CHARACTERISATION OF THE COLLAGEN BINDING DOMAIN OF GELATINASE A: INVOLVEMENT OF SPECIFIC RESIDUES IN THE FIBRONECTIN TYPE II MODULES IN SUBSTRATE RECOGNITION

by

TODD ROBERT MOORE

B.Sc., The University of British Columbia, 1999

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENT FOR THE DEGREE OF
MASTER OF SCIENCE
in
THE FACULTY OF GRADUATE STUDIES

(Department of Oral Biological and Medical Sciences)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April 2001

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Oral Biological
and Medical Sciences
The University of British Columbia
Vancouver, Canada
Date ....April 27, 2001.....

Abstract

The matrix metalloproteinase (MMP) family of endopeptidases can collectively degrade many components of the extracellular matrix. Their proteolytic activities have been implicated in normal processes such as extracellular matrix turnover and certain pathological conditions such as periodontitis, arthritis, and tumour metastasis.

Based upon domain composition gelatinase A is separated from the other MMPs by the insertion of three contiguous fibronectin type II modules into its catalytic domain. Since it was determined that a recombinant domain consisting of the three fibronectin type II modules bound to native type I collagen this domain was termed the collagen binding domain (CBD). The function of the CBD is to donate substrate binding exosites to allow for broader enzyme specificity. Considering that the CBD contains exosites for binding native/denatured collagen, this domain was subjected to site-directed mutagenic studies to elucidate essential residues involved in collagen binding.

The binding properties of the collagen binding domain to denatured type I collagen (gelatin) was investigated using a recombinant protein constructed of the second and third fibronectin type II module (rCBD23). Ten mutations were performed within the rCBD23 protein. Since the binding of substrate to the CBD is via a hydrophobic pocket, the mutations F264A, F264Y, F266A, F266Y, F322A, F324A, F322Y, F324Y, F264A/F322A, and F266A/F324A were introduced into rCBD23 in order to determine the effect of removing hydrophobic character.

It was found that there was a decrease in the binding of the mutant proteins that was proportional to the distance between the mutated residue side chain and a strictly conserved tryptophan at the base of the hydrophobic pocket. Complete abrogation of gelatin binding was observed in the double mutant F266A/F324A. The observed effect was proposed to be the result of destabilisation of the strictly conserved tryptophan that forms the base of the hydrophobic pocket.

These studies have furthered our knowledge and understanding of the interactions of CBD with substrate. The present study, combined with results from other studies, could be used to synthesize compounds that are specific to the CBD and are able to irreversibly inhibit the binding of CBD to substrate. A drug that would preferentially block binding of the CBD to collagen is proposed to reduced the metastasis of tumour cells by abolishment of type IV collagen binding. The overall effect is decreased tumour migration from the primary site of development and therefore substantially decreasing tumour progression.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

BSA:	Bovine serum albumin
C domain:	Carboxyl-terminal domain
CBD:	collagen binding domain
CD:	Circular dichroism
cDNA:	Complementary DNA
COL:	collagenous domains
COSY:	chemical shift correlated spectroscopy
DMSO:	dimethyl sulfoxide
DTT:	Dithiothreitol
ECL:	Enhanced chemiluminescence
ECM:	extracellular matrix
EGF:	Epidermal growth factor
FACITs:	fibril-associated collagens with interrupted triple helicies
FPLC:	Fast protein liquid chromatography
GAGs:	glycosaminoglycans
Gelatin:	Denatured collagen
FN:	fibronectin
KDa	kilodaltons
MCP-3:	monocyte chemoattractant protein-3
MMPs:	matrix metalloproteinase
MT-MMP	Membrane-type MMP
NC:	noncollagenous domains
Nm	Nanomenter

NMR:	Nuclear magnetic resonance
r:	Recombinant
PAGE:	Polyacrylamide gel electrophoresis
PBS:	phosphate-buffered saline
PMSF:	Phenylmethylsulfonyl fluoride
TGF-β	Transforming growth factor-β
TIMP:	tissue inhibitor of metalloproteinase
TPA:	12-O-tetradecanoylphorbol-13-acetate
SDS:	Sodium dodecyl sulphate

Acknowledgements

The road to this junction in my life has been long and very challenging. As long ago as elementary school I remember the below average grades and the wonderment of my teachers when they saw that I was underachieving at everything I tried to do scholastically. The one thing that remained is they all believed. They believed that I could do this and they were right. From Mr. Brendzy in grade 5, to Mr. Rootman in grade 6, and to Mr. Baptie in grade 11 and 12, they all saw a spark inside that at some point in time would ignite.

My path into biochemistry was paved during my studies at Langara Community College when my instructor Mike Holmwood took an afternoon to enlighten me as to what courses I needed to take in order to learn about the complexities of life at the molecular level.

My curiosity for science and biochemistry blossomed when I became a member of the Overall laboratory. Within the fertile confines of the Overall lab, Dr. Chris Overall took me under his wing, kindled my inquisitiveness, and showed me that there is a plethora of knowledge to be extracted from our world and I thank him. My many thanks are extended to all of the members of the Overall laboratory for their guidance during my time within the Overall laboratory. To Gayle for helping to supervise my 449 project, to Angus for keeping the lab energized, to Andrea for being my "CBD buddy", to Dr. B for keeping me inline, to Eric for "keeping it real", to Dennis for helping me with my project and being my buddy, to Clive for his tonnes of knowledge, and to Heidi (Boo) for our invigorated discussions and for being there whenever I needed her.

The most important thanks go out to my parents and sister for their understanding during my growing years. They stoically endured all of the above times in my life and are therefore to be congratulated for their ever enduring presence during my years of study and also must too celebrate in the closure of this chapter of the book that is my life. *vene vidi vici.*

Introduction

Extracellular Matrix Components

It has now been determined that the extracellular matrix (ECM) constitutes many diverse complex structures that provide a mechanical scaffold for cellular adhesion and migration. This mechanical scaffold determines the histochemical substructures specific to every organ thus providing cells with the correct biological information for growth and differentiation (Aumailley and Gayraud, 1999). Intense recent work dedicated to the elucidation of important structures and functions of the ECM has now further established that the ECM profoundly influences the major cellular processes of growth, differentiation, migration, and apoptosis (Boudreau and Jones, 1999). The ECM is composed of a variety of versatile proteins, glycoproteins, and polysaccharides that are secreted locally and assembled into an organized meshwork in close association with the surface of the cell that produced them. In connective tissues, the ECM is frequently more abundant than the cells that it surrounds and thus is a major determinant of the physical properties of the tissue. Collagen fibres form the architectural framework of the vertebrate body with varying amounts being found in different organs and structures such as skin and bone, where they are found to be the major component. In contrast, in other tissues such as the brain and spinal cord, connective tissue is only a minor component of the organ (Aumailley and Gayraud, 1999).

In most connective tissues, the matrix molecules are secreted predominantly by fibroblasts (Streuli, 1999). There are two main classes of molecules in the ECM: (1) the fibrous proteins that perform either a structural function (collagen and elastin) or an adhesive role (fibronectin and laminin) and (2) the ubiquitously distributed glycosaminoglycans (GAGs) and proteoglycans that perform many roles within the extracellular space (Hay, 1991). However there are approximately 140 molecules that comprise the entire ECM, plus many that also have variants based on alternative splicing.

Collagenous components

General Structure and Biosynthesis

The most abundant structural component of the ECM is collagen. Collagen is secreted by connective tissue cells as well as a variety of other cell types (Alberts *et al.*, 1994). The characteristic feature of collagen is its long, stiff, triple helical structure in which three collagen polypeptide α chains are wound around each other into a coiled-coil conformation. Collagen has an unusual amino acid composition in which glycine, proline and hydroxyproline are the dominant amino acids. Further characterization of the α chains shows that these amino acids are arranged into a repetitious tripeptide sequence Gly-X-Y, that forms the collagenous (COL) domains and noncollagenous portions (NC domains) of variable length and location. Within the repetitious motif, X is frequently Pro and Y is frequently an hydroxyproline. The placement of a glycine at every third position allows each individual α chain to pack very tightly, therefore creating a formidable challenge to proteolytic attack. The repeating Pro residues exclude

the possibility that the polypeptide chains in collagen could adopt an α-helical or β-sheet conformation. Instead, individual collagen polypeptide chains assume a poly-L-proline-II helical secondary structure that aggregates into three-stranded cables with a right-handed twist (Zubay *et al.*, 1995). Based on differing primary sequence, 34 different α chains have been cloned and sequenced, several of them being differentially spliced, to yield even more diversity within the collagen superfamily. Up to now 19 different combinations of α chains (collagen types I-XIX) have been either identified or predicted to exist within the superfamily of vertebrate collagens (Aumailley and Gayraud, 1999) (Table 1).

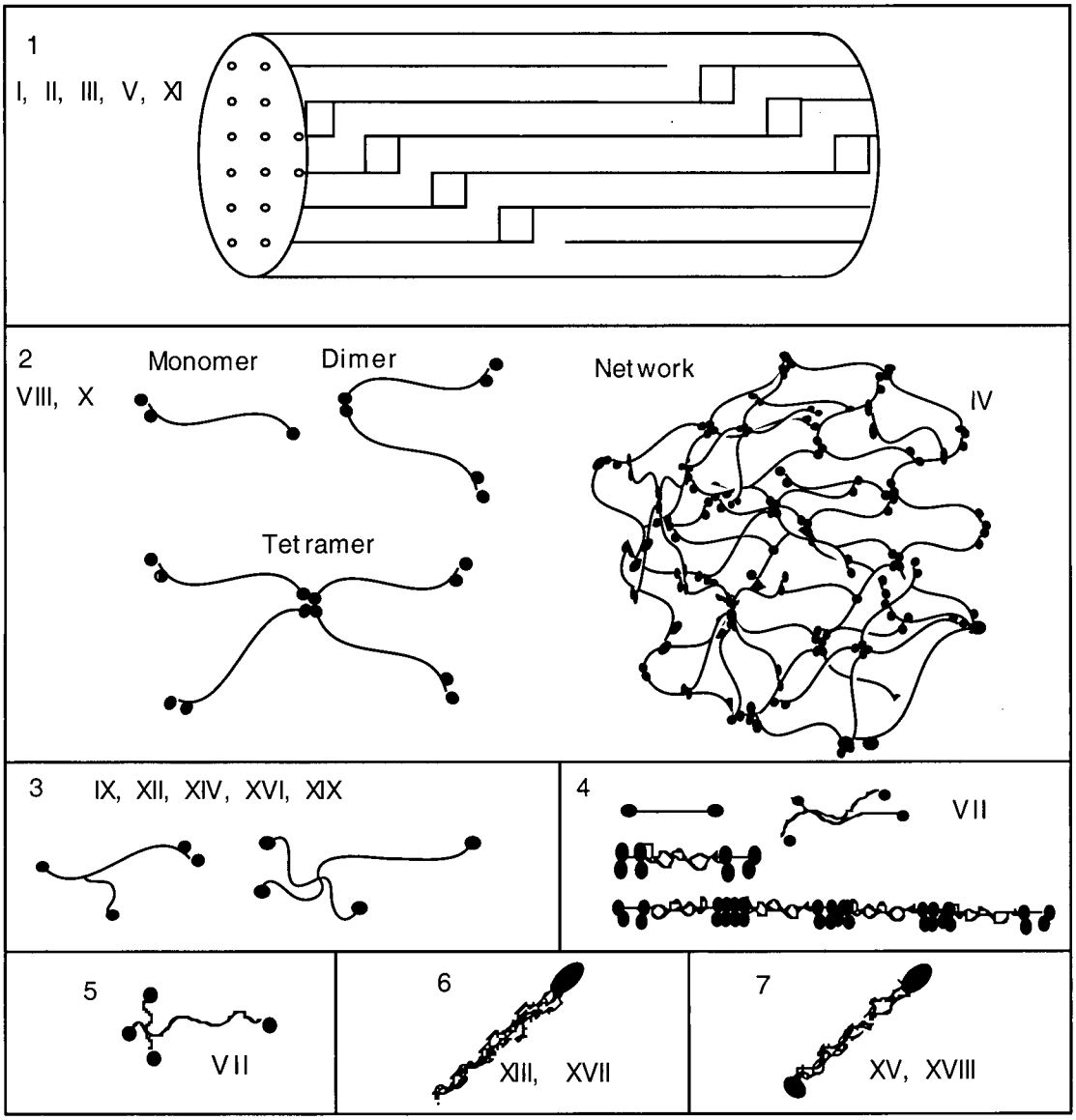
Collagen Families

Collagens have the ability to form highly organized polymers. The different collagen types can be grouped into several classes: (1) the fibril-forming collagens (types I, II, III, V, and XI), (2) the network forming collagens (basement membrane type IV and types VIII and X), (3) the collagens that associate with the surface of fibrillar collagen and thus are referred to as the fibril-associated collagens with interrupted triple helicies (FACITs; that include types IX, XII, XIV, XVI and XIX), (4) the collagen that forms beaded filaments (type VI), (5) the collagen that forms anchoring fibrils for basement membranes (VII), (6) the collagens with transmembrane domains (XIII and XVII), and (7) the recently named multiplexins (multiple triple-helix domain and interruptions, XV and XVIII) (Prockop and Kivirikko, 1995) (Figure 1). For the focus of this thesis the two classes of collagens will be reviewed are: 1) the fibrillar collagens (with special

Table 1: The Collagen Family and Subfamilies

Type	Molecular Composition	Tissue Distribution
Fibrillar collagen		
I	$[\alpha_1(I)]_2\alpha_2(I)$	Most connective tissue
II	$[\alpha_1(II)]_3$	Cartilage, Vitreous humor
III	$[\alpha_1(III)]_3$	Extensible connective tissues
V	$[\alpha_1(V)]_2\alpha_2(V)[\alpha_1(V)\alpha_2(V)\alpha_3(V)]$	Tissues containing type I
XI	$[\alpha_1(XI)\alpha_2(XI)\alpha_3(XI)]$ and chimeras between $\alpha(V)$ and $\alpha(XI)$	Tissues containing type II
Network-forming Collagen		
IV	$[\alpha_1(IV)]_2\alpha_2(IV)$	Basement Membranes
VIII	?	Many tissues, especially endothelial
X	?	Hypertrophic cartilage
Fibrillar-associated collagen with interrupted triple helicies		
IX	$[\alpha_1(IX)\alpha_2(IX)\alpha_3(IX)]$	Tissues containing type II
XII	$[\alpha_1(XII)]_3$	Tissues containing type I
XIV	$[\alpha_1(XIV)]_3$	Tissues containing type I
XVI	$[\alpha_1(XVI)]_3$	Many tissues
XIX	?	Rhabdomyosarcoma cells
Beaded filament		
VI	$[\alpha_1(VI)\alpha_2(VI)\alpha_3(VI)]$	Most connective tissues
Anchoring Fibrils		
VII	$[\alpha_1(VII)]_3$	Anchoring fibrils
Transmembrane-containing domains		
XIII	?	Many tissues
XVII	$[\alpha_1(XVII)]_3$	Skin hemidesosomes
Multiplexins		
XV	?	Many tissues
XVIII	?	Many tissues, especially liver and kidney

Figure 1



Schematic representing the different structure of collagen molecules. (1) Fibrillar collagens - Collagen fibrils align themselves in a staggered fashion along the axis of collagen fibril, (2) Network-forming collagens - The collagen fibres organise themselves according to their NC domains to form a mat-like structure, (3) FACITs - These collagens are found on the surface of the complexed fibrillar collagens, (4) Beaded filament collagens, (5) Anchoring fibrils - anchors the basement membrane to the underliyng type IV collagen and laminin of the ECM, (6) Collagens with transmembrane domains, and (7) The multiplexins. Figure adapted from Prockop and Kivirikko (1995)

interest dedicated to type I collagen) and 2) network forming collagens (primarily basement membrane type IV collagen).

