite degenerate motif search algorithm, and several of the substrate sequences appeared in diverse ECM proteins (Table 2). Thus, screening of a second generation library constructed using pentapeptide consensus information yielded substrates that are hydrolyzed more rapidly by MT1-MMP than the first generation pentapeptide substrates.

Characterization of Substrate Cleavage Kinetics

The cleavage rate of individual substrates were initially measured and ranked for cell-displayed substrates (Table 1) as previously described (Boulware and Daugherty, 2006). Substrates from the 10 mer focused library exhibited up to 4-fold enhanced hydrolysis rates on the cell surface relative to 5 mer substrates. Soluble protease activity probes (i.e., protease beacons) were constructed using fluorescent proteins optimized for FRET studies as previously described (Nguyen and Daugherty, 2005). Protease beacons were constructed for three decapeptide substrates (C-10.1-Y, C-10.2-Y, C-10.7-Y), the most rapidly hydrolyzed pentapeptide substrate (C-5.1-Y), and two substrates identified using either oriented peptide libraries (C-MPL-Y) (Turk et al., 2001) or substrate phage display (C-A42-Y) (Kridel et al., 2002). The lowest concentration of MT1-MMP that the FRET beacon can significantly detect was 0.3 nM (see Figure S1 available online). Extended ten-residue substrates were cleaved 4- to 6-fold faster than the most rapidly cleaved 5 mer PQGLL and previously reported peptide substrates (C-A42-L, C-MPL-Y) (Figure 2A and Table 3), as measured using fluorimetry.

Since the most rapidly cleaved substrate identified from the random library was identical (5/5 amino acids) to a known human type-1 collagen cleavage site PQGLL (Sato et al., 2005), substrate hydrolysis rates were also measured for human collagenases MMP-1, 2, 8, 9, 13, and MT2-MMP. Although selectivity was not explicitly favored during our screens, the most rapidly cleaved substrate (C-10.1-Y) exhibited selectivity for MT1-MMP over other collagenases, with the exception of MMP-9 (Table 3; Table S2). Kinetic constants (k_{cat}/K_M) measured using FRET substrates correlated with those measured for the corresponding substrate displayed on the cell surface, although the absolute values measured using the FRET beacons were roughly 6-fold higher. The extended FRET substrate C-10.1-Y exhibited 3- to 14-fold selectivity for MT1-MMP over the other MMPs analyzed with the exception of MMP-9.

To investigate whether the optimized substrates might be suitable for in vivo imaging or prodrug applications, their stability was measured in human plasma (Figure 2B). In a 10-fold dilution of plasma, the limit of sensitivity for the FRET assay, substrate cleavage did not occur during a 4 hr incubation, indicating that the MT1-MMP activity was completely inhibited by human plasma even when diluted by 10-fold (10% plasma). The P-value was <0.0001 when the MT1-MMP activity curves of 1% and 10%

Table 2. Candidate MT1-MMP Substrates Identified Using CLiPS/ScanProsite

Substrate Sequence	Human Protein	Site	SWISS-PROT #
PQGLL	Collagen α-2 type I	863-867	P08123
	Endoglin	296-300	P17813
	Keratin type II	486-490	Q9NSB4
	Otogelin	930-934	Q6ZR10
	Agrin	1405-1409	O00468
PQGLR	Collagen α-1 type VIII	233-237	P27658
	Collagen α-3 type IX	419-423	Q14050
	Interleukin-11 receptor α	221-225	Q14626
	Interleukin-23 subunit α	79-83	Q9NPF7
	Integrin β-4	103-107	P16144
	LRP-6	607-611	O75581
PSGLR	Fibronectin type III domain	10-14	Q9H6D8
	Transforming growth factor β1	3-7	P01137
	LRP-3	685-689	O75074
PKGLL	Collagen α-1 type XXIV	787-791	Q17RW2
	Platelet glycoprotein V	186-190	P40197
WGGLR	Integrin α-5	744-748	P08648
APLGLQ	Collagen α-3 type IX	497-501	Q14050
	Thrombomodulin	217-221	P07204
LPSGL	LPR-3	684-688	O75074
	Lumican	199-204	P51884
PLGIA	Collagen α −2 type I	896-900	P08123
	Collagen α −1 type III	946-950	P02461

Candidate substrates have not been experimentally validated.

