Introduction

Limited proteolysis, or proteolytic processing, affords a precise pathway for the control of protein function in addition to more traditional roles of nonselective protein breakdown and degradation. In recent years this represents a major shift in conceptualization of the in vivo role of proteases. Although activation of complement and clotting factors by precise processing by serine proteases has long been recognized, it is perhaps true to state that this concept gathered momentum in the '90s by the recognition of the pivotally important role of caspases, a family of intracellular cysteine proteinases, in apoptosis (Earnshaw et al., 1999). More recently, the matrix metalloproteinase (MMP) family is also being increasingly recognized as playing pivotal processing roles in a variety of cell processes by activating or inactivating other proteins and thereby initiating or terminating a wide repertoire of cellular processes. Proteolysis regulates the activity of many proteins resulting in enzyme activation or inactivation, conversion between functional states (e.g., receptor agonist to antagonist), or shedding from cell surfaces. The pericellular and plasma membrane environment is rich in proteolytic potential that modulates cell responses and differentiation by altering cell interactions and signaling after extracellular matrix remodelling, and facilitates migration of host response cells, regenerating tissue cells, and metastatic tumor cells. Thus, proteolytic regulation of cellular function and tissue integrity occurs in processes such as apoptosis, immune responses, cell migration and neuronal outgrowth, and hormone/cytokine processing. Not surprisingly, proteolytic activity therefore underlies several foetal and childhood developmental abnormalities and diseases. Hence, proteases and their substrates are important potential targets for new drug development.

Key words: Degradome/Degradomics/Exosite/Gelatinase A/MCP/MMP-2/SDF.

Review

Discovery of Chemokine Substrates for Matrix Metalloproteininases by Exosite Scanning: A New Tool for Degradomics

Increasingly it is being recognized that matrix metalloproteinases (MMPs) are important processing enzymes that regulate cellular behaviour and immune cell function by selective proteolysis of cell surface receptors and adhesion molecules, cytokines and growth factors. These functions will likely prove to be as important in vivo as the proposed roles of MMPs in pathological matrix degradation. To screen for new protease substrates we have reported a novel ‘exosite scanning’ strategy that utilizes protease substrate-binding exosite domains as yeast two-hybrid baits. We discovered that the chemokine monocyte chemoattractant protein-3 (MCP-3) binds the hemopexin C domain of gelatinase A (MMP-2) leading to its efficient cleavage, converting an agonist to a potent receptor antagonist. We have now found that other MMPs cleave MCP-1, MCP-2, MCP-3, MCP-4, SDF-1α and SDF-1β indicating that the intersection between the chemokine and MMP families is broad with important implications for the control of inflammatory and immune processes. Use of engineered substrates with altered exosite binding affinities further revealed the power of exosites in dictating proteolytic specificity – either directing cleavage of non-preferred sites or in other cases virtually eliminating proteolysis of readily accessible scissile bonds. Hence, bioinformatic searches for protease substrates based on scissile bond preference will only reveal a subset of substrates unless the influence of exosites is considered.

Key words: Degradome/Degradomics/Exosite/Gelatinase A/MCP/MMP-2/SDF.
cation of genomic and proteomic approaches to characterize proteases, their substrates and inhibitors on a system-wide scale in an organism (Figure 1). Accordingly, the term 'degradome' refers to the complete set of proteases expressed by a cell, tissue or organism at a particular time, in addition to defining the natural substrate repertoire of a particular protease in an organism (López-Otin and Overall, 2002).

Since the identification of proteolytic targets is much sought after for the development of new drugs, considerable efforts are being expended in developing screening technologies to identify new proteases and their substrates. Many screens have been devised to search for novel substrates including libraries of covalent inhibitors (Nazif and Bogyo, 2001), combinatorial substrate libraries (Harris et al., 2000), phage displayed peptide libraries (Deng et al., 2000), and mixture based libraries (Turk et al., 2001). These tend to be based upon indirect identification of substrates through definition of scissile bonds, often then relying on bioinformatics searches of databases to identify potential substrate proteins. However, the identification of a potential cleavage bond does not necessarily identify substrates that are relevant in vivo. New technologies are urgently required to identify in vivo substrates, their cleavage products in vivo, and the responsible protease. Our work has focused on the role of matrix metalloproteinases (MMPs) in biological processes and in developing screening techniques for biologically important MMP substrates in vivo.