Fibril-Forming Collagens

The fibril-forming collagens are similar in size and structure. These collagens are synthesized and secreted as larger precursor molecules, the procollagens, and are subsequently N- and C-terminally processed by specific extracellular proteinases to collagens. This processing results in formation of mature collagen molecules that contain large (300 nm) triple-helical domains of approximately 1000 amino acids that are terminated by very short NC sequences, the telopeptides. In a process termed fibrillogenesis, the collagen molecules assemble into cross-striated fibrils where each molecule is displaced about one-quarter of its length relative to its nearest neighbor (Figure 1). This results in the formation of two major tissue-specific fibrillar polymers: the type II collagen containing fibrils typical of cartilage and the collagen I containing fibrils of interstitial connective tissues. The other types of fibril forming collagens have a more varied tissue distribution (Prockop and Kivirikko, 1995) (Table 1).

Network-Forming Collagens

The network-forming collagens include the family of type IV collagens that constitute basement membranes, and the respective type VIII and X collagens of endothelial and hypertrophic cartilage. The COL domain of a type IV collagen molecule is longer than that found in the fibril-forming collagens and consists of ~1400 amino acids of the -Gly-X-Y- motif that are frequently interrupted by short

NC sequences. The N-terminus of a collagen molecule contains a small NC domain and the C-terminus contains a larger more significant NC domain of ~230 amino acids. Type IV collagen molecules are capable of self assembly into net-like structures whereby the collagen monomers dimerize at the C-terminal and tetramerize at the N-terminus. In addition to these end-to-end interactions, the triple-helical domains intertwine to form supercoiled structures (Prockop and Kivirikko, 1995) (Figure 1).

Noncollagenous components -

Glycosaminoglycans

The glycosaminoglycans (GAGs) are unbranched polysaccharide chains comprised of repeating sugar-aminosugar disaccharide subunits that are highly negatively charged. The GAGs can be broken into four main groups based on their residue linkage, residue number and the location of the sulphate groups: (1) hyaluronan, (2) chondroitin and dermatan sulphate, (3) heparan sulphate and heparin, and (4) keratan sulphate. Due to the large number of negatively charged sulphate groups, positively charged cations (Na^+) are attracted to the GAGs and in turn set up an osmotic gradient that causes water to diffuse into the extracellular space thus creating hydrostatic turgor pressure. Therefore, within the ECM the GAGs adopt a highly extended conformation that occupies a very large volume relative to their mass. The GAG chains fill most of the extracellular space. Except for hyaluronan, all of the GAGs are found covalently attached to a core protein in the form of a proteoglycan (Alberts *et al.*, 1994).

Proteoglycans

Proteoglycans can be considered to constitute a distinct subset of noncollagenous glycoproteins containing GAG side chains that range in weight from 10 to 600 kDa (Table 2). The associated GAG side chains of proteoglycans perform a major transient role in chemical signaling between cells by constituting extracellular gels of varying pore size and charge density that serve as a selective sieves which regulate the trafficking of macromolecules and cells according to their size and/or charge (Aumailley and Gayraud, 1999). It has been shown that several growth factors can be bound by either the GAG side chains or the core protein of proteoglycans as illustrated respectively by fibroblast growth factor binding to heparan sulphate (Moscatelli, 1987) and TGF-β binding to the core protein of decorin (Fukushima, *et al.*, 1993). Proteoglycans have also been shown to bind and regulate the activities of proteases and protease inhibitors via different mechanisms including: sequestration, steric hindrance, pooling, protection from proteolytic degradation, and alteration of local concentration for more effective presentation to cell surface receptors (Streuli, 1999).

Glycoproteins

In addition to collagens and proteoglycans, many glycoproteins are building blocks of the ECM (Table 2). Elastin and fibrillins are ubiquitous proteins of connective tissues that form the so-called elastic fibres, in which elastin corresponds to the amorphous material and fibrillins, together with other small proteins, constitute the associated 10-12 nm microfibrils (Aumailley and Gayraud,

Table 2: Structural glycoproteins and proteoglycans of the ECM

Glycoproteins

- Interstitial connective tissue
  - Fibronectins
  - Tenascins
  - Fibrillins
  - Elastin
  - Microfibril-associated
  - Matrilins
  - Thrombospondins
- Basement Membrane
  - Laminins
  - Nidogen/entactin
  - Fibulin

Proteoglycans

- Small leucine-rich proteoglycans
  - Decorin
  - Biglycan
  - Fibromodulin
  - Lumincin
  - Epiphycan
- Modular proteoglycans (hyaluronan- and lectin-binding)
  - Aggreacan
  - Versican
  - Neurocan
  - Brevican

1999). Fibrillins are a family of proteins consisting almost entirely of epidermal growth factor motifs.

The best studied ECM glycoprotein is fibronectin (FN). The molecule has two subunits (250-280 kDa) disulphide bonded to each other to form a dimer with two 50 nm arms (Figure 2). The amino terminus of FN contains three types of repetitive modules: 12 modules of type I homology, 2 modules of type II homology, and 15-17 modules of type III homology. The latter contributes up to half of the molecule and is present in variable numbers due to the complex alternative splicing of a single FN gene thus giving rise to at least 20 variants in humans (Schwarzbauer and Sechler, 1999). As a dimeric ligand, fibronectin can induce receptor clustering by binding two or more integrins. These integrin-FN clusters create a relatively high local concentration of fibronectin at the cell surface thus helping to promote FN self association and FN-cytoskeletal interactions. Collaborations between the fibronectin matrix and growth factors can regulate cell differentiation and migration (Schwarzbauer and Sechler, 1999).

The most abundant glycoproteins of basement membranes are the laminins (Figure 3). Laminins have been implicated in many biological processes, including cell adhesion, migration, and differentiation (Hay, 1991). Laminins are constructed from three genetically distinct polypeptides, the α, β, and, γ chains (Aumailley and Gayraud, 1999). After assembly of the three distinct polypeptides the final mass of the laminin molecule can reach one million Daltons (Hay, 1991). Laminin has a number of binding sites including, one for type IV collagen, one for

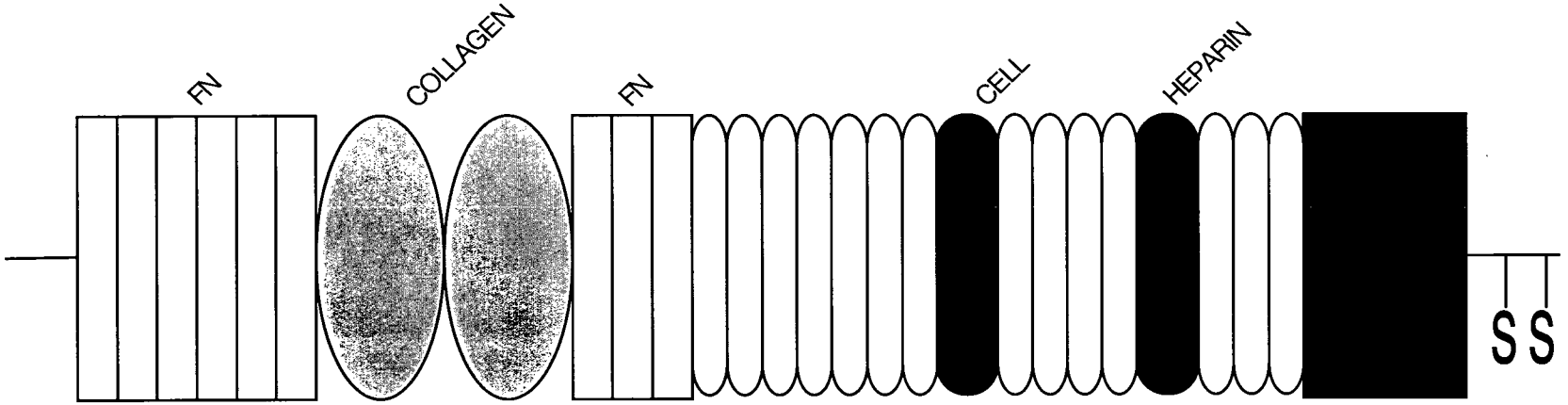
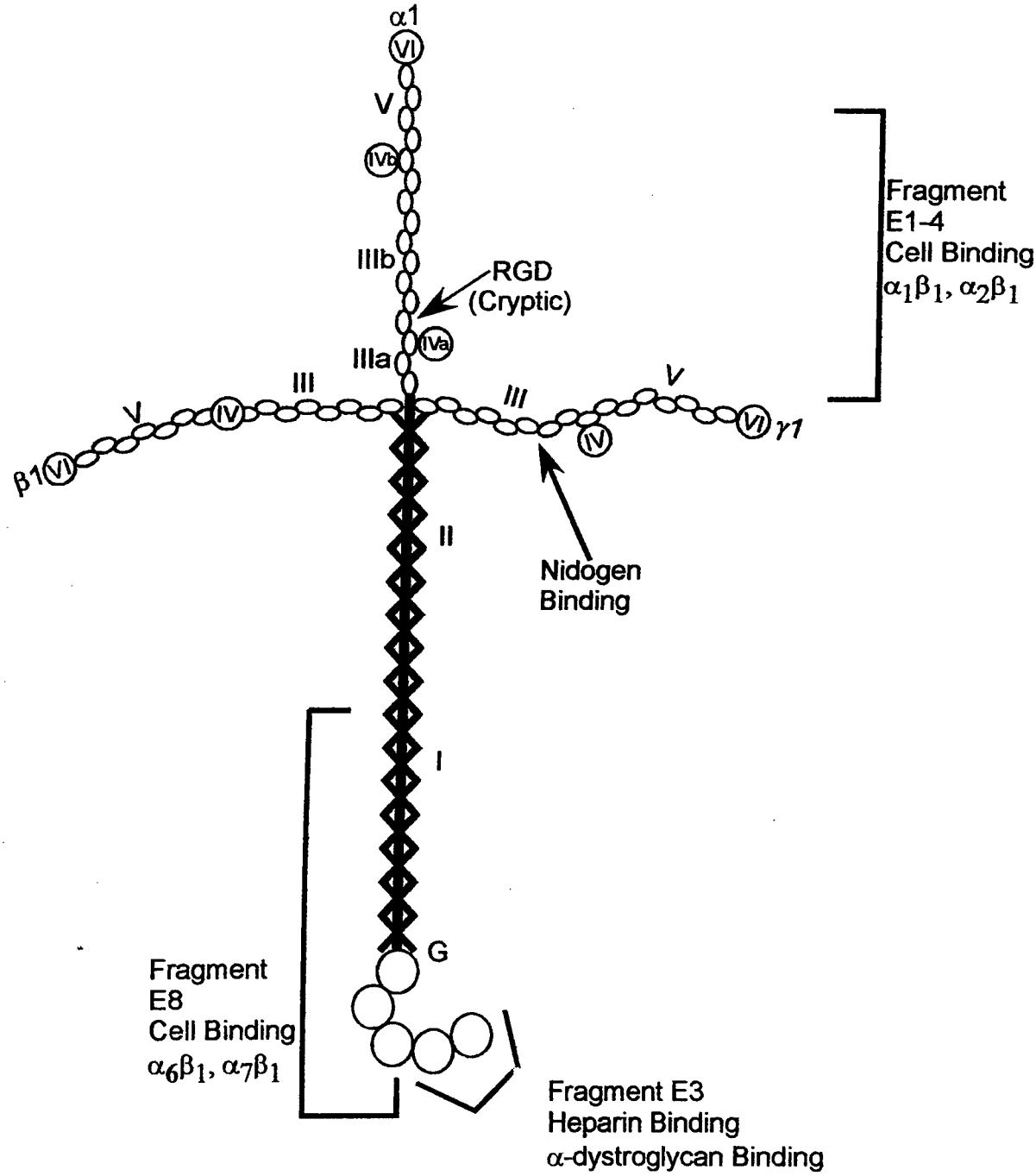


Figure 2

Schematic representation of fibronectin. The fibronectin dimer is formed through interchain disulphide bonds at the -COOH terminus (SS). Each subunit of the dimer contains 12 units of type 1 homology (□), 2 units of type II homology (0), and 15-17 units of type III homology (0). Sets of repeating units make up the respective FN, collagen, and heparin binding sites. The amino terminal type I repeats form an essential assembly domain that binds FN.

Figure 3



The structure laminin of is detailed above. In laminin 5 the globular domain at the C-terminus of the molecule binds to surface-associated integrins to help facilitate hemidesmosome association.

heparan sulphate, one for entactin, and two or more to laminin binding proteins on the cell surface (Alberts *et al.*, 1994).

ECM Remodelling

Perhaps most intriguing are the dynamics involved in ECM remodelling. A somewhat static three dimensional description of ECM structure only begins to describe the levels of complexity involved. Caution must be observed when thinking about the ECM because most often we are only looking at a small volume within the extracellular space. The ECM components act in concert to regulate many processes such as cellular differentiation, morphogenesis, motility, and wound healing. The ECM can affect cell behaviour in two main ways. One mechanism is to directly regulate cell behaviour through cell-ECM interactions via either growth factor modulation of cellular responses or receptor mediated signaling. The second mechanism is through the harbouring of factors that regulate growth, survival, and differentiation (Streuli, 1999). This mechanism exploits the use of remodeling enzymes to control the release of matrix-bound growth factors that subsequently control cellular differentiation. Indeed, a large number of enzymes are involved in ECM remodeling, including the tissue serine proteases and the large family of matrix metalloproteinases (MMPs). These enzymes act as broad spectrum proteases for major ECM degradation events that occur during tissue remodeling (Streuli, 1999).