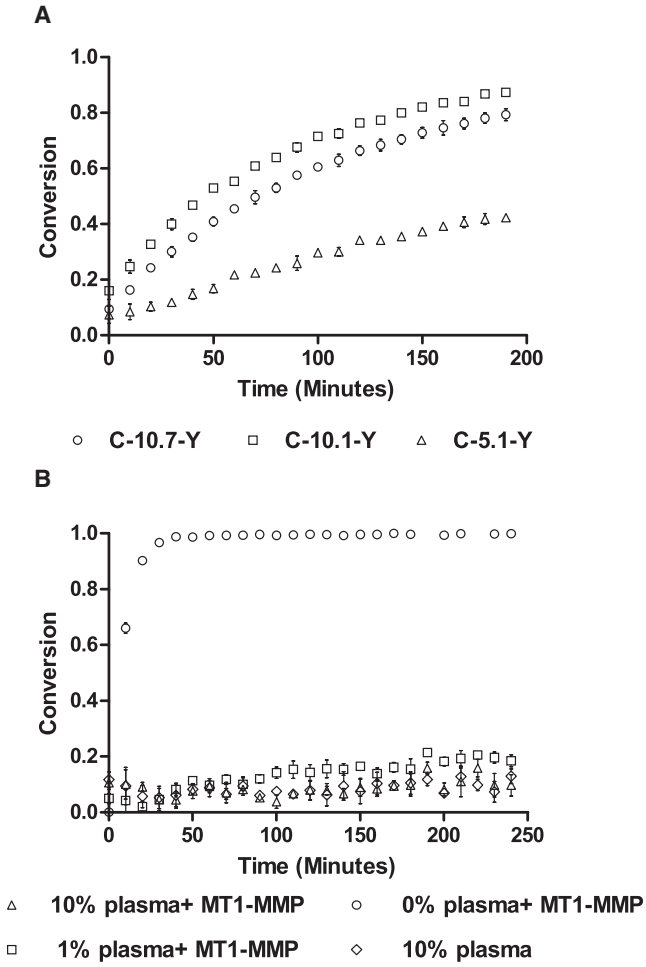

Figure 2. Measurement of MT1-MMP Activity Using Protease Beacons

(A) The mean extent of conversion of the FRET substrates was measured with respect to time after incubating with 2.5 nM MT1-MMP.

(B) The mean extent of conversion of C-10.1-Y was monitored over time after incubating with 50 nM MT1-MMP and different concentrations of human plasma at 37°C. See also Figure S1.

plasma was compared using two-way ANOVA analysis, indicating that the hydrolysis rates are statistically significant and MT1-MMP activity was detected only when plasma was diluted by at least 100-fold. Thus, the FRET substrates were stable in plasma and MT1-MMP activity was completely inhibited by 10% plasma likely due to the presence of TIMPs or other protease inhibitors in human plasma. Finally, since imaging or prodrug studies would benefit from cross-reactivity with mouse MT1-MMP, substrate conversion of C-10.1-Y by mouse and human MT1-MMP was measured and the decapeptide substrate was cleaved at an equivalent rate when incubated with either protease (Figure S2).