Matrix Metalloproteinases Are Processing Enzymes

MMPs are an important family of twenty-three human tissue and leukocyte endoproteases that traditionally are considered to degrade the extracellular matrix in inflammation and a broad range of pathologies (reviewed by Overall, 1991; Nagase and Woessner, 1999). Increasing evidence strongly implicates MMP activity in the regulation of diverse cellular and physiological processes, but many of these biologically important substrates are unknown. The collagens alone efficiently cleave native interstitial collagens. Although several MMPs including the stromelysins and matrixins cleave denatured collagen (gelatin), gelatinolysis is most efficiently performed by gelatinase A (MMP-2) and gelatinase B (MMP-9). MMP-2 also degrades native collagen types I, IV, VII, X, and XI, elastin, laminin 5, entactin, galectin-3 and fibronectin (see www.clip.ubc.ca for a comprehensive updated list of MMPs and their substrates). Thus, gelatinases are likely critical links in the proteolytic cascade responsible for the degradation of tissues in many pathological processes although direct evidence for the role of MMPs in tissue degradation is limited (Sodek and Overall, 1988). Indeed, even recent studies tend to focus on the tissue degradative aspects of MMP activity in disease, not recognizing the effects of MMP processing that can activate or inactivate cytokines and growth factors or shed cell surface molecules: MMPs mediate the release and activation of cytokines such as Fas ligand (Powell et al., 1999; Fingleton et al., 2001), TNF-α (Gearing et al., 1994), MMP activity sheds fibroblast growth factor receptor-1 (Levi et al., 1996) and the heparin-binding epidermal growth factor (Suzuki et al., 1997), activates IL-8 (Van den Steen et al., 2000), and MMPs cleave and inactivate insulin-like growth factor binding proteins (Fowlkes et al., 1994), the CC chemokine monocyte chemoattractant protein (MCP)-3 (McQuibban et al., 2000), MCP-1, -2 and -4 (McQuibban et al., 2002), and the CXC chemokine stromal cell-derived factor (SDF)-1α and β (McQuibban et al., 2001). In view of the number of these recent examples of new MMP substrates, it is very likely that MMPs will be discovered to be important processing enzymes of an even more diverse range of many classes of bioactive substrates. The challenge is to identify these substrates, particularly those that may only be transiently expressed and present in pm or nm amounts in vivo.

Matrix Metalloproteinase Subsites and Exosites

The protease active site performs the two-fold functions of binding substrate and catalyzing peptide bond hydrol-
ysis. The efficiency of these actions defines protease substrate specificity. Like most other MMPs, MMP-2 is a multidomain enzyme that shares similar primary and tertiary structures with other MMPs (reviewed by Bode et al., 1999). MMP-2 and MMP-8 contain a unique 174-residue fibronectin type II module triple repeat (Coller et al., 1988; Wilhelm et al., 1988) inserted in a loop 16–19 Å from the active site Zn\(^{2+}\) and like all MMPs, except matrielysin (MMP-7), endometase (MMP-28), and CA-MMP (MMP-23), have a terminal hemopexin carboxyl (C) domain. The catalytic domain of MMP-2 consists of a 5-stranded, β-sheet flanked by three α-helices (A–C) (Morgunova et al., 1999). The MMP active site is formed by α-helix B containing the HExXH motif, a loop on the surface of the catalytic subdomain lying downstream from this motif that contains the third Zn\(^{2+}\)-coordinating histidine residue and methionine-turn, and β-strand IV that forms an antiparallel β-sheet with bound substrates. MMPs have virtually identical zinc environments (reviewed by Bode et al., 1999). Therefore, this points to the importance of individual amino acid substitutions in the active sites of the different MMPs in delineating the substrate-binding S\(_{1}\)–S\(_{4}\)' subsites flanking the catalytic Zn\(^{2+}\) ion, which recognize complimentary P\(_{1}\)–P\(_{4}\)' residues in the substrate.