Matrix Metalloproteinases

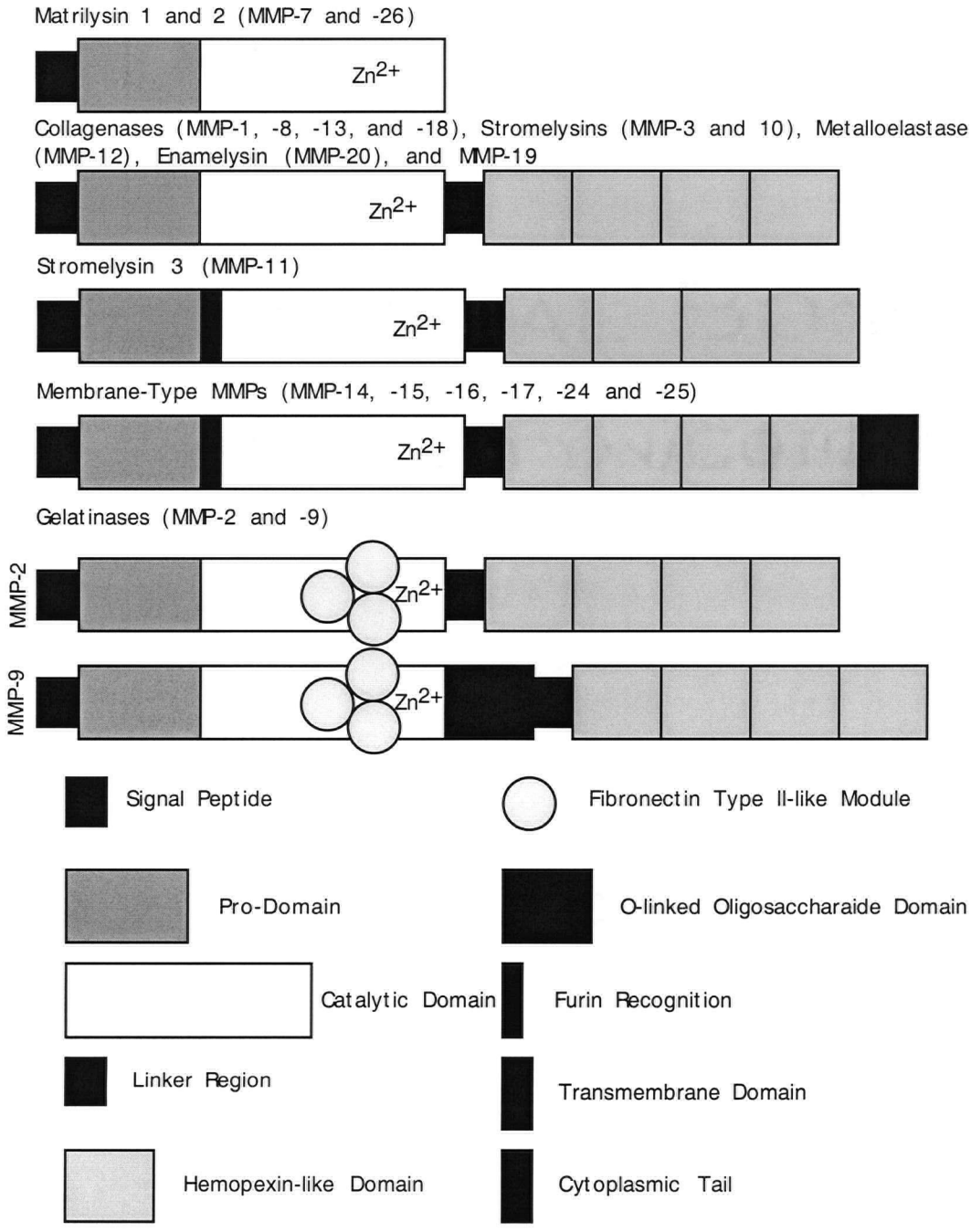
Physiology and Pathology

The matrix metalloproteinases (MMPs) are a diverse group of zinc containing endopeptidases that are capable of degrading many components of the extracellular matrix. MMPs have been implicated in a wide range of physiological processes such as: normal wound healing, bone remodeling, uterine resorption, trophoblast implantation and angiogenesis (Nagase and Woessner, 1999). Alternatively, MMPs have also received a tremendous amount of work devoted towards elucidating their involvement in many pathological processes such as chronic inflammatory and degenerative diseases as well as tumor metastasis (Parks and Mecham, 1998). Perturbations in collagen turnover and ECM degradation are signature features of periodontitis, arthritis, renal diseases, and the metastatic spread of cancer cells (Overall and Sodek, 1987, Bresnihan *et al.*, 1999, Fontana and Delmas, 2000, and Constigan *et al.*, 1995). The proteolytic activities of MMPs are precisely controlled during activation and are inhibited by endogenous inhibitors such as the α-macroglobulins and tissue inhibitors of metalloproteinases (TIMPs) (Nagase and Woessner, 1999).

MMP Families and Domain Structure

To date 23 different human MMPs have been identified and or cloned. The MMP family shares significant sequence homology and a common multi-domain organization (Figure 4). All of the MMPs are synthesized as prepropeptides with an approximately 20-amino acid signal peptide to direct intracellular trafficking.

Figure 4



Schematic representation of the MMP domain structure. Illustrates the increasing complexity of the MMP family through genetic recombination of individual domains.

Except for the furin-activated membrane-type MMPs (MT1-6 - MMPs) and stromelysin-3, MMPs are secreted as zymogens being maintained in a latent state by an ~ 80 residue N-terminal pro-domain (Bode *et al.*, 1999). The propeptide domain contains the uniquely conserved sequence PRCG(V/N)PD whereby the absolutely conserved Cys chelates the catalytic zinc thus maintaining enzyme latency (this Cys has been referred to as the "cysteine switch") (Nagase and Woessner, 1999). Immediately following the propeptide is an approximately 170 amino acid catalytic domain, which contains the catalytic zinc, a structural zinc and two to three structural calcium ions (Bode *et al.*, 1999). The active site, like all proteinases, performs the two-fold function of binding substrate and catalyzing peptide bond hydrolysis (Overall, 2000). The efficiency of these actions defines the substrate specificity of the proteinase by determining enzymic activity towards particular substrates. The catalytic domain contains the highly conserved zinc-binding sequence HEXXHXXGXXH and a conserved methionine which forms a unique 'Met-turn' structure characteristic of the metzincin superfamily (Bode *et al.*, 1999). The catalytic activity of the MMPs is contributed by a water molecule that is polarised by a conserved glutamic acid residue positioned at the bottom of the active site cleft (Crabbe *et al.*, 1994). Except for the matrilysin's (MMP-7 and MMP-26) all of the MMPs are secreted with a C-terminal hemopexin domain. The C-terminal hemopexin domain is approximately 210 amino acids long and has an ellipsoidal disk shape. The hemopexin domain has an overall four bladed β-propeller structure of pseudo-fourfold structure, with each blade consisting of four anti-parallel β-strands and an β-helix (Bode *et al.*, 1999). Unequivocal evidence implicating the C-domain in

substrate recognition and subsequent cleavage has been demonstrated through the C-domain interactions of the collagenases (MMP-1,-8, and -13), where the C-domain has been shown to be required for cleavage of native triple helical collagen (Murphy and Knauper, 1997) and in gelatinase A for the cleavage of chemokines (McQuibban *et al.*, 2000). Gelatinase A and B (MMP-2 and MMP-9, respectively) are unique in that they have three contiguous fibronectin type II repeats inserted into their catalytic domains. This domain exhibited high affinity binding to gelatin and was initially termed the gelatin binding domain (Bányai and Patthy, 1991). It was later found by Steffensen *et al.* that the fibronectin type II modules of gelatinase A also bound avidly to type I collagen and thus lead the authors to term this domain as the collagen binding domain (CBD) (Steffensen *et al.* 1995). Adopting the terminology of Steffensen, in this thesis the recombinant form of the CBD will be termed rCBD123, with the r designation referring to recombinant protein and 123 referring to a construct containing modules 1, 2, and 3. In the gelatinase A molecule, the domain is simply referred to as the CBD. As will be described in greater detail later, the function of the CBD is to donate substrate binding exosites to help confer substrate specificity to the gelatinases (Bode *et al.*, 1999) and to possibly function in triple helicase activity (Overall, 2000).

Gelatinase A

Gelatinase A (also known as MMP-2, 72-kDa gelatinase and 72-kDa type IV collagenase; EC 3.4.24.24) was first described by Liotta *et al.* in 1979 as an enzyme secreted by a metastatic murine tumor that was capable of degrading

soluble type IV collagen into 1/4 N-terminal and 3/4 C-terminal fragments (Liotta, *et al.*, 1979). Approximately ten years later a cDNA expressed clone of the type IV collagenase was constructed and the expressed product was found to have a molecular weight of 72 kDa (Collier *et al.*,1988). Further characterisation of the expressed protein lead researchers to classify the 72-kDa collagenase into the matrixin family of proteinases and subcategorised it as the second MMP to be discovered. Gelatinase A is unique in that it has a ubiquitous tissue distribution, is uncharacteristically activated at the cell surface (Overall and Sodek, 1990), and it is constitutively expressed by many cell types, including gingival fibroblasts (Parks and Mecham, 1998).

Regulation

Regulation of gelatinase A occurs both transcriptionally and post-transcriptionally. Unlike other MMPs, transcription of gelatinase A is not readily induced by agents such as 12-O-tetradecanoylphorbol-13-acetate (TPA) (Overall *et al.*, 1991) or interleukin 1α (IL-1a), both of which have been shown to induce transcription at gene and promoter elements. The promoter regions of gelatinase A show marked differences from the other MMPs in that this region lacks the well known transcription activator sequences for activator protein-1 and polyomavirus enhancer A-binding protein 3. This region also contains a unique noncanonical TATA box, as well as a putative enhancer element located -223 to -422 relative to the translational start site (Parks and Mecham, 1998). Unlike other MMPs, the gelatinase A promoter also lacks upstream TGF-β inhibitory elements. Overall *et al.* (1991) found that gelatinase A transcription was not suppressed but slightly

upregulated by TGF-β. The presence of an activator protein-2 element contributes to cell-type specific expression of both gelatinases.

Cellular regulation of progelatinase A activation is very important in the initiation of ECM turnover. Post-transcriptionally, gelatinase A regulation occurs via increased mRNA stability. Evidence for this was found by Overall *et al.* (1991) where it was discovered that addition of TGF-β, which normally reduces proteolytic activity through reduced proteinase synthesis and increased TIMP expression, increased gelatinase A secretion by human fibroblasts and rat bone cells. After addition of TGF-β it was found that total cellular gelatinase A mRNA was found to increase approximately 1.5- to 2.2-fold. This increase in gelatinase A mRNA was attributed to a concomitant increase in gelatinase A stability ($t_{1/2}$ increase from 46 to 150 h), hence providing evidence for post-transcriptional regulation of gelatinase A expression.

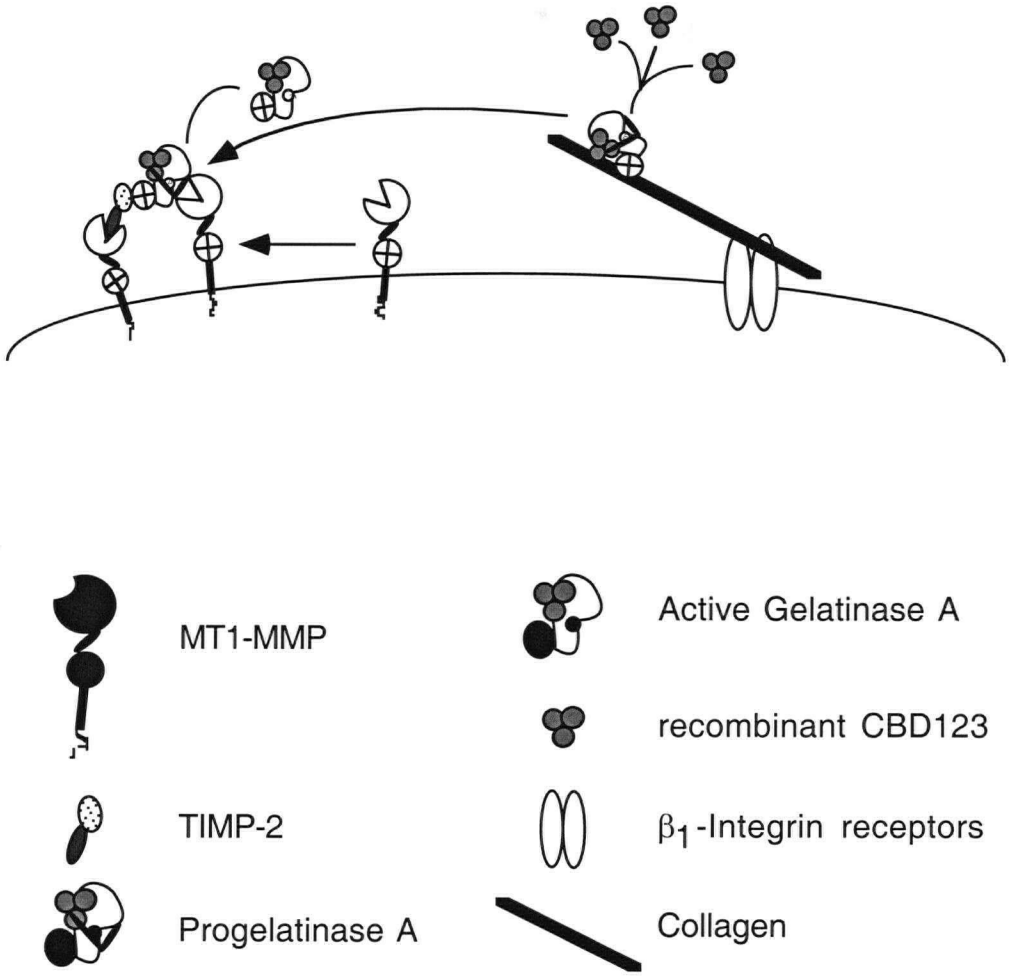
While most active MMPs are inhibited by TIMP binding to their active sites, extracellular progelatinase A is often found specifically complexed with the endogenous inhibitor TIMP-2. Site-directed and deletion mutant studies have localised the TIMP-2 binding site on MMP-2 to charged residues within the C-terminal domain (Overall *et al.*, 1999 a,b, Butler *et al.*, 1999). It has been found that negative charges on the C-terminal tail of TIMP-2 (and possibly TIMP-4) help facilitate TIMP binding to the C-domain of progelatinase A, thus conferring binding specificity to progelatinase A (Bigg *et al.*, 1997, 2001). The progelatinase A/TIMP-2 association regulates cell-surface activation of progelatinase A via a membrane-associated activator membrane-type 1-MMP (MMP-14), that is C-

terminally bound to the cell membrane (Sato *et al.*, 1996). This work stemmed from the observation that the lectin concanavalin A induced the endogenous activation of gelatinase A (Overall and Sodek, 1990). Through interactions with the N-terminus of TIMP-2, membrane-bound MT1-MMP localises the progelatinase A/TIMP-2 complex at the cell surface. The trimolecular complex of progelatinase A/TIMP-2/MT1-MMP allows for a second active MT1-MMP to proteolytically cleave the N-terminal propeptide of progelatinase A at Asn 37-Leu 38 to give rise to a 64 kDa intermediate that is autoproteolytically cleaved to fully active gelatinase A (Woessner and Nagase, 2000) (Figure 5). Upon addition of rCBD to concanavalin-treated fibroblasts there is an increase in the amount of gelatinase A activation that was attributed to increases in progelatinase A shuffling into the cellular activation pathway via displacement of progelatinase A bound to the cell surface through β_1-integrin/collagen/CBD interactions (Steffensen *et al.*, 1998) (Figure 5).