Measurement of Endogenous MT1-MMP Activity in Human Tumor Cell Lines

Given the improved cleavage rates of the extended substrates identified here relative to the reported MT1-MMP substrates (C-A42-Y, C-MPL-Y), we hypothesized that endogenous MT1-MMP activity of tumor cells could be quantified using substrate probes exhibiting FRET. The extent of conversion of all of the substrates did not exceed background when incubated for 4 hr with MT1-MMP deficient breast carcinoma cells (MCF-7). However, human fibrosarcoma cells (HT-1080) expressing MT1-MMP mediated roughly 70% conversion in 4 hr for the optimized substrates (Figure 3), while conversion of previously reported substrates (C-A42-Y, C-MPL-Y) did not rise above 20%, making activity detection difficult. Nonspecific hydrolysis by the possible presence of soluble proteases, shed or vesicular MT1-MMP could not be detected since serum-free conditioned supernatants did not mediate detectable cleavage under the same assay conditions. In addition to MT1-MMP, HT-1080 cells have been reported to express other MMPs including MT2-MMP and MMP-2 (Ito et al., 2010; Sato and Takino, 2010). Importantly, MT1-MMP was solely responsible for protease beacon activation by HT1080, since activation was completely inhibited by a selective, high affinity anti-MT1-MMP antibody (DX-2400) (Devy et al., 2009) (Figure S3). Again, plasma addition strongly inhibited substrate cleavage by cell lines, though activity was detectable at up to 5% plasma (Figure S3). Thus, in cell-based assays, the enhanced substrates provided at least 6-fold increased sensitivity relative to previously reported substrates (C-A42-Y, C-MPL-Y), enabling detection of endogenous MT1-MMP activity.

Table 3. Second-Order Rate Constants for MT1-MMP FRET Substrates

Substrate	P6	P5	P4	P3	P2	P1	P1′	P2′	P3′	P4′	P5′	P6′	P7′	k_{cat}/K_M (μM^{-1} Min^{-1}) MT1-MMP	MMP-1	MMP-8[a]
C-10.1-Y	S	L	A	P	L	G	L	Q	R	R				3.9 ± 0.1	0.28 ± 0.01	0.25 ± 0.01
C-10.2-Y	W	M	K	P	Q	G	M	R	L	M				3.3 ± 0.2	0.21 ± 0.01	0.67 ± 0.03
C-10.7-Y	V	G	E	P	R	G	M	L	A	G	S	L	K	3.3 ± 0.2	0.39 ± 0.01	0.47 ± 0.04
C-A42-Y		S	G	R	I	G	F	L	R	T	A			0.56 ± 0.04	0.13 ± 0.01	- - - -
C-MPL-Y			I	P	E	S	L	R	A					0.76 ± 0.08	0.99 ± 0.01	1.41 ± 0.05
C-5.1-Y				P	Q	G	L	L						1.04 ± 0.06	0.35 ± 0.01	1.15 ± 0.06

[a] Active site titration was not performed for MMP-8.
Standard deviation values are derived from triplicate measurements. See also Table S2.

DISCUSSION

Matrix metalloproteinases exhibit similar active site structures that can account for the high similarity of short peptide substrates for different MMP family members and the difficulty in designing small molecule inhibitors that are selective for particular MMPs (Hooper, 1994; Stocker et al., 1995). For instance, several MMPs recognize peptide substrates that contain the P-x-x-Φ motif (where Φ is a hydrophobic amino acid) similar to those found in gelatins and collagens (Chen et al., 2002; Deng et al., 2000; Kridel et al., 2001). The extended substrates identified here, exhibited significantly increased rates of hydrolysis by MT1-MMP when compared to shorter substrates. In previous studies that involved screening of random peptide libraries for MT1-MMP substrates, substrates with the highest cleavage rates exhibited poor selectivity and were cleaved at similar or greater rates by nontarget proteases (Kridel et al., 2002; Ohkubo et al., 1999). However, activity enhancement via substrate extension yielded substrates that were preferentially cleaved by MT1-MMP when compared to several closely related MMP family members. Even so, our results indicate that substrate extension provides a straightforward approach to increase substrate contacts with the protease surface neighboring the catalytic cleft to improve substrate recognition and hydrolysis rates.