In addition to active center subsites, other equally important determinants of protease specificity lie on discrete substrate binding domains located outside the active site (Overall, 2001). We termed these ‘exosites’ (Stefensen et al., 1995; Overall et al., 2000a) and have extensively studied and defined their pivotal role for cleavage of non-collagenous substrates by MMPs (Stefensen et al., 1995; Wallon and Overall, 1997; Overall et al., 2000a; McQuibban et al., 2000, 2001, 2002). The importance of MMP substrate binding domains was first demonstrated for collagenolysis (Clark and Cawston, 1989). Since the width of the active site cleft of MMPs is ~5 Å, only a single collagen α-chain can be bound and cleaved at a time. Therefore, the ~15 Å-diameter collagen triple helix must first locally unfold by an enigmatic ‘triple helicase’ mechanism (reviewed in Overall, 2002) to allow scission of the α1 and α2-chains. The hemopexin C domain of collagenase binds native collagen (Clark and Cawston, 1989; Murphy et al., 1992; Bigg et al., 1994). Hemopexin C domain deletion ablates collagenolysis but not catalytic competence; truncated collagenase still degrades synthetic peptide substrates and casein, but not native type I collagen (Clark and Cawston, 1989; Chung et al., 2000). Substitution of the two Cys residues disrupts the hemopexin C domain structure and causes the loss of type I collagenolysis, but not that of casein degradation (Windsor et al., 1991). Clamping of collagen to the active site by the domain may ‘splay’ the collagen (Bode et al., 1996). However, the MMP-2 hemopexin C domain does not bind native or denatured collagen types I, IV, V, or X substrates (Murphy et al., 1994; Wallon and Overall, 1997; Overall et al., 2000), leading us to propose a novel model for MMP-2 triple helicase activity (Overall, 2001) to account for the fundamental difference in molecular tendency of collagenolytic activity between the collagensases and gelatinases. Removal of the hemopexin C domain from stromelysin-1 (Okada et al., 1986; Koklitish et al., 1991; Allan et al., 1991; Murphy et al., 1992) or MMP-2 (Murphy et al., 1992; O’Connell et al., 1994) did not modify known substrate specificity. What then was the role for the hemopexin C domain in noncollagenolytic MMPs? The gelatinases bind tissue inhibitors of metalloproteinases (TIMPs) to the hemopexin C domain (Ward et al., 1991; Bigg et al., 1997; Overall et al., 1999) and this was thought to be a primary function of the domain. However, our recent investigations have revealed the importance of the hemopexin C domain in binding non-collagenous substrates. We showed that recombinant MMP-2 hemopexin C domain binds fibronectin (Wallon and Overall, 1997), a well-characterized substrate of the enzyme, and chemokines in interactions that are critical for efficient catalysis (McQuibban et al., 2000, 2001, 2002) by decreasing the $K_\text{m}$ and efficiently increasing $[S]_\text{total}$ compared to $[S]_\text{bulk}$ (Overall, 2001). Hence, the MMP hemopexin C domain appears to play important roles in both preparing complex substrates, such as collagen, for cleavage and tethering noncollagenous substrates to MMPs for more efficient proteolysis. As described here we also provide evidence that exosites may shield or exert a negative influence on substrate cleavage. That is, our discoveries overall lead us to suggest that exosite domain function extends beyond substrate recognition and binding and may also repel or sterically block potential substrates and so reduce the likelihood of cleavage of even readily available scissile bonds.