Substrate Specificities

Gelatinase A is capable of cleaving a number of peptide bonds such as Gly-Val, Gly-Leu, Gly-Ile, Gly-Glu, Gly-Asn, and Gly-Ser in gelatin to produce small peptides. Besides gelatin and soluble types I, IV and V collagen, gelatinase A can also cleave type VII and X collagen, and elastin. Interesting to note is that both gelatinase A and B can only cleave pepsin-solubilised type IV collagen, both enzymes being unable to cleave native type IV collagen. These findings seem to raise some doubt about whether the gelatinases are true "type-IV collagenases" *in vivo.* Indeed, while the first cleavage site of soluble type IV collagen by

Figure 5



Schematic representation of geltinase A activation. First, an MT1-MMP binds to the N terminus of TIMP-2 (1). The free -COOH terminus of the TIMP is available to interact with the C-domain of progelatinase A (2). These interactions help to position progelatin-ase A at the cell surface for activation by a second active MT1-MMP (3). Upon MT1-MMP dependent gelatinase A cleavage, active gelatinase A is released from the cell surface (4). Upon addition of rCBD123, pericellularly-bound gelatinase A is released from type I collagen Steffensen *et al.*, (1998).

gelatinase A occurs 1/4 of the distance from the amino terminus, the cleavage site within full-length native type IV collagen remains to be determined (Parks and Mecham, 1998).

In addition to the ability of gelatinase A to degrade native ECM structures such as fibronectin and vitronectin, gelatinase A has been shown to possess β-secretase activity, being able to cleave the β-secretase site within amyloid protein precursor 695. Gelatinase A is also capable of cleaving laminin-5, which, through interactions with integrins, is essential for adhesion of epithelial cells to the basement membrane. Cleavage of laminin-5 liberates a pro-motility cryptic site that induces cells to migrate (Parks and Mecham, 1998). Gelatinase A functions most of the time to modulate the structure of the ECM therefore regulating processes such as cell migration, development, and morphogenesis.

Metastasis

Three essential events have been defined in tumour migration: (1) tumour cell adhesion to ECM components, (2) ECM degradation by proteolysis, and (3) tumour cell migration into the degraded area (Kleiner and Stetler-Stevenson, 1999). Benign epithelial tumours are always characterised by an intact basement membrane that encapsulates the neoplastic epithelium from the connective tissue stroma, whereas malignant epithelial tumors have an ill-defined basement membrane that forms an incomplete barrier which facilitates the spread neoplastic cells (Parks and Mecham, 1998). This ability of metastatic tumour cells to initially traverse the encapsulating basement membrane and to subsequently cross the subepithelial basement membrane of the vascular

system allows the tumour cells to indiscriminately circulate throughout the body. Indeed, the ability of tumour cells to degrade the type IV collagen contained within the basement membrane is paramount in the determination of metastatic potential.

Evidence for the enha nced expression of gelatinase A in human tumours is derived from experimental studies correlating gelatinase A expression to tumour grade (Davies *et al.*, 1993). Immunocytochemical and *in situ* hybridization studies have shown that there is a local increase in gelatinase A expression surrounding many human tumours (Rooprai *et al.*, 1998). Accordingly, benign proliferative disorders of these tissues usually show a low or negative immunoreactivity to antibodies directed against gelatinase A (Still *et al.*, 2000). Upon tumour cell migration, gelatinase A has been shown to play an important role in angiogenesis (Kleiner and Stetler-Stevenson, 1999). These findings have been substantiated by the use of a gelatinase A knockout mouse where it was found that there was both a decrease in tumour metastasis and angiogenesis, thus illustrating the *in vivo* potential of gelatinase A (Itoh *et al.*, 1998). However, there were very few phenotypic differences in the knockout mouse, which is likely a reflection of the omnivorous potential of the MMPs.

Gelatinase A-Mediated ECM Remodelling

Regulation of extracellular matrix degradation by gelatinase A is governed by the net balance of progelatinase A secretion, expression, activation, and local TIMP concentration. The fact that increased gelatinase A secretion around tumours precedes the acquisition of an invasive phenotype suggests that the activation of

gelatinase A makes a significant contribution to an invasive phenotype (Parks and Mecham, 1998). To support this idea, Nomura *et al.* (1996) used gelatin zymography to show that active forms of gelatinase A and B were more frequently detected in human gastric carcinoma tissue, and that the activation of the zymogen form of gelatinase A, but not that of gelatinase B, correlated well with the degree of local invasion and lymphatic permeation. To further this notion, regulation of gelatinase A activity by TIMP-2 can serve as a means to regulate the extracellular activity of gelatinase A. As described before, TIMP-2 selectively binds to progelatinase A and inhibits gelatinase A. Consequently, TIMP-2 can suppress invasion, metastasis, neovascularisation, and growth of some rodent and human tumours (Kleiner and Stetler-Stevenson, 1999).

Exosites

One of the keys for proteases to exhibit a high specific activity (k_{cat}/K_M) is the ability to bind efficiently to substrate (small K_M). In order to increase binding avidity, enzymes contain discrete binding subsites, termed "exosites", that are far removed from the catalytic site (Overall, 2000). Exosites are found within modules or domains of unrelated proteins that have, through the process of gene transposition, inserted themselves into distinct polypeptides, thus conferring a protein greater evolutionary potential by functioning to increase substrate recognition and therefore the spectrum of the proteinase. Indeed, gelatinase A has evolved to contain several exosites that function in substrate recognition. Recently it was discovered through the use of a yeast-2-hybrid screen that the C-domain of gelatinase A interacts with the chemokine monocyte chemoattractant

protein-3 (MCP-3) subsequently facilitating gelatinase A-dependent cleavage of MCP-3 to MCP-3 (5-76) that antagonizes chemotaxis (McQuibban *et al.*, 2000). Gelatinase A and B are also structurally separated from the MMP family by the substrate binding exosites that are located on the three contiguous fibronectin type II modules that are inserted into their catalytic domains (Bányai and Patthy, 1991)

Fibronectin Type II Modules

Evidence for the involvement of the fibronectin type II modules of gelatinase A in the binding of gelatin was first described by Bányai and Patthy (FEBS lett. 1991). This paper demonstrated for the first time that a recombinant protein consisting of only the three fibronectin type II repeats from gelatinase A could bind gelatin. Similarly, it was shown by Collier *et al.* that the gelatin binding site of gelatinase B resides within its fibronectin type II repeats (Collier *et al.*, 1992). Bányai *et al.* subsequently purified wild-type fibronectin type II modules, deletion mutants lacking either the first, second, or third module (DELβgalcoll 23, DELβgalcoll 13, and DELβgalcoll12, respectively), and the single modules (DELβgalcoll 1, DELβgalcoll 2, and DELβgalcoll 3). From these studies, they found that each fibronectin type II module was capable of saturable binding to a gelatin-Sepharose 4B column. Scatchard analysis of binding assays showed that gelatin-Sepharose possessed a range of binding sites with different affinities for the recombinant proteins. Comparison of association constants illustrated marked differences in recombinant protein affinity for gelatin. Recombinant proteins containing two of the fibronectin type II modules bound more avidly than

any of the individual modules. Correspondingly, wild-type domain competed off both of the single deletion mutants, thus displaying the highest affinity for gelatin. This suggested that the three fibronectin type II modules of gelatinase A cooperate in gelatin binding.

The important question raised was whether the fibronectin type II modules acted singly to bind gelatin, i.e. single binding sites on each of the modules, or, do the modules spatially orient themselves so as to form a cooperative binding cleft. Bányai *et al.* addressed this question through competition experiments where it was found that pairs of non-overlapping fragments, such as DELβgalcoll 13/DELβgalcoll 2 or DELβgalcoll 23/DELβgalcoll 1, were able to compete each other off gelatin. This suggested to the authors that different type II units may bind to the same sites on gelatin and that different fibronectin type II modules do not have unique binding sites and that there was some promiscuity in their binding to gelatin (Bányai *et al.*, 1994). Indeed, Steffensen *et al.* (1995) showed through the use of CNBr fragments of gelatin that the CBD bound to multiple sites on the α-chains.

Fibronectin Type II Module Deletion Mutant Studies

To further assess the role of the fibronectin type II domain Murphy *et al.* (1994) looked at this from the perspective of full-length gelatinase A. The properties of the gelatinase A deletion mutant $\Delta_{V191\text{-}Q364}$ (representing removal of all the fibronectin type II repeats) were compared with those of full-length gelatinase A. Firstly, activation of full-length gelatinase A and the deletion mutant $\Delta_{V191\text{-}Q364}$ both occurred through the single canonical intermediate stage. The activity of both

proteins towards the synthetic substrate McaPLANvaDpaAR was found to be very similar. This illustrates that complete removal of the catalytic domain-inserted fibronectin type II repeats does not reduce catalytic activity towards an active site-binding substrate. The deletion mutant $\Delta_{V191-Q364}$ was shown to have only 50% of the activity of full-length gelatinase A towards β-casein, only 10% of the activity of full-length gelatinase A towards gelatin (Murphy *et al.*, 1994), and a total loss of elastinolytic activity (Shipley *et al.*, 1996). Enzyme-linked immunosorbent assays decisively proved that the binding of gelatinase A to type I collagen could be localised to the fibronectin type II repeats since the deletion mutant was unable to bind to native type I collagen coated wells whereas full-length gelatinase A avidly bound type I collagen (Murphy *et al.*, 1994). Through deletion mutant and fibronectin competition assays Allan *et al.* (1994) provided further evidence implicating the role of the fibronectin type II domains in binding type I and IV collagen. It was shown that collagenase 1 and stromelysin 1 could be simultaneously bound to gelatinase A-bound type I collagen. Therefore the authors concluded that gelatinase A has different binding specificities than stromelysin 1 and collagenase 1, both of which bind collagen fibrils through their C-terminal hemopexin domains (Allan *et al.* 1994). These data fully substantiated the role of the fibronectin type II repeats in affording gelatinase A the ability to bind gelatin.

Fibronectin Type II Module Characterisation

Characterisation of rCBD123 substrate binding showed that the fibronectin type II domain was capable of binding denatured type I collagen in the micromolar range (Steffensen *et al.* 1995). It was also shown that rCBD123 avidly bound elastin, heparin, native type I, V, and X collagens denatured type II, IV, V, and X collagens, but neither native type IV and V collagen, fibronectin, reconstituted basement membrane, laminin nor SPARC, all of which are substrates of gelatinase A (Steffensen *et al.* 1995., Overall *et al.* 1997., and Abbey *et al.*, 2001). Native type I collagen, which is not efficiently degraded by gelatinase A, competed with gelatin for a shared binding site on rCBD123. rCBD123 also displaced full-length gelatinase A bound to native type I collagen (Steffensen *et al.*, 1995, 1998). If each of the three modules contained a single collagen binding site, it was plausible to suggest that rCBD123 could bind more than one molecule of collagen. To test this, Steffensen *et al.* incubated [14C]-glycine-labelled collagen in a molar excess of rCBD123. Complexes of rCBD123 bound to the radiolabelled ligand were then found to bind unlabelled native or denatured type I collagen coated on microwell plates. This demonstrated for the first time that rCBD123 possessed at least two collagen-binding sites that could be simultaneously occupied by two collagen molecules. The binding of rCBD123 to α1(I) collagen cyanogen bromide fragments shows that there are multiple binding sites contained within the α1(I) collagen fragment (Steffensen *et al.*, 1998). These findings indicated that there was a potential for the fibronectin type II

modules to function in facilitating enzyme localisation in connective tissue matricies.

Fibronectin Type II Module Structural Determination

Recent work on the fibronectin type II repeats of gelatinase A has focused on determining: 1) the overall structure of the three modules and 2) the residues involved in the binding cleft and/or site(s). This body of work was based on previous studies that were used to model fibronectin type II modules to proteins that share high primary sequence homology. Fibronectin type II modules are a common structural motif containing two disulphide bridges linking Cys residue pairs of the first and third Cys residues and the second and fourth Cys residues. A distant homology has been discovered between fibronectin type II modules and the three-disulphide kringle units found in thrombolytic and fibrinolytic enzymes. This suggests that these two types of modules evolved from a common ancesteral protein. Interestingly, with the exception of the gelatinases, the fibronectin type II modules appear only as single modules or in pairs. Two fibronectin type II modules are found in fibronectin (Owens *et al.*, 1986), bovine seminal fluid protein-109 (Esch *et al.*, 1983) and bovine seminal plasma protein BSP-30 (Calvette, J.J., 1996) - both collagen binding proteins. A single fibronectin type II module is found in the mannose macrophage receptor precursor (Taylor *et al.*, 1990), phospholipase A2 receptor (Ishizaki *et al.*, 1994), mannose 6-phosphate receptor (Morgan *et al.*, 1987), hepatocyte growth activator (Miyazawa *et al.* 1993), and blood coagulation factor XII (Hageman factor)(McMullen and Fujikawa, 1985) (Table 3).