Quantitative activity-based substrate library screening combined with bioinformatic analysis identified a group of candidate physiological substrates localized in the ECM and on cell surfaces. In vitro methods for protease specificity characterization, although useful for active site specificity analysis, are often insufficient to unambiguously identify physiological substrates. For example, solution phase positional scanning or oriented peptide libraries can quickly reveal the full spectrum of subsite preferences, but do not identify cooperative effects nor individual preferred substrates that implicate physiological substrates (Schneider and Craik, 2009; Turk et al., 2001). Substrate phage (Deperthes, 2002) and CLiPS methods (Boulware and Daugherty, 2006) can implicate physiological substrates, primarily for relatively specific enzymes that cleave a smaller number of substrates. Here, screening for rapid

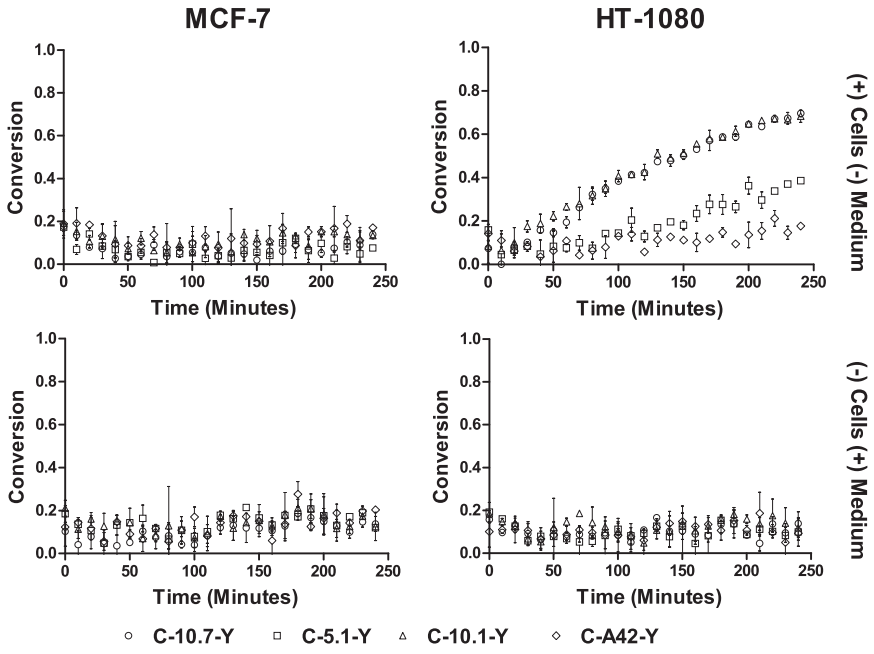

Figure 3. Measurement of MT1-MMP Activity in Tumor Cells Using Protease Beacons
MT1-MMP FRET beacons were incubated with MCF-7 and HT1080 cells or conditioned media and substrate conversion was monitored over time at 37°C. See also Figures S2 and S3.

cleavage yielded pentapeptide substrates that are identical to motifs in several human collagens, including types (I, III, VIII, IX, etc.). Use of ScanProSite revealed that PQGLR is identical to a known substrate within human type IX collagen (α3) and type VIII collagen (α1). Substrates PQGLL and PIGLR occur in type I collagen (α2) and type XI collagen (α1), respectively (Sato et al., 2005). Furthermore, PMPLMA is similar to the sequence PSPII, a cleavage site for proMMP-2 activation (Ohkubo et al., 1999). In addition to collagens, the optimal pentapeptide PQGLL substrate motif occurs 51 times in the human genome and conspicuously occurs in a variety of ECM proteins including keratin type II, agrin (a laminin-associated protein of the basal lamina (Remacle et al., 2006)), and endoglin (a major endothelial cell glycoprotein involved in cell migration (Garcia-Pozo et al., 2008)). Moreover, the extended consensus motif PxGLxxR is present in multiple integrins, disintegrin, and metalloprotease (ADAM) family proteases whose cleavage could activate an invasive program in cascade fashion. Thus, extended substrate consensus data suggested an array of candidate physiological substrates present within the ECM. Even so, our results suggest that collagenolysis is a primary function of MT1-MMP.