‘Exosite Scanning’ by Yeast-Two Hybrid Screens

In order to search for new MMP substrates we developed a highly novel screen to identify protease substrates using, for the first time, the yeast two-hybrid system (McQuibban et al., 2000). The catalytic domain of a protease as ‘bait’ would cleave ‘library’ substrates in the yeast host cell and so eliminate any transcription signal; therefore, we devised a strategy that utilized a potential exosite domain as bait. Although the MMP hemopexin C domains had not been previously described as contributing to catalysis of non-collagenous proteins by MMPs, we hypothesized that proteins which bind the hemopexin C domain of MMP-2 in the yeast 2-hybrid system may be substrates of the enzyme. We term this approach ‘exosite scanning’. We first needed proof-of-principal that this system would be amenable for screening extracellular targets containing disulfide bonds. The yeast two-hybrid system is thought to be primarily useful for intracellular proteins and not for extracellular proteins containing disulfide bonds. However, we reasoned that disulfide bonding is often required for protein stability in harsh environments and that the inherent fold of the protein may still form in the absence of stabilizing cross-links under
conditions that do not stress the protein. Hence, the yeast growth conditions at 30°C may permit at least some proteins that normally are functional at 37°C to adopt their correct conformation in the absence of disulfide bond formation. TIMP-2 is known to bind the hemopexin C domain of proMMP-2 (Ward et al., 1991; Overall et al., 1999). Therefore, we transfected yeast cells with the appropriate constructs expressing β-Gal DNA binding and activator domains fused with the TIMP-2 C domain, which contains 3 disulfide bonds, or the MMP-2 hemopexin C domain, which contains a single disulfide bond. Vigorous colony growth and strong β-gal activity confirmed that productive transcription complexes had formed in the transfected yeast cells on complex formation between the MMP-2 hemopexin C domain and the TIMP-2 C domain constructs (Overall et al., 2000b; McQuibban et al., 2000). Therefore, we reasoned that this system was suitable as a screen for extracellular disulfide-containing protease substrates using a disulfide-containing domain as bait (McQuibban et al., 2000).

**Exosite Scanning Identification of MCP-3 as a MMP Substrate**

Exosite scanning for substrates in a human gingival fibroblast cDNA library using the hemopexin C domain of MMP-2 as a yeast two-hybrid bait resulted in a full-length ‘hit’ that identified monocyte chemoattractant protein (MCP)-3 as a potential interactor (McQuibban et al., 2000). MCPs are CC chemokines containing a canonical disulfide bond duplex originating from a CC sequence 10 residues from the N-terminus. As members of a ~54-member super-family of chemoattractant cytokines responsible for directing and maintaining leukocyte traffic throughout the body, MCPs are pivotal stimulators of monocyte and lymphocyte chemotaxis and function. Chemokine receptor usage specifies cell type migration and receptor affinities dictate potency. MCPs are produced locally in tissues for the directed migration of leukocytes to sites of host challenge in infection, inflammation and cancer. The MCP family targets multiple leukocyte subsets (monocytes, basophils, eosinophils, dendritic cells, and NK cells) whereas in the initial phases of inflammation several CXC chemokines are important for attracting PMNs. The development of inflammatory lesions is absolutely dependent upon chemokine expression and inflammatory/immune cell responsiveness. However, the mechanisms of clearance of chemokine gradients that lead to inflammation resolution are very poorly understood.

Our biochemical analyses showed that the chemokine MCP-3 represents an entirely novel substrate class not previously described for MMPs. By chemical cross-linking and binding studies we provided direct evidence that the 76-amino acid residue MCP-3 binds the hemopexin C domain of MMP-2 with a dissociation constant (K_d) of 0.4 × 10^{-8} M. Electrospray ionization time-of-flight mass spectrometry and N-terminal sequencing of the MCP-3 degradation fragments revealed that the scissile bond was Gly^4—Lle^5 — the preferred scissile bond in gelatin for this enzyme. We designated this cleavage product MCP-3 (5–76). Enzyme kinetic analyses revealed that MMP-2 cleavage of MCP-3 was extremely efficient and with a k_cat/K_m of ~800 μm⁻¹s⁻¹, MCP-3 is a considerably better substrate than gelatin. In contrast, removal of the hemopexin C domain reduced the turnover rate to ~500 μm⁻¹s⁻¹ revealing the importance of this exosite in potentiating cleavage. Moreover, addition of exogenous hemopexin C domain substantially reduced cleavage of MCP-3 by MMP-2, whereas addition of exogenous recombinant collagen binding domain (CBD) comprised of the three fibronectin type II modules of MMP-2 (Steffensen et al., 1995) had no effect. Thus, the hemopexin C domain of MMP-2 plays an important role in the binding of MCP-3 substrate, an interaction that markedly enhances catalysis.