Table 3: Primary Sequence Alignment of Selected Fibronectin Type II-Like Repeats

```
           D-----GGN-DGEPCVFPFIYNGKKYSSCTTEGRSDGFLWCSTTANYDRDGKWGFCPDE-
----------------------|---------|---------|---------|---------|---------|
---------------------10--------20--------30--------40--------50--------60
----------------------|---------|---------|---------|---------|---------|
FN Type I   VTQT.YGGNSNGEPCVLPFTYNGRTFYSCTTEGRQDGHLWCSTTSNYEQDQKYSFCTDHT
FN Type II  LVQT.RGGNSNGALCHFPFLYNNHNYTDCTSEGRRDNMKWCGTTQNYDADQKFGFCPMAA
hGelA_CBD1  VVR.VKYGNADGEYCKFPFLFNGKEYNSCTDTGRSDGFLWCSTTYNFEKDGKYGFCPHE.
hGelA_CBD2  ALFT.MGGNAEGQPCKFPFRFQGTSYDSCTTEGRTDGYRWCGTTEDYDRDKKYGFCP.ET
hGelA_CDB3  AMSTV.GGNSEGAPCVFPFTFLGNKYESCTSAGRSDGKMWCATTANYDDDRKWGFCPDQ.
hGelB_CBD1  VVPT.RFGNADGAACHFPFIFEGRSYSACTTDGRSDGLPWCSTTANYDTDDRFGFCPSER
hGelB_CBD2  LY.T.RDGNADGKPCQFPFIFQGQSYSACTTDGRSDGYRWCATTANYDRDKLFGFCPT.R
hGelB_CBD3  ADSTVMGGNSAGELCVFPFTFLGKEYSTCTSEGRGDGRLWCATTSNFDSDKKWGFCPDQG
PDC1 AA     D..........EECVFPFVYRNRKHFDCTVHGSLFP..WCSLDADYV..GRWKYCAQRD
PDC2 AA     DY.........AKCVFPFIYGGKKYETCTKIGSMWMS.WCSLSPNYDKDRAWKYC....
BSFPA31_AA  D..........NKCVFPFIYGNKKYFDCTLHGSLFL..WCSLDADYT..GRWKYCTKND
BSFPA32_AA  DY.........AKCVFPFIYEGKSYDTCIKIGSTFMNYWCSLSSNYDEDGVWKYC....
HF Bull     ..........GEPCHFPFQYHRQLHHKCIHRGRPGPQPWCATTPNFEKDQRWAYCLEPK
HGF ACTIVA  .........EDGRPCRFPFRYGGRMLHACTSEGSAH.RKWCATTHNYDRDRAWGYCVEA.
PLPA2 Rec   .........AHGTPCMFPFQYNQQWHHECTREGREDNLLWCATTSRYERDEKWGFCPDPT
M6P Rec     ..........DGEPCVFPFVFNGKSYEECVVESRA..RLWCATTANYDRDHEWGFCKHST
```

Legend. Primary sequence alignment of selected fibronectin type II-like modules. The residues in bold illustrate the conserved sequence position of the conserved hydrophobic pocket residues. The highlighted area illustrate the regions where the existence of similar aromatic sequence homology that lead to the selection of the present mutations.

Constantine *et al.* (1992) first determined the solution structure and ligand binding properties of PDC-109 domain b by NMR spectroscopy. This paper loosely outlined the core structure of PDC-109b as being formed of two antiparallel β sheets and two irregular loop regions at the top and bottom of the molecule. The cavity formed by the backbone is largely filled by a cluster of aromatic residues, including Tyr 11, Tyr 30, Tyr 37, Trp 42, and Tyr 45, all of which are solvent-accessible and define an exposed hydrophobic surface (note: for consistency I will refer to the residue number as being counted from the beginning of each module as illustrated in Table 3). This was the first description of a putative hydrophobic binding surface. The residues Phe 9, Thr 20, and Ile 22 form the foundation for the aromatic cluster. Conclusions regarding the binding site structure were derived from ligand-induced resonance shifts observed via NMR. The Trp 30 H6 and H7 resonances were most sensitive to 3,3-dimethylbutylamine (an N-terminal leucine mimic). This residue was therefore hypothesized to be either in direct contact with the ligand or indirectly affected by a change in orientation of the Tyr 11 and Tyr 37 rings, both being hypothesized to be likely disturbed by direct ligand contact. The side chains of Thr 20, Ile 22, and Ala 40 were hypothesized to surround the putative binding surface and therefore any disturbances experienced by these residues are likely to be secondary effects.

Bányai *et al.* (1996) were the first group to model the structure and domain-domain interactions of the fibronectin type II modules from gelatinase A to bovine seminal fluid protein PDC-109. From circular dichroism (CD) spectra it was determined that the fibronectin type II domain of gelatinase A consisted of

approximately 32% β-sheet and 19% β-turn with no detectable α helix. βgalcoll 1, βgalcoll 2, and βgalcoll 3 were estimated to contain 30%, 30%, and 31% β-sheet, and, 28%, 27%, and 23% β-turn structures, respectively. The estimated secondary structures of the fibronectin type II repeats were, as expected, very similar to that of the second type II domain of bovine seminal fluid protein PDC-109 domain b. CD spectroscopy, differential scanning microcalorimetry, and urea-induced denaturation of recombinant βgalcoll 23 and βgalcoll 123 were used to show that the thermal stability of the second type II module is significantly increased by its interactions with the first and third modules. It was postulated from these results, that the tandem modules permit little flexibility in their relative orientation at physiological temperatures. Bányai *et al.* further proposed that all three fibronectin type II modules contribute to binding of a single substrate molecule. It must be noted that this hypothesis differed from the observation of Steffensen *et al.* (1995), where it was shown that rCBD123 was capable of binding to more than one molecule of collagen. Bányai *et al.* further proposed a model whereby the three fibronectin type II modules form an extension of the substrate binding cleft of gelatinase A (Bányai *et al.*, 1996).

Until 1997, the solution structure of the first human fibronectin type II module had not yet been determined. Pickford *et al.* determined the structure of the first type II module from human fibronectin by 2D NMR spectroscopy and found that the structure was very similar to that of the shorter fibronectin type II module from PDC-109 domain b. In both the first fibronectin type II module and PDC-109b, the two double-stranded β sheets are oriented approximately perpendicular to

each other, with the cleft between them being occupied by the side chains of the invariant and highly conserved aromatic residues. The disulphide bonds were in close proximity to one another, and were located on the opposite face of the second aromatic cluster β sheet. Based upon primary sequence homology, the invariant Phe 18 and Trp 39 (corresponding to Phe 9 and Trp 30 of PDC-109b) were completely buried in the fibronectin type II structure and constituted the core of the molecule. The highly conserved residues Leu 13, Tyr 20, Phe 25, Tyr 46, Tyr 52, and Phe 54 were packed around this core, with the sidechains of the four residues Tyr 20, Tyr 46, Tyr 52, and Phe 54 (corresponding to Tyr 11, Tyr 35, Trp 43, and Tyr 45 in PDC-109b) clustered on one face of the module forming a solvent-exposed hydrophobic surface. When the authors closely examined the NMR-derived model the presence of a possible ligand-binding pocket in the hydrophobic surface was noted with the Trp 39 indole ring forming the pocket floor (Pickford *et al.*, 1997). This was the first description of the hydrophobic surface as being a hydrophobic pocket and has lead to further work aimed at elucidating the contact residues. However, not just hydrophobic interactions are involved in binding to gelatin. In the absence of chaotropes, both full-length and a 42 kDa fibronectin fragment that contains the fibronectin type II repeats bound gelatin and could be eluted from immobilised gelatin by a reduction in pH. This suggested direct involvement of charged aspartate and glutamate residues in binding. Elution by the mild denaturant dimethylformamide at concentrations lower than that required for elution by guanidine, suggested an important role for hydrogen bonding in substrate binding (Pickford *et al.*, 1997).

Sticht *et al.* (1998) continued working with fibronectin type II domains and subsequently determined the solution structure of the glycosylated second type two module of fibronectin. The averaged structure of the second fibronectin type II module was found to contain a short α helix and three antiparallel β sheets, all of which partly enclose a cluster of aromatic residues. The topology of the second fibronectin type II module was found to be similar to that of the first fibronectin type II module. In both fibronectin type II modules, the distance between the N and C termini is short, but relative to the first fibronectin type II module, the N terminus of the second fibronectin type II module is shifted towards residues Gly 11 and Cys 14, thus forming a double-stranded antiparallel β-sheet (Sticht *et al.*, 1998). As is now becoming a general trend in the overall structure of the fibronectin type II and type II modules, many of the highly conserved aromatic residues in the fibronectin type II module form a solvent-exposed hydrophobic surface. As with the first fibronectin type II module, it was found that there was a depression within the hydrophobic surface of the second type II module, at the bottom of which is the invariant tryptophan residue (Trp 39). Sticht *et al.* continued to model the first and second fibronectin type II modules together. After screening 300,000 randomized module pairs for favourable non-covalent interactions there was no single lowest energy model obtained. Although it was not possible to unambiguously ascertain a single modelled fibronectin type II module pair, one consistent feature regarding gelatin binding arose during modelling procedures. First, in all analyzed structures a minimum distance of 14.2 Å is observed between the core Trp residues of the two fibronectin type II modules, which, according to the authors, suggests that

ligand-binding hydrophobic pockets form discrete binding sites. This was in stark contrast to Bányai *et al.* who had very shortly before proposed that the three fibronectin type II modules of gelatinase A acted together in forming an extended binding pocket (Bányai *et al.*, 1996). The notion that there are discrete hydrophobic binding sites contained within fibronectin type II modules helps to support the findings by Steffensen *et al.*, where it was shown that rCBD123 was capable of binding two separate molecules of collagen simultaneously (Steffensen *et al.*, 1998).

Even with the flourish of activity dedicated to determining the solution structure of the first and second fibronectin type II modules, the determination of the solution structure of one or all of the fibronectin type II modules from gelatinase A remained to be completed. In 1999, Briknarová *et al.* determined, for the first time, the solution structure of the second fibronectin type II module from gelatinase A. It was found that the module consisted of two short double-stranded antiparallel β sheets arranged approximately perpendicular to each other and three large irregular loops. The first β sheet and the loops were arranged around the second β sheet, forming a large cavity filled with the aromatic sidechains of Phe 16, Phe 18, Phe 20, Tyr 25, Tyr 37, Trp 39, Tyr 46, Tyr 52, and Phe 54. Phe 18 was totally buried within the molecule and Phe 20, Tyr 37, Trp 39, Tyr 46, Tyr 52, and Phe 54 formed an extended hydrophobic surface, whereas Phe 16 faced the other side of the molecule. The disulphide bridge and both the N and C termini were located at the back of the β sheet, on the other side of the molecule (Briknarová *et al.*, 1999).

To assay for changes in spectral character upon substrate complexation, Briknarová *et al.* used a synthetic peptide with the consensus sequence $(PPG)_6$ and monitored the chemical-shift changes induced by $(PPG)_6$ binding via 2D chemical shift correlated spectroscopy (COSY) and ^{1}H-^{15}N heteronuclear shift correlated spectroscopy experiments. Residues with the most perturbed backbone amide resonances were limited to a band that ran diagonally across the front side of the second fibronectin type II module and comprised the aromatic cluster and surrounding areas. Backbone amide resonances from aromatic residues from the front side of the molecule were found to be more sensitive to peptide binding than resonances of aromatic residues that are internal or face the back. It was concluded that the bound peptide extended along the aromatic cluster, interacting with Phe 20, Tyr 37, Trp 39, Tyr 46, Tyr 52, and Phe 54 on the front face of the module. Comparing ligand binding residues of the second fibronectin type II module of gelatinase A to that of the earlier reported interacting residues in PDC-109b and the first and second fibronectin type II modules, continues to highlight the involvement of a spatially conserved hydrophobic pocket with the consensus sequence - $(F/Y)^{20}$-W^{39}-Y^{46}-$(Y/F)^{52}$-F^{54}).

Until now, much of the work with the fibronectin type II modules has been focused on determining the overall structure of the molecule. Indeed, structural studies have lead researchers to infer specific residue involvement in ligand binding, but little work has been afforded to elucidating the direct involvement of ligand-interacting residues. Tordai and Patthy addressed this issue by performing site-directed mutagenesis of the second fibronectin type II module of

gelatinase A. The mutations were modeled from the proposed hydrophobic binding surface of PDC-109 domain b and included residues: R19L, R19L/R38L, Y25A, Y37A, R38L, Y46A, D49A, K50G, K50R, and Y52A of the second fibronectin type II module of gelatinase A. During chracterisation of the mutants, it was found that the mutants could be divided into three categories based upon their structural and functional integrity. The first category, which contained the correctly refolded mutants R19L, R38L, K50G, K50R, and R19L/R38L, showed no impaired affinity for gelatin or alteration of their Tm and denaturation midpoints. These data lead the authors to conclude that these residues are neither involved in substrate recognition nor structural integrity. The second group of mutants, Y25A, Y46A, D49A, and Y52A, were structurally compromised as determined by CD spectroscopy and therefore were devoid of any gelatin binding (Tordai and Patthy, 1999). Indeed, the authors were unable to make any conclusions about the interactions of these residues with gelatin substrate, however, the incorrect folding of these mutants is likely attributable to a loss of hydrophobic character and therefore supported the importance of a structural hydrophobic core. The third category of mutants, comprising only Y37A, was correctly folded as determined by CD spectroscopy and thermal and chemical denaturation and showed severely depleted gelatin affinity. The authors concluded that the only plausible reason for the reduction in gelatin affinity was that this residue is directly involved in gelatin recognition. This was the first direct evidence implicating a contact site in the fibronectin type II module of gelatinase A. From the modelled structure, this residue was placed on the right hand rim of the hydrophobic pocket and has, for the first time, provided direct experimental

evidence for the involvement of the hydrophobic pocket in ligand binding. Since the authors were only using a modelled structure, questions arose as to where exactly in the fibronectin type II module did this residue lie.