CLiPS provided a highly effective tool for substrate discovery and optimization, owing to quantitative library analysis and screening using FACS. Substrate phage, proteome-derived database-searchable peptide libraries, positional scanning combinatorial libraries and CLiPS have been applied to identify substrates but typically have not been used for directed evolution (Boulware and Daugherty, 2006; Deperthes, 2002; Harris et al., 2000; Schilling and Overall, 2008). CLiPS has been shown to enable substrate identification for both promiscuous and highly specific proteases including caspase-3, enteropeptidase (Boulware and Daugherty, 2006), staphylopains A and B from *Staphylococcus aureas* (Dubin et al., 2008; Stec-Niemczyk et al., 2009), karolysin, an MMP-like protease from a periodontal pathogen (Karim et al., 2010), and here, MT1-MMP. CLiPS has also enabled directed evolution of substrate activity for the tobacco etch virus TEV nuclear inclusion protease, which identified substrates with high sequence similarity to the native TEV substrate (Boulware et al., 2010). For MT1-MMP, flow cytometric population analysis enabled efficient identification of the concentration and reaction time that would yield only the most rapidly cleaved substrates. Although not favored explicitly during screening, selectivity could be further evolved by counter screening to remove substrates that are cleaved by other proteases.

Detecting and measuring the activity of proteases including MMPs in vivo and in ex vivo tissues remains a challenge. Proteolytic activity is usually detected using gelatin or collagen zymography, in situ zymography or protease beacons (Snoek-van Beurden and Von den Hoff, 2005). However, protease activity probes frequently suffer from insufficient selectivity and activity, and can be expensive to synthesize. Zymography methods do not generally identify the protease species hydrolyzing the substrate, since they report the activity of a variety of proteases that can degrade gelatin or collagen including many MMPs, cathepsins, and stratum corneum thiol protease (Hashimoto et al., 2006; Platt et al., 2006; Watkinson, 1999). Furthermore, reliable identification of the active MMP species in zymograms can be difficult due to the fact that MMPs can form complexes (Snoek-van Beurden and Von den Hoff, 2005). In an attempt to address this limitation, several protease imaging probes or protease beacons have been designed to detect individual protease activities in vitro and in vivo (Chen et al., 2009; Jiang et al., 2004; Law and Tung, 2009; Wunder et al., 2004). However, frequently utilized chromogenic and fluorogenic substrate probes lack prime side amino acids that influence activity and selectivity. The FRET beacons developed here using FRET-optimized fluorescent proteins yielded a large dynamic range (20-fold) to enable highly sensitive MT1-MMP detection. Thus, the present study demonstrates that extended substrate peptides can provide critical gain of function for the detection, localization, and quantification of activity of specific proteases in vitro, providing a new tool for basic and applied studies of proteolytic enzymes.

SIGNIFICANCE

Given the diverse roles of MT1-MMP in normal physiological and pathological events, especially in cell invasion, metastasis, and angiogenesis, several imaging probes have been designed to report MT1-MMP activity. However, the utility of these probes is limited by their low sensitivity and selectivity for MT1-MMP. Here, improved MT1-MMP substrates were developed using CLiPS to iteratively evolve activity. The best substrates identified exhibited substantially greater hydrolysis rates than previously identified peptide substrates, and preferential cleavage by MT1-MMP over several other family members. These extended substrates, when incorporated into FRET beacons, enabled direct detection of endogenous MT1-MMP activity in tumor cell lines. Thus, the present substrate discovery approach should have utility for developing improved protease-activity probes for a variety of in vivo and ex vivo applications.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents

Human fibrosarcoma (HT1080) and breast carcinoma (MCF-7) cell lines were obtained from American Type Culture Collection (ATCC). Tumor cells were cultured in Minimum Essential Medium (Eagle) or RPMI-1640 from ATCC supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 50 μg/ml streptomycin (Invitrogen, Carlsbad, Ca). Bacterial experiments were performed with *E. coli* strain MC1060 and grown in LB medium supplemented with 34 ug/ml chloramphenicol. Soluble catalytic domains of matrix metalloproteinases: MMP-1, MMP-2, MMP-8, MMP-9, MMP-13, and MT1-MMP were obtained from BIOMOL International LP (Plymouth Meeting, PA). MT2-MMP was obtained from Chemicon (Ca). Ni-NTA resin was purchased from QIAGEN. Streptavidin-R-phycoerythrin (SAPE) was from Invitrogen. BPER-II Bacterial Protein Extraction Reagent was from Pierce (Rockford, IL). Oligonucleotides were from Operon Biotechnologies (Huntsville, AL) and restriction enzymes were from New England Biolabs (Ipswich, MA). Human (ID 6046773) and Murine (ID 30658833) MT1-MMP cDNA were purchased from Open Biosystems. DX-2400 anti-MT1-MMP antibody was provided as a generous gift from Dyax (Cambridge, MA). Galardin GM-6001 was purchased from Enzo life Sciences (Plymouth Meeting, PA).