**MMP-Cleaved MCP-3 Is a CCR Antagonist**

The effects of cleavage of the N-terminal tetrapeptide from MCP-3 were then investigated. In calcium flux and trans-well migration assays, MMP-2-mediated cleavage of MCP-3 not only resulted in loss of bioactivity as evident from the abrogation of CC receptor (CCR)-induced intracellular calcium mobilization and loss of cell chemotaxis, but also generated a potent receptor antagonist for native MCPs that bind CCR-1, -2 and -3. Moreover, this effect is amplified since cleaved MCP-3 antagonizes other chemokines that bind these receptors such as macrophage inflammatory protein-1α. Hence, MCP-3 (5–76) was shown to be a broad spectrum CCR antagonist in vitro (McQuibban et al., 2000). In mouse models of inflammation, the MMP-cleaved MCP-3 almost totally eliminated monocyte infiltration in subcutaneous blisters in a concentration-dependent manner and, by fluorescent activated cell sorting analysis, reduced mononuclear infiltration in zymosan-induced peritonitis by 40% up to 4 h after administration of the antagonist. Importantly, the pathophysiological relevance of this was also demonstrable in man. We isolated MMP-2/MCP-3 complexes from synovial fluid of arthritis patients and conclusively identified the cleaved MCP-3 fragment in rheumatoid synovial fluids using a neoepitope antibody strategy, providing direct evidence for the involvement of MMPs in inactivating MCP-3 in human disease. In more recent work (McQuibban et al., 2002), we have found that other MMPs (including MMP-1, MMP-3, MMP-13, and MMP-14) cleave MCP-3 but important MMPs expressed by leukocytes (MMP-8 and MMP-9) do not. This implies that in vivo leukocytic cells migrate without destroying their cognate chemoattractant gradients, lending credence to the notion that this is an important intersection between the MMP family and immune cell signaling networks. Thus, not only are MMPs induced in the stroma by cytokines in inflammation and cancer, but
these enzymes can in turn down-regulate chemokine activity to dampen inflammatory and immune processes in a self-attenuating feedback loop.

Although inflammatory chemokines are important for the development of inflammation where it is desirable to promote immune responses and to remove damaged tissue or bacteria, in chronic inflammation chemokines are often detrimental. The continued chemokine-directed influx and activation of inflammatory and immune cells in chronic inflammatory lesions leads to tissue destruction and loss of function. The inappropriate release of proteins such as MMPs from resident tissue cells and infiltrating inflammatory cells upon cytokine stimulation causes pathological connective tissue destruction. The conventional treatment to ameliorate these lesions is to reduce the inflammatory response by anti-inflammatory agents such as corticosteroids, ibuprofen, and cyclosporin. We suggest that in chronic inflammatory diseases the natural generation of endogenous chemokine antagonists by MMP activity is not sufficient to resolve chronic inflammation because of the concurrent inappropriate excessive stimulation of chemokine release. However, exogenously administered broad-spectrum chemokine antagonists may prove effective in breaking the cycle of excessive chemokine release and leukocyte attraction. Although low molecular weight chemokine receptor inhibitors are attractive candidates and have provoked considerable interest by pharmaceutical companies, modified chemokines are also very attractive because they retain binding specificity and bind with very high affinity at nm concentrations to receptors. By competing with natural ligands, MMP-cleaved forms of chemokines block binding and signaling and so have the potential to therapeutically modulate inflammatory diseases.

Multiple MMPs Cleave MCP-1, -2, -3 and -4

Following our presentation of the first evidence for matrix metalloproteinase cleavage and inactivation of any chemokine in vivo we have screened other chemokines that may be MMP substrates. We have found that MCP-1, MCP-2 and MCP-4 are efficiently cleaved by the MMP stromelysin-1 at position 4–5 and that collagenase can also cleave MCP-1 and MCP-4 (McQuibban et al., 2002). Notably, for MCP-4, proteolysis proceeds further generating MCP-4 (8–75). In all cases, MMP cleavage results in loss of agonist activity, although for MCP-1 this was not complete with approximately 20% agonist activity retained. Moreover, all cleaved MCPs were antagonists for their cognate receptors as measured in chemotaxis assays using CCR-transfected B19 300 pre-B cells. Agonist potency of MCP-1 (5–76), MCP-4 (8–75) and MCP-3 (5–76) were compared in vivo utilizing a carrageenan-induced inflammation model in the rat paw. Twenty-four hours after the induction of inflammation, either PBS or chemokine antagonist were administered to the paw and the oedema measured 12 h later. Compared with the rat paws injected with PBS that experienced a continued increase in inflammatory oedema, MCP-1 (5–76) and MCP-4 (8–76) reduced this by ~66%. However, it was striking to see that the broad spectrum CCR antagonist MCP-3 (5–76) reduced inflammation ~1.4-fold to a level below that at the time of antagonist injection. Hence, precise processing of all MCPs by multiple members of the MMP family results in loss of agonist activity and the generation of receptor antagonists that have the demonstrated potential to control inflammatory responses in vivo.