In June of 1999, the X-ray crystal structure of gelatinase A was resolved to 2.8 Å. This allowed researchers to directly examine the gelatinase A molecule, hence allowing for a determination of the spatial orientation of the several domains found within gelatinase A. The structure of gelatinase A confirmed that the fibronectin type II modules were comprised of a pair of β sheets, each composed of two antiparallel strands, connected through a short α helix. It was also shown that the two β sheets form a hydrophobic pocket that is accessible from the outside of the molecule. A cis-proline following the first β sheet was part of a hairpin turn that oriented the surrounding aromatic side chains into the hydrophobic pocket. Interestingly, it was found that in full-length gelatinase A the propeptide residue Phe 37 was inserted into the hydrophobic pocket of the third fibronectin domain. The propeptide F37 was also found to interact with the third fibronectin type II module via a hydrogen bond and a salt bridge. It was surmised by the authors that this interaction mimicked the interaction between the third fibronectin type II module and gelatin that based on biochemical evidence is predicted to have all three kinds of interactions (Pickford *et al.*, 1997). The final piece of evidence uncovered the relative spatial orientation of the hydrophobic binding sites located in each of the three fibronectin type II modules. It was found that the hydrophobic binding sites did not orient themselves towards each other to form an extended binding cleft, but on the contrary, they turn outwards

as in what the author termed "a three-pronged fishhook" (Morgunova *et al.*, 1999).

Aim of Present Work

The specific aim of this thesis is to identify new amino acid residues in the CBD that are involved in collagen binding in order to derive the collagen binding consensus site. New sites for mutagenesis have been selected from 1) the results of our first round of mutagenesis (Steffensen *et al.*, 2001) and 2) a 3D model of the CBD made using a "protein threading" algorithm and energy minimization (Steffensen *et al.*, 2001). The mutation K263A showed reduced collagen binding. This identified a loop on the CBD between two conserved cysteines that is important for collagen interaction. Of note, in this loop CxFPFxFxGxxYxS(/Sx)C is the longest conserved intra-cysteine sequence in all three CBD modules of gelatinase A and B; except that in gelatinase B the placement of the Ser residues is YSxC. Lys263 is the nonconserved x in CBD2 after the first Cys. This highlights this region as an important part of the molecule. The requirement for DMSO to elute bound CBD from collagen indicates that hydrophobic interactions are involved in binding. The only other absolutely conserved aromatic residues in the CBD are a Trp adjacent to the third Cys and a Phe in the GCFP sequence at the end of this domain. However, their placement adjacent to the conserved Cys residues would indicate a structural role for these residues.

In order to assess the role of conserved phenylalanine residues in gelatin binding, we used a CBD23 recombinant protein since the individual modules

proved to be difficult to express and purify. We mutated the phenylalanines at positions 264 and 266 in module 2 and the corresponding residues in module 3, 322 and 324, to alanines (numbered in relation to the full-length enzyme and both in positions 16 and 18 in Table 3). This allowed for a determination of the effect of reducing the aromatic character of this site by exchanging the large hydrophobic side chains of phenylalanine for the smaller methyl groups of alanine. Secondly, we mutated the phenylalanines to tyrosines in order to conserve the aromatic character and to determine the effect of introducing a phenolic hydroxyl group that has the potential to hydrogen bond. Finally, we introduced corresponding double mutations, F264A/F322A and F266A/F324A, into both of the conserved CBD modules. The double mutations were predicted to remove residual binding contributed by the unmutated wild type module in the paired constructs and unequivocally allow for the assessment of the role of these residues in gelatin binding.

Chapter 2

Materials and Methods

Site-Directed Mutagenesis - The *Escherichia coli* strain DH5α was used as a host strain for pGYMX-CBD23 plasmid isolation and propagation. Using pGYMX-CBD123 as a template, pGYMX-CBD23 was constructed by PCR amplification of the respective region as described previously (Abbey *et al.*, 2001). The following mutations were introduced into the third collagen binding module of gelatinase A: F322A, F322Y, F324A and F324Y using the mutagenic primers: F322A 5′ - GCC CAG GAA AGT GAA GGG G***GC*** GAC ACA GGG GGC - 3′ and 5′ - GCC CCC TGT GTC ***GC***C CCC TTC ACT TTC CTG GGC - 3′; F322Y 5′ - GCC CAG GAA AGT GAA GGG G***T***A GAC ACA GGG GGC - 3′ and 5′ - GCC CCC TGT GTC T***A***C CCC TTC ACT TTC CTG GGC - 3′; F324A 5′ - GCC CAG GAA AGT G***GC*** GGG GAA GAC ACA GGG GGC - 3′ and 5′ - GCC CCC TGT GTC TTC CCC ***GC***C ACT TTC CTG GGC - 3′; and F324Y 5′ - GCC CAG GAA AGT G***T***A GGG GAA GAC ACA GGG GGC - 3′ and 5′ - GCC CCC TGT GTC TTC CCC T***A***C ACT TTC CTG GGC - 3′. DH5α was the cell line transformed with all mutagenesis products for the purposes of preparing plasmid stocks for sequencing and further experiments. The primers used for sequencing included HIEGR, M13 Reverse and M13-17 Forward. Correctly exchanged nucleotides were determined by dideoxysequencing. Similar procedures were followed by Andrea Connor for the preparation of corresponding mutations within the second collagen binding domain of gelatinase A (F264A, F264Y, F266A, and

F266Y). Using the mutated F264A and F266A pGYMX-CBD23 constructs and the above F322A and F324A primers, the double mutants F264A/F322A and F266A/F324A were produced by Dennis Lee. The mutants F322A, F324A, F322Y, F324Y, F264A/F322A, and F266A/F324A were purified and characterized by myself as described below.

Purification of Recombinant Gelatinase A Domains and Antibodies – rCBD23 (Ala^{249}-Gln^{364}) of human gelatinase A was expressed in *Escherichia coli* BL21 gold and purified by Ni^{2+}-chelate and gelatin Sepharose chromatography. A 5 ml seed culture of *pGYMX-CBD23*-transfomed BL21 Gold cells was grown overnight at 37 °C in LB media (1% (w/v) Bactotryptone, 0.5% (w/v) Yeast extract, 1% (w/v) NaCl, pH 7.5) with 40 μg/ml ampicillin. 7 x 600 ml superbroth cultures (1% (w/v) Bactotryptone, 0.8% (w/v) Yeast extract, 1% (w/v) NaCl, 0.001% glycerol (v/v), pH 7.4) containing ampicillin (40 μg/ml), were each inoculated with a 1/1000 dilution of the seed culture. The cultures were incubated for 24 h at 37 °C. Cells were harvested by centrifugation (7,000 x g for 10 minutes at 4 °C). The cells were suspended in a total of 100 ml of lysis buffer (20 mM Na_2HPO_4, 50 mM NaCl, 2 mM MgCl, 5 μg/ml DNase, 1 mg/ml lysozyme, 1 mM PMSF, pH 8.0) and incubated, with agitation, at 37 °C for 2 h. The sample was then sonicated, on ice, for 3 x one minute intervals. Inclusion bodies were removed by centrifugation (15,000 x g for 15 min at 4°C). Inclusion bodies were dissolved in 200 - 300 ml of solubilization buffer (10 mM Tris, 8 M Urea, pH 8.0) and stirred vigorously for 4 – 18 h. Upon pellet dissolution, 30 ml of Ni^{2+}-charged chelating Sepharose was added to the solubilization solution. The resin was batch-loaded

for 2 h to overnight at 4 °C. The batch-loaded resin was recovered by centrifugation at 500 x g for 5 minutes at 4 °C. The resin was washed repeatedly with chromatography buffer (CB)(100 mM Na_2HPO_4, 0.5 M NaCl, 8 M urea, pH 8.0) until the supernatant was clear. The resin was packed into a Kontes glass column (3 cm x 15 cm) and washed with 100 ml of CB. The column was then washed with 250 mL of CB + 1 M NaCl and the 250 mL of CB + 1 M NaCl, pH 6.0. The column was re-equilibrated with CB and subsequent elutions were performed by FPLC (Biochem-Pharma). Non-specifically bound protein was pre-eluted in CB + 10 mM imidazole. Specifically bound rCBD mutant proteins were eluted with a 500 mM imidazole step gradient. Peak fractions were pooled and diluted three-fold with CB and the sample was placed in a dialysis bag (6 – 8 kDa cutoff). Protein was refolded against a 2 x volume of Vollers buffer (18.2 M Na_2CO_3, 24 mM $NaHCO_3$, 0.273 mM cysteine/2.73 mM cystine, pH 10.0), with aeration, for 2 h at room temperature. After refolding the sample was dialyzed against a 2 x volume of phosphate-buffered saline (PBS)(140 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.5 mM KH_2PO_4) plus 0.273 mM cysteine/2.73 mM cystine, pH 7.4, with aeration, for 2 h at room temperature. Refolded protein was exhaustively dialyzed against PBS to remove urea. Correctly refolded protein was selected for by using gelatin Sepharose affinity chromatography. The refolded sample was applied to a PBS-equilibrated 7 ml gelatin Sepharose column. Specifically bound protein was eluted in a 20 ml 0 – 10 % dimethyl sulfoxide (DMSO) gradient. Fractions containing rCBD23 proteins were dialyzed against PBS at 4 °C (2 x 2 h). Protein samples were aliquoted, frozen, and then stored at –20°C. Polyacrylamide gel electrophoresis (PAGE) in 15% gels was

used to follow the purification of recombinant protein. Electrospray mass spectroscopy was performed on a SCIEX API 300 (Perkin-Elmer) mass spectrometer was used to assess fidelity of gene expression. Rabbit polyclonal antibody (α-His_6) raised against the NH_2-terminal His_6 fusion tag on the recombinant proteins was affinity purified as before (Steffensen *et al.,* 1998).

Microwell Substrate Binding Assays for the Determination of Gelatin Affinity – 96 microwell plates were coated with 0.5 μg/well of denatured type I collagen (gelatin). Unbound gelatin was removed by PBS + 0.05 % Tween 20 (v/v) washes and the plates were blocked with 2.5 % (w/v) BSA in PBS for 1 h at room temperature. After further washes with PBS/Tween, wild-type and mutant protein preparations were clarified by centirifugation at 10,000 x g for 5 min at 4 °C and quantitated at 280 nm immediately before use. Samples were serially diluted in 1 x PBS, from 5 μM to 0.6 pM and allowed to react for 1.5 h at room temperature. Based upon the aromatic content of the protein, the protein concentrations were determined using extinction coefficients (expressed as protein concentration in mg/mL per unit of absorption at 280 nm) calculated using the software program Protean (Lasergene). After incubation, the plates were washed with PBS/Tween. Bound recombinant protein was detected with an αHis_6 antibody dilution (1:500 in PBS + 0.01 % Tween 20 + 0.25 % BSA), followed by a reaction with alkaline phosphate-conjugated goat anti-rabbit antibody (Bio-Rad) (1:1000 PBS dilution + 0.01 % Tween 20 + 0.25 % BSA). Both incubations were for 1 h at room temperature and plates were washed with PBS/Tween 20. For quantitation, *p*-nitrophenyl phosphate disodium (Sigma) was added as a substrate. To ensure linearity of the assay, the colour intensity was determined at various times by

measuring the absorbance (405 nm) in an automated plate reader. Negative controls for non-specific binding consisted of reaction mixtures substituting respective rCBD23 proteins with recombinant horse heart myoglobin. The best curves were fitted to the data using SigmaPlot 2000 (SPSS). Absorption values that were decreased at high recombinant protein concentrations were disregarded when fitting the curve.

FPLC Analysis to Assay for Gelatin Affinity - On an FPLC machine a gelatin Sepharose (1 mL) (Pharmacia) column was packed in a Kontes column (1 x 10 cm) and equilibrated in PBS. The absorption baseline was established with PBS. Recombinant proteins were clarified by centrifugation at 10,000 x g for 5 min at 4 °C and quantitated at 280 nm immediately before use. Individually, 500 µl samples of proteins (50 µg) were sequentially loaded onto the column, allowed to transiently bind, and then eluted with DMSO. The column was loaded, washed and eluted at a flow rate of 2.5 ml/min. After loading, the column was washed with approximately 5 mL of PBS to reestablish a zero absorbance baseline after the flow-through peak. Bound protein was eluted with a 20 ml 0 – 10% DMSO gradient. Differential elution of bound protein was observed via superposition of FPLC traces.

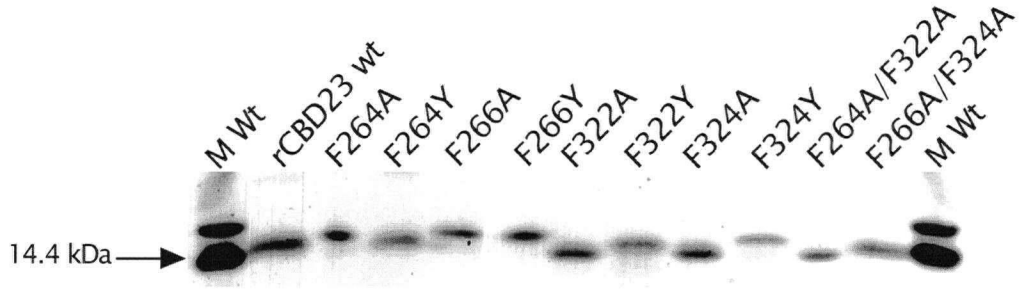
Chapter 3

RESULTS

Recombinant Protein Expression - To investigate the role of specific CBD residues in substrate recognition, we used the pGYMX-CBD23 construct to express the deletion mutant rCBD23 (lacking CBD1) (Abbey *et al.*, 2001). As illustrated by SDS-PAGE analysis, wild-type and mutant rCBD23 proteins were expressed in *E. coli* and purified to homogeneity (Figure 6). Fidelity of gene expression, confirmation of N-terminal methionine processing, and sample homogeneity was determined by mass spectrometry (Table 4).