Construction of Substrate and Plasmids

The two color CLiPS 5 mer random library (pBSGXP2) previously described (Boulware et al., 2010) was used to enrich for cleavable peptides for

MT1-MMP. Plasmid (pBSGXP2-2) was constructed with a streptavidin-binding peptide (WVCHPMWEVMCLR) (Rice et al., 2006), and linker (GGSGQSGLGSGSGSGSGQSG) on the N terminus of CPX, and binding peptide of SLP-76 (PAPSIDRSTKPPL, P2) on the C terminus. Plasmid pBSGXP2-2 was constructed by using the pBSGXP2 (Boulware et al., 2010) as a template and was amplified with primers 1 and 2 (Table S1) to remove the *Sfi*I site upstream of the SA-PE binding peptide. The PCR fragment was then amplified with primers 3 and 4 to append a *Sfi*I site downstream of the SA-PE binding peptide. Similarly, a 10 mer focused library of the form x-x-x-P-x-(G/P)-(L/M/I)-x-x-x was constructed with the pBSGXP2 as a template for PCR with primers 5 and 6. The PCR product was digested with *Sfi*I, ligated into similarly digested pBSGXP2-2, and transformed into electro-competent *E. coli* yielding 8×10^7 independent transformants.

The CyPet-YPet caspase substrate plasmid (pBCcpY) was used as a template plasmid to construct the MT1-MMP cleavable FRET substrates (Nguyen and Daugherty, 2005). The CyPet gene in pBCcpY was replaced with another having optimized codons for *E. coli* expression (Codon Devices). FRET substrates were constructed to encode the MT1-MMP substrates by amplifying YPet using the new plasmid template with forward primer 7 (all substrates except the 10.7) and 8 (for 10.7) to add a GGSQSAG linker on the N terminus of YPet, and reverse primer 9. The PCR fragment was amplified with reverse primer 1 and forward primers 10, 11, 12, 13, 14, and 15, to encode the substrates represented in 10.1, 10.2, 10.7, 5.1, a substrate reported in (Kridel et al., 2002), and a substrate reported in Turk (Turk et al., 2001), respectively. The PCR fragments were then digested with KpnI and *Sph*I and ligated with the similarly digested plasmid (pBCcpY-2) to yield the plasmid C-10.1-Y, C-10.2-Y, C-10.7-Y, C-5.1-Y, C-A42-Y, and C-MPL-Y.

Genes encoding the catalytic domains of mouse and human MT1-MMP (aa Y112-R299) were amplified with forward primers 16 and 17 and reverse primers 18 and 19, respectively, to append a *Sfi*I restriction site upstream of the C-terminal hexa-histidine tag. The resulting PCR products were amplified with forward primers 16 and 17 and reverse primer 20 to add a *Sfi*I restriction site at the C terminus. The PCR fragments were digested with *Sfi*I and ligated into a similarly digested plasmid (pBCcpY-2).