MMP Cleavage of SDF-1α and SDF-1β

We have also commenced an examination of CXC chemokines for cleavage by MMPs. Recently we reported that stromal cell-derived factor-1α and β are novel substrates for a number of MMPs including MMP-1, MMP-2, MMP-3, MMP-9, MMP-13 and MMP-14 (McQuibban et al., 2001). Once again cleavage specifically occurred at position 4–5, which abrogated agonist activity, but did not generate a CXC receptor-4 (CXCR4) antagonist. This indicated that the N-terminal 4 residues of SDF-1α contribute markedly to the total binding affinity for the receptor. Since CXCR4 is expressed on CD34+ and B cells, we assessed the effects of MMP cleavage of SDF-1α on cell migration of pre-B NALM-6 and CD34+ haematopoietic stem cells. In both cases we found a total loss of chemoattractant activity. We proposed that GCSF induction of stem cell mobilization from bone marrow may result from loss of the SDF-1α retention and homing signal rather than through MMP degradative effects such as penetration of basement membrane. This would lead to stem cell egress from the marrow. CXCR4 is also the coreceptor for T-tropic HIV strains. By binding CXCR4, SDF-1α is known to protect against infection. We found that MMP-cleaved SDF-1α had lost its ability to protect CD4+ cells from HIV infection (McQuibban et al., 2001). Interestingly, HIV is known to induce MMP expression in cells (Johnston et al., 2000) and so this may prove to be the mechanism of action for this virulence factor. Hence, our data suggests that MMP inhibitor drugs may prove efficacious in slowing HIV infection rates.

Exosites: Powerful Modulators of MMP Activity

To examine the mechanistic aspects of exosite binding on MMP function we utilized engineered chemokine substrates. MCP-3 binds the hemopexin C domain of MMP-2 and that of MT1-MMP, which we have also shown cleaves MCP-3 (McQuibban et al., unpublished), indicating that exosite usage is likely to be a general phenomenon and an important determinant of proteolytic activity throughout the MMP family. In contrast, MCP-1 is neither cleaved by MMP-2 nor is it bound by the hemopexin C domain and it lacks the Gly-Ile scissile bond at position 4–5. The N-terminal 10-amino acid residues of the...
Exosite scanning revealed for the first time the utility of the yeast two-hybrid system as a novel tool for the discovery of protease substrates (McQuibban et al., 2000).

Although this approach lacks high throughput utility, a significant advantage of genomic screens over proteomic approaches is that they tend to minimise negative bias against rare proteins that may be expressed in very small quantities in vivo or only transiently. In addition to using exosite domains as yeast-two hybrid baits, we have also used the MMP catalytic domain as bait in two novel approaches. We hypothesized that substrates will bind inactivated catalytic domain mutants without cleavage to produce a positive transcription response in yeast cells or a stable complex in solution from which the bound substrate can be identified by tandem mass spectrometry. Using inactive catalytic domain capture (ICDC) with inactive MT1-MMP catalytic domain as a yeast-two hybrid bait we have identified known MMP substrates such as collagen and laminin-5 as well as growth factors, not previously recognized as MMP substrates, as interactor proteins. This new approach should enable screening for substrates of multidomain proteases that do not require exosite binding or for proteases not having exosite domains. Thus, a distinct advantage of genomic screens to search for new protease substrates can be seen to be derived from the ability to detect low abundance proteins such as cytokines and cell surface proteins. Bioactive molecules that initiate or modulate signaling cascades and that are cleaved by proteases should prove to be some of the most biologically important protease substrates that are processed in the body. The identification of these molecules and the responsible proteases will likely prove to be a continuing challenge, but one that should provide great insight into the normal mechanisms of regulating cell behaviour and its aberration in disease.

References