Microwell Substrate Binding Assays for the Determination of Gelatin Affinity - The relative affinity of wild-type and mutant rCBD23 for gelatin was determined through the use of ELISA-based assays. It was found that the apparent dissociation constant for wild-type rCBD123 binding to immobilized gelatin was approximately 1×10^{-8} M, whereas the binding of wild-type rCBD23 was approximately 8×10^{-8} M (Figure 7). The reduction in binding of approximately one order of magnitude is supported by the earlier findings of Steffensen *et al.* (1998). This decrease supports the hypothesis that cooperativity is involved in the binding of the CBD to substrate. The mutants F264A and F322A exhibited a 2.5 - 3 fold reduction in binding, $K_d \sim 0.2 \times 10^{-6}$ and 0.24×10^{-6}, respectively. Binding of the corresponding Phe→Tyr mutants was similar to that of the wild-type rCBD23 protein (Figure 7). The mutants F266A and F324A showed a greater reduction in gelatin binding as observed by a respective 6-fold and 13-

Figure 6



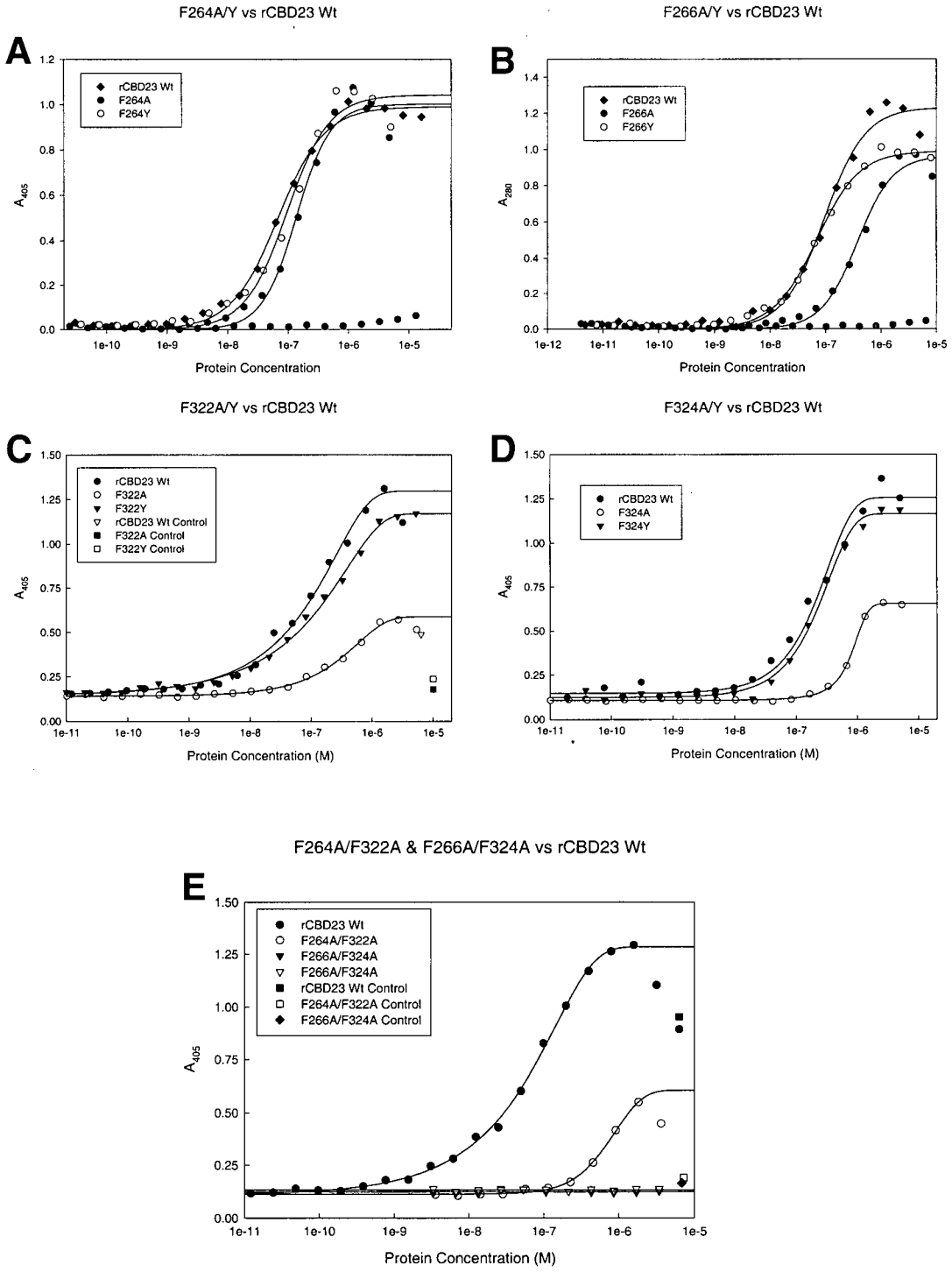
SDS-PAGE analysis of purified protein samples after gelatin Sepharose chromatography. The samples were electrophoresed under reducing conditions on a 12.5 % Tris-Tricine gel.

Table 4: Electrospray MassSpectrometry of Recombinant Proteins

Recombinant Protein	Expected M_r (Da)		Found M_r (Da)	Δ Da
	+ Met	- Met		
rCBD12 Wt	15 094	14 963	14 961	2
rCBD23 Wt	14 680	14 549	14 547	2
F264A	14 604	14 473	14 477	-4
F264Y	14 696	14 565	14 564	1
F266A	14 604	14 473	14 477	-4
F266Y	14 696	14 565	14 566	-1
F322A	14 604	14 473	14 470	3
F322Y	14 696	14 565	14 567	-2
F324A	14 604	14 473	14 472	1
F324Y	14 696	14 565	14 566	-1
F264A/F322A	14 528	14 397	14 397	0
F266A/F324A	14 528	14 397	14 398	-1

Electrospray mass spectrometry of recombinant proteins. Masses of the recombinant proteins were measured and compared to values calculated from the amino acid composition minus 4 Daltons for the loss of hydrogens during disulphide formation. rCBD12 and mutants F264A, F264Y, F266A, and F266Y were purified by A. Connor. The mutant F264A/F322A was purified by D. Lee.

Figure 7

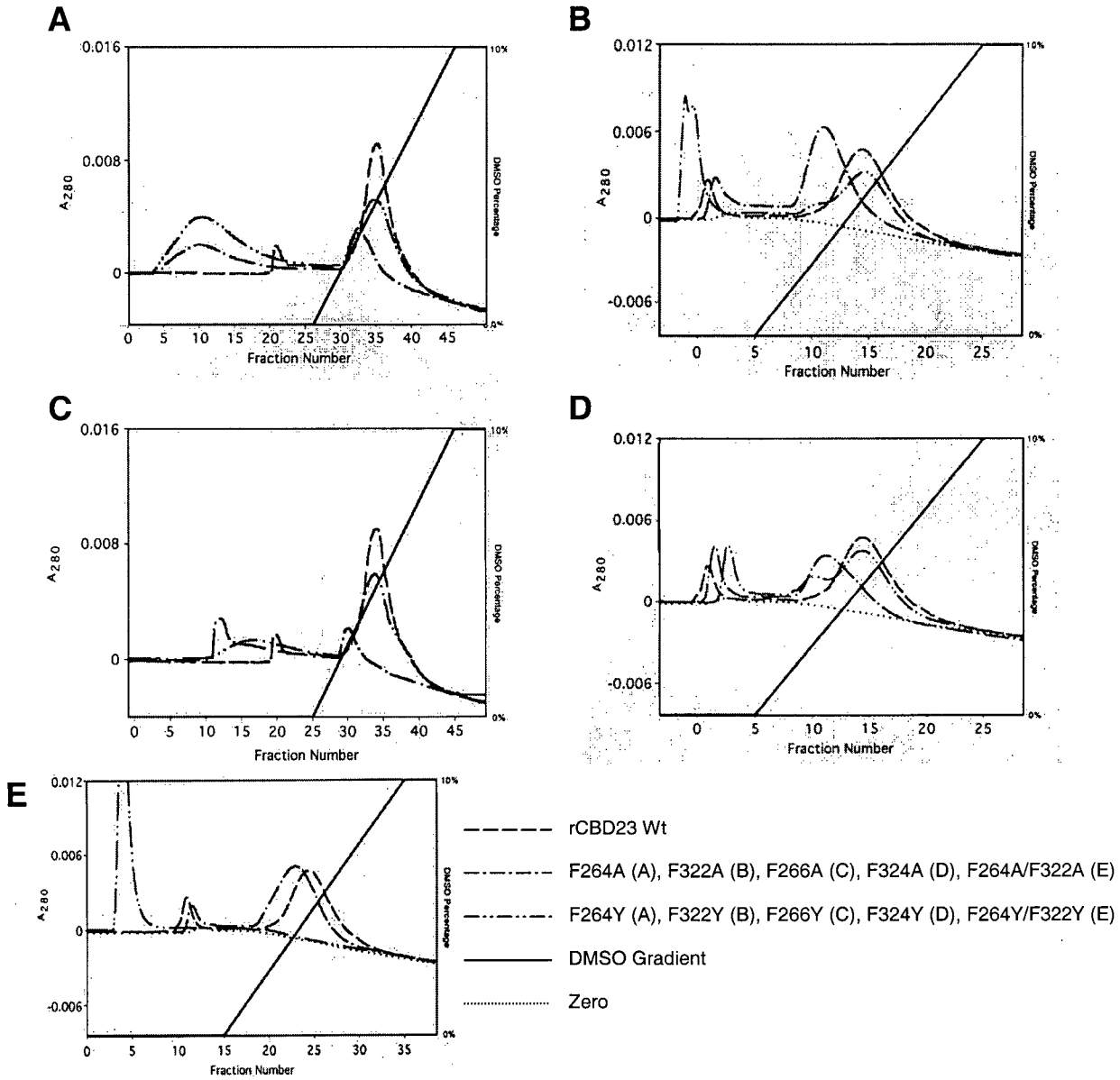


Plots of the microwell substrate binding assays were used to determine the apparent dissociation constants. The figure shows that: (1) single alanine mutations had reduced affinity for denatured type I collagen, (2) tyrosine mutations maintained gelatin binding, (3) reduced gelatin binding was observed for F264A/F322A and (4) abrogated gelatin binding was observed for the F266A/F324A mutant. Representative horse heart myoglobin controls are shown in panels C and E. Plots A and B were made by A. Connor.

fold reduction in gelatin binding versus wild-type rCBD23 (Figure 7). Again, binding was essentially unaltered in the corresponding Phe→Tyr mutants (Figure 7). In order to assess the contribution of the non-mutated domain (ie. CBD3 in the F264A or F266A single mutants and CBD2 in the F322A or F324A single mutant proteins), the corresponding double mutations F264A/F322A and F264A/F324A were made. The F264A/F322A mutant showed that binding affinity to gelatin was reduced (K_d ~ 0.46 x 10^{-6}) relative to the single mutants F264A and F322A (K_d ~ 0.2 x 10^{-6} and 0.24 x 10^{-6}), but not more than the single mutants F266A and F324A (K_d ~ 0.5 x 10^{-6} and 1.1 x 10^{-6}). The importance of positions 266 and 324 in gelatin binding was shown by the double mutant F266A/F324A which showed no affinity for gelatin (Figure 7).

Differential Elution of Wild-Type and Mutant rCBD23 from a Gelatin Sepharose Column - In order to further assess the ability of mutant domains to bind gelatin, we exploited the hydrophobic interactions during binding of fibronectin type II modules to gelatin. DMSO disruption of these interactions allows for the comparison of elution profiles. Recombinant proteins were loaded onto a 1 ml gelatin Sepharose column and were eluted with a 0 - 10 % DMSO gradient. The differential elution of mutant and wild-type rCBD23 proteins was compared by superposition of FPLC absorbance profiles (Figure 8). F264A and F322A eluted in 3.5 % and 3.0 % DMSO respectively. The elution profiles of the mutants F264Y and F322Y were very similar to that of wild-type rCBD23, revealing that the introduction of a hydroxyl group, which has the potential for hydrogen bond formation, did not alter again gelatin binding. The mutants F266A and F324A eluted at lower DMSO concentrations, 2.7 % and 3.0 % respectively, than the

Figure 8



DMSO mini-column elution profiles. 50 μg of each protein was added to 1 mL gelatin Sepharose columns. The elution of the respective proteins is illustrated above. This figure illustrates that removal of hydrophobic character reduces the percentage of DMSO required to elute bound protein. Panel A shows the eluting concentrations of F264A and F264Y were found to be 3.3 and 4.3% respectively. The eluting concentration of wild-type rCBD23 was determined to be 4.2%. Panel B shows the eluting concentrations of F266A and F266Y were found to be 2.4 and 4.2% respectively. These data illustrate that removal of F266 hydrophobic character had a greater effect than removal of F264 hydrophobic character. Panel C shows the eluting concentrations of F322A and F322Y were found to be 2.8 and 4.7%, respectively. The eluting concentration of wild-type rCBD23 was determined to be 4.7%. Panel D shows the eluting concentrations of F324A and F324Y were found to be 3.0 and 4.6%, respectively. These data illustrate that removal of F322 hydrophobic character had a greater effect than removal of F324 hydrophobic character. Panel E shows that the mutant F264A/F322A avidly bound gelatin Sepharose, whereas the second double mutant F266A/F324A showed no affinity for gelatin Sepharose. Panels A and C were made by A. Connor.

F264A and F322A mutants. Again, introduction of hydroxyl groups in the mutants F266Y and F324Y did not significantly alter the elution profile relative to that of the wild-type domain since the core hydrophobicity of the molecule was not perturbed. This result reiterates the previous observations for mutants F264 and F322 illustrating that the reduction of hydrophobic character decreases gelatin affinity and that introduction of phenolic hydroxyls does not alter binding. Involvement of the wild-type module in binding gelatin, that renders interpretation of the single Phe mutants complex, was addressed by applying the double mutants to a gelatin Sepharose column. The mutant F264A/F322A unexpectedly eluted at a DMSO concentration greater than that of any of the single Ala mutants (DMSO % = 4.1). The second double mutant did not bind gelatin and was recovered only in the flow through (Figure 8). This is in agreement with our ELISA results where F266A/F324A did not bind gelatin.