Identification of Peptide Substrates for MT1-MMP Using CLiPS

To screen the 5 mer random library, the overnight bacterial culture was subcultured 1:50 for 2 hr at 37°C and then induced with 0.1% (w/v) L-(+)-arabinose for 3 hr at room temperature. Approximately 10^8 cells were washed with reaction buffer, and then pelleted. The reaction buffer was 50 mM Tris-Cl (pH 7.5) supplemented with 20 mM NaCl, 2mM $CaCl_2$, and 10 μM $ZnCl_2$. The pelleted cells were then resuspended in 20 μl of reaction buffer and the volume was then divided in half. The first sample was incubated with 80 nM MT1-MMP and the second sample was incubated in the absence of enzyme, and both reaction samples were incubated at room temperature with shaking for 2 hr. The samples were then diluted 1:100 in phosphate buffered saline (PBS pH 7.4) to impede the reaction, then resuspended in PBS supplemented with 50 nM SA-PE and 250 nM *Alaj*GFP-Mona SH3 to label the cells and were incubated at 4°C for 1 hr. The labeled cells were then washed with PBS and analyzed on a FACSAria (BD Biosciences) with 488 nm excitation and fluorescence at 530 and 576 nm was measured. A total of eight sorts were performed, alternating between isolating for cells that properly display substrates while excluding potential *Alaj*GFP-Mona SH3 ligands in the absence of MT1-MMP (green+ red+) and then sorting cells with cleaved substrates after incubating with MT1-MMP (green+ red–). Cells from the final round of sorting were plated to isolate single clones for DNA sequencing. The 10 mer focused library was screened similarly, with the exception that the cells were incubated with MT1-MMP (60 nM) at room temperature for 1 hr with shaking, and after labeling the cells. A total of seven sorts were performed; four for cleavage and three for display, while alternating between sorting for display and for cleavage.

Characterization of the Activity of Cell Displayed Substrates

To measure the extent of conversion of cell displayed substrates, the overnight bacterial cultures of the clones from the final sort of the 5 mer random library were subcultured as before in duplicate samples, and substrate display was induced for 3.5 hr at room temperature. Cells were washed and resuspended in reaction buffer (10 μl) and the volume was then divided in half (each 5 μl), the first sample was incubated with 80 nM MT1-MMP and the second sample was incubated without enzyme (10 μl final reaction volume), and both reaction samples were incubated at room temperature with shaking for 45 and 75 min. Cells were washed and labeled with 50 nM SA-PE. The labeled cells were again washed in PBS and their fluorescence was measured by flow cytometry. Conversions for 10 mer substrates were similarly measured in duplicate after 20 and 40 min incubations at room temperature. Conversion was calculated using the expression:

$$conversion = \frac{F_{R-} - F_{R+}}{F_{R-} - F_0}, \quad (1)$$

where F_{R-} is the red fluorescence after incubation without the enzyme, F_{R+} is the red fluorescence after incubation with the enzyme, and F_0 is the fluorescence of unlabeled cells. It has been reported that the K_M of MT1-MMP for a short peptide substrate is ~10 μM (Toth et al., 2002). The Michaelis-Menton equation can then be simplified into a linear first order equation since the concentration of substrates expressed on the surface of the cells ([S]) is approximately 0.08 μM and $K_M >> [S]$. Thus, the second-order rate constant can be expressed as

$$\frac{k_{cat}}{K_M} = -\frac{Ln[(1 - Conversion)]}{[E] \cdot t}, \quad (2)$$

where [*E*] is the enzyme concentration, and *t* is the time at which the conversion measurement was taken (Boulware and Daugherty, 2006). The reported k_{cat}/K_M values are averages at the two different time points.

Measurement of MT1-MMP Activity Using Protease Beacons

The concentrations of active MMP-1, MMP-2, MMP-9, MMP-13, MT1-MMP, and MT2-MMP were determined by active site titration with MMP tight inhibitor Galardin. To determine the second-order rates constants of the substrates in soluble form, bacterial cultures expressing the C-terminal hexa-histidine tagged protease beacons were subcultured 1:100 in 40 ml volume for 2 hr at 37°C, and then induced with 0.2% (w/v) L-(+)-Arabinose for 18 hr at room temperature. Expressed protein was extracted from cells using B-PER II reagent and was further purified using affinity chromatography by using Ni-NTA and following the suggested protocol. The concentration of the protein was measured by using a standard curve of fluorescence intensity at 527 nm as a function of YPet concentration when excited with 514 nm. The hydrolysis reaction for each peptide was performed in triplicate in 100 μl total volume using the same reaction buffer with 120 nM FRET substrate. To monitor the cleavage of the peptides, the fluorescence emission at 485 and 527 nm was measured every 5 min upon excitation with 433 nm using a Tecan Safire fluorescence spectrophotometer (Tecan, Durham, NC). To determine the minimum concentration of MT1-MMP needed for hydrolysis detection, C-10.1-Y substrate was incubated with different concentrations of MT1-MMP (25 nM, 8 nM, 2.5 nM, 0.92 nM, and 0.3 nM) were incubated with C-10.1-Y at 37°C for 4 hr. The substrates were also treated with 8 nM MMP-1, 8 nM MMP-8, and 2.5 nM MT1-MMP at room temperature for 4 hr to analyze substrate selectivity. In a separate assay, the FRET substrates were also treated with 2.2 nM MMP-2, 3.9 nM MMP-9, 2.3 nM MMP-13, 3.2 nM MT2-MMP, and 1.5 nM MT1-MMP at 37°C for 4 hr to analyze substrate selectivity. The second-order rate constants (k_{cat}/K_M) were then calculated by fitting the substrate conversion to the simplified Michaelis-Menton equation (Equation 2). The extent of conversion of the FRET substrates at different time points was determined using the following equation:

$$Conversion = \frac{FR_i - FR_t}{FR_i - FR_{min}}, \quad (3)$$

where FR_i is the initial FRET ratio (527:485 nm), FR_{min} is the minimum FRET ratio, and FR_t is the FRET ratio at a particular time point after subtracting the background fluorescence. The second-order rate constant (k_{cat}/K_M) was determined by fitting to Equation 2. To determine whether endopeptidases present in heparinized human plasma could hydrolyze the FRET substrates, individual substrates (120 nM) were incubated in 10% plasma in 100 μl total volume using the same reaction buffer at 37°C for 4 hr. To determine if human plasma influences the activity of MT1-MMP, C-10.1-Y was incubated with 10%, 1%, and 0% plasma with 50 nM MT1-MMP in 100 μl reaction volume using same reaction buffer at 37°C for 4 hr. The hydrolysis of the substrates was monitored by measuring the emission fluorescence at 485 and 527 nm

as described (Boulware and Daugherty, 2006). To determine whether the peptide substrates were cross-reactive with murine MT1-MMP, both mMT1-MMP and hMT1-MMP catalytic domains were expressed recombinantly in *E. coli* then extracted from cells using B-PER II reagent and purified using Ni-NTA. The expressed proteases were then incubated with 1 nM hMT1-MMP (Biomol) for 1 hr at 4°C and 30 min at 37°C to auto-activate the proteases (Koo et al., 2002). Then 120 nM of C-10.1-Y was incubated with 10 μM of the expressed MT1-MMP. The FRET substrate was also incubated with the reaction buffer and 1nM MT1-MMP to verify that hydrolysis was not a result of the added protease. Substrate conversion was monitored by measuring the fluorescence emission at 485 and 527 nm.

Measurement of MT1-MMP Activity in Tumor Cell Lines Using Protease Beacons

To measure tumor cell-associated substrate cleavage, HT-1080 or MCF-7 cells were washed with reaction buffer and cultured in RPMI-1640 (without FBS or phenyl-red indicator) for 18 hr. Cells were then scraped and pelleted. The medium was collected and the cells were washed twice with 5 ml of reaction buffer. The FRET substrates at 120 nM final concentration were then incubated with the tumor cells in 100 μl reaction volume using the same reaction buffer at 37°C for 4 hr in duplicate. In another assay, the substrates were incubated with tumor cell conditioned media in duplicate to determine if any soluble protease can cleave the substrates. To determine whether hydrolysis is due to MT1-MMP and not other MMPs, HT-1080 cells were incubated with a highly selective anti-MT1-MMP antibody antagonist at 90 nM DX-2400 for 1 hr at 4°C. The cells were then incubated with 120 nM of C-10.1-Y at 37°C in 100 μl reaction volume. To determine if plasma can inhibit proteases produced by the tumor cells, the FRET substrate C-10.1-Y was incubated with HT-1080 cells with the following dilutions of human plasma in duplicate: 0%, 1%, 5%, and 20%, and hydrolysis was monitored as described above.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and two tables and can be found with this article online at doi:10.1016/j.chembiol.2010.12.017.

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