CHAPTER 4

DISCUSSION

It has been shown that there is a distant homology between type II domains and the three-disulphide kringle units of thrombolytic and fibrinolytic enzymes (Patthy *et al.* 1984). The non-proteolytic kringle domains are known to mediate protein-protein interactions (Trexler and Patthy, 1983). It was found that the fibronectin type II domain bound collagen and similarly bovine seminal fluid protein PDC-109b bound to gelatin. Through the use of NMR studies, the precise structure of PDC-109b was elucidated which contributed to a better understanding of the fibronectin type II domains (Constantine *et al.*, 1992). It was found that the molecule contained a hydrophobic surface that was hypothesized to be a putative binding surface for gelatin (Constantine *et al.*, 1992). Further NMR studies of the first fibronectin type II domain showed that there was indeed a hydrophobic cluster on one side of the molecule and that the cluster contained a putative hydrophobic binding pocket (Pickford *et al.*, 1997). Five surrounding hydrophobic residues and one strictly conserved tryptophan at the bottom of the pocket forms the hydrophobic pocket on the fibronectin type II domain. The general principle that a conserved primary sequence infers information about the amount of conserved tertiary structure allowed for modelling of the second fibronectin type II module of gelatinase A to PDC-109b (Tordai and Patthy, 1999). Until now there has been a limited number of studies looking at the direct involvement of residues contained within the hydrophobic pocket. Therefore, in

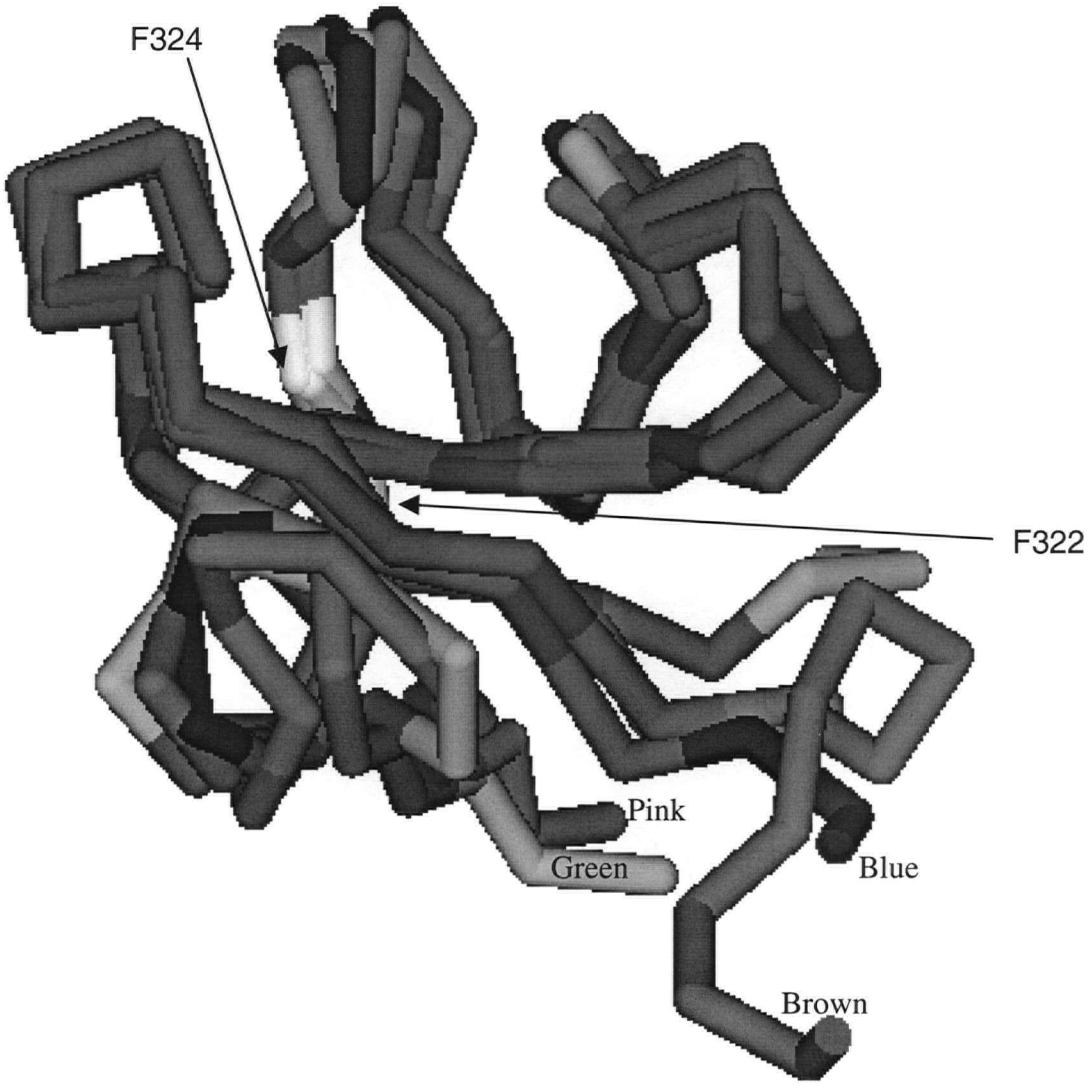
the present work we undertook the challenge to systematically illustrate the roles of key amino acids in gelatin binding.

The Overall laboratory has previously demonstrated that chemical modification of the CBD shows that lysine residues are solvent exposed and that one or more of the residues is involved in heparin and gelatin binding (Steffensen *et al.*, 2001). Since rCBD12 showed weaker collagen binding, the lysine residues in CBD modules 2 and 3 were targeted for alanine substitution to detect binding sites. Total loss of heparin binding by the K357A mutation confirmed the importance of this site in heparin binding. A second mutant protein, K263A, showed reduced levels of binding at saturation to type I collagen without a concurrent change in the apparent dissociation constant. Neither of the mutants with altered heparin or type I collagen affinity nor the K330, K343A, or K298/299A mutants displayed any changes in binding to denatured types IV or V collagen, or elastin. This indicates that the interaction of K263 is ligand specific (Steffensen *et al.*, 2001). The K263A mutation identified a loop on the CBD between two conserved cysteines that is important for collagen interaction. Of note, this loop, CxFPFxFxGxxYxS(/Sx)C (Table 3) is the longest conserved intra-cysteine sequence in all three CBD modules of gelatinase A and B; except that in gelatinase B the placement of the Ser reidues is YSxC. The requirement for DMSO to elute bound CBD from collagen indicates that hydrophobic interactions are involved in binding. This highlights these conserved phenylalanine sites (Table 3).

Using the VAST search service offered by the National Center for Biotechnology Information, we were able to superimpose the three CBD domains over the fibronectin type II domain (Figure 9). It can be seen that there is a high degree of structural homology between the three CBD molecules and FN. Morgunova *et al.* have presented crystallographic data that orients the hydrophobic pockets within each of the CBDs on the outside of what has been likened to a three-prong fishhook (1999). Upon looking at the superimposition of these individual domains it can be seen that the hydrophobic pocket is strictly conserved, with only slight variation in the backbone regions outside the hydrophobic pocket.

In our present work we made the mutants F264A, F264Y, F266A, F266Y, F322A, F322Y, F324A, F324Y, F264A/F322A and F266A/F324A. These mutations were designed to remove the large aromatic side chains of the phenylalanines by exchanging the benzyl side chain of phenylalanine to the smaller methyl side chain of alanine. Alanine mutations are typically conservative and do not disrupt the structure. These mutations were targeted against the first two conserved Phe residues in the conserved sequence CxFPFxFxGxxYxS(/Sx)C that has been implicated to be involved in gelatin binding (Steffensen *et al,*. 2001) (Table 3). Our mutagenesis plan preceded the report of the structure of gelatinase A and this has subsequently enabled us to determine the exact orientation of the phenylalanine residues within the CBD. The F264 and F322 residues appear to be more buried within the β-sheet backbone of the CBD molecule and as such would be predicted to have a lesser effect on binding and possibly play more of a role in contributing to the structural stability of the molecule. Indeed, we still observed binding in these alanine mutants that was slightly reduced relative to

Figure 9



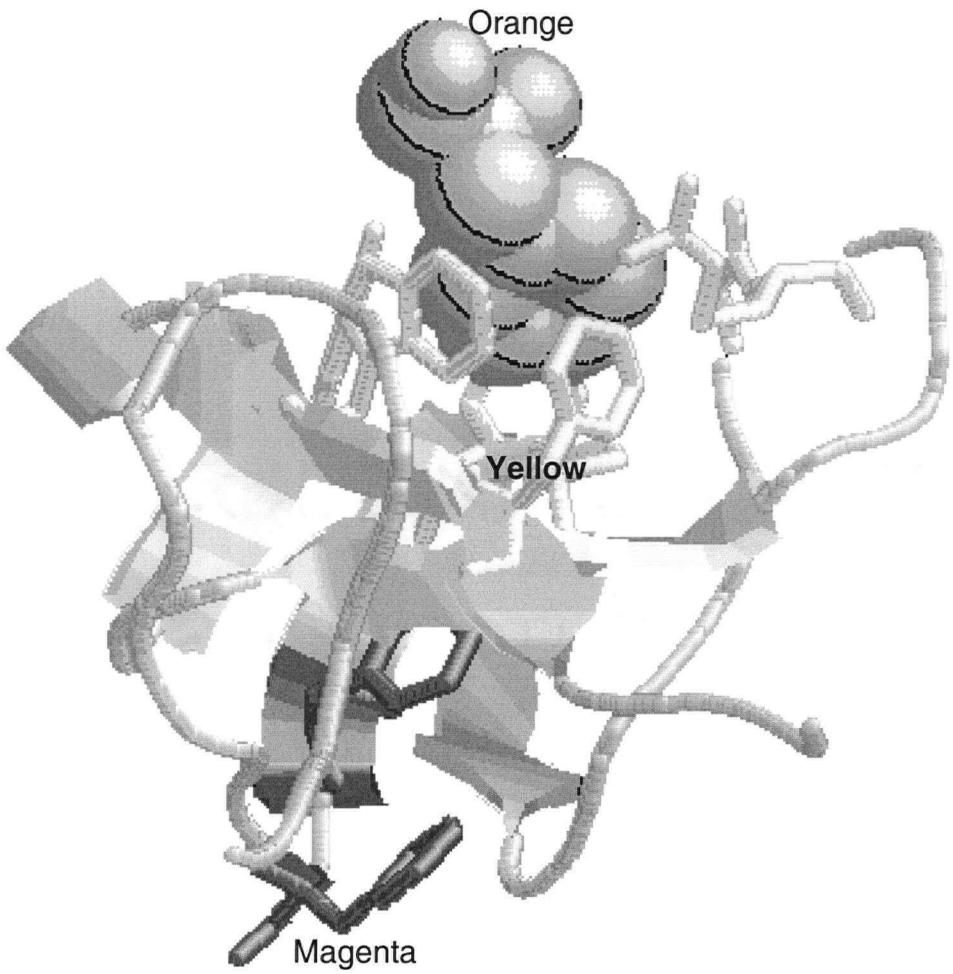

```
FN Type II vlvQT rggnsnGALC HFPFLynnhN YTDCTSEgRR DNMKWCGTTQ NYDADQKFGF Cpma
hGelA_CBD1 qvvRV kygnadGEYC KFPFLfngkE YNSCTDTgRS DGFLWCSTTY NFEKDGKYGF Cphe
hGelA_CBD2 talFT mggnaeGQPC KFPFRfqgtS YDSCTTEgRT DGYRWCGTTE DYDRDKKYGF Cpet
hGelA_CDB3 tamST vggnseGAPC VFPFTflgnK YESCTSAgRS DGKMWCATTA NYDDDRKWGF CPdq
```

Three dimensional structural alignment of the first (green), second (blue), and third (pink) fibronectin type II-like modules of gelatinase A onto the first fibronectin type II domain (brown). This figure illustrates that there is a high degree of three dimensional conservation in the fibronectin type II-like modules. The red regions equate to highly conserved spatial organization.

that of the wild-type domain. This indicated that mutations, which alter the hydrophobic core, did not induce a fatal disruption of molecular stability. Nonetheless we consider it possible that the removal of aromatic character at this position by the Phe→Ala mutations could reduce hydrophobicity within the binding pocket by destabilisation of the Trp located at the bottom of the binding pocket.

Microwell substrate binding assays demonstrated that the wild-type rCBD123 bound gelatin more avidly than wild-type rCBD23 (Figure 7). Abbey *et al.* (2001) found that there was a 10-fold decrease in the binding of rCBD12 to denatured type I collagen. Tordai and Patthy (1994) discovered that removal of the middle domain of CBD123 (CBD13) caused there to be a decrease in the K_a. This illustrates that all of the three CBD modules facilitate the binding of the CBD to substrate. Deletion mutant studies using a solid phase assay have lead to the observation that the loss of a CBD module accounts for a reduction of approximately one order of magnitude. The mutants F264A and F322A showed that the mutated domain contributed to some of the binding as there was an approximate 2.5 - 3 fold reduction in binding for each of the proteins. If there were no contribution by the mutated domain, ie. if it were improperly folded, the single wild-type domain would bind and therefore account for an ~10-fold reduction in binding. The F264A and F322A mutants show reduced, but not abrogated, binding within the mutated domain. This result was attributed to the fact that these residues are buried well within the molecule and that they are far removed from the Trp at the bottom of the hydrophobic pocket (closest distance between the Trp and the Phe rings being 9.2 and 8.7 Å, respectively)(Figure 10).

Figure 10



Crystal structure of the third fibronectin type II module from gelatinase A. Yellow residues highlight the conserved hydrophobic pocket that interacts with F37 of the propeptide (orange). The magenta residues are F322 and F324, with F322 being located further away from the hydrophobic pocket than F324.

Tyrosine mutants were used to determine the effects of a conservative mutation on the module structure. A binding curve similar to that of wild-type rCBD23 was seen for the corresponding Phe→Tyr mutants (Figure 7) indicating that the introduction of a buried phenolic hydroxyl group did not perturb the hydrophobic pocket-gelatin interaction. Possibly these mutants will bind other substrates with altered affinity, but this has yet to be tested.

The mutants F266A and F324A showed a greater reduction in gelatin binding as observed by a respective 6-fold and 13-fold reduction in gelatin binding versus wild-type rCBD23 (Figure 7). The F266A mutants apparent dissociation constant showed that there is some residual binding by the mutated domain, conversely the F324A mutant had a greater than 10-fold reduction in binding. The greater than 10-fold reduction in binding by F324A could be attributed to a disruption of substrate contact by the wild-type domain. Perhaps the mutant domain indirectly hinders substrate interactions with the unmutated module (Figure 7). The residues F266 and F324 are in close proximity to the Trp at the bottom of the hydrophobic pocket compared with F264 and F322 and therefore we suggest have a more profound effect on gelatin binding (closest distance between the Trp and the Phe rings for F266 is 4.4 Å and F324 is 4.1 Å)(Figure 10).

The 5.4-fold reduction in binding of the F264A/F322A mutant showed that binding was reduced relative to the single mutants F264A and F322A but not more than the single mutants F266A and F324A. This further illustrated that the F264A and F322A mutants had reduced binding and that the apparent K_d's for the single mutants represented the average of the individual apparent K_d's in the

bimodular mutants of rCBD23. The reduction in binding for the single mutants F266A and F324A was very similar to the reduction in binding when comparing rCBD123 to either rCBD12 or rCBD23. Individual mutations essentially eliminated binding within that module. Indeed, introduction of both mutations into the rCBD23 protein totally eliminated gelatin affinity (Figure 7).

Differential elution of wild-type and mutant rCBD23 from gelatin Sepharose exploited the property of DMSO in disrupting hydrophobic interactions. The discrepancy in binding of the F322A and F324A mutants in the ELISA assays and the gelatin Sepharose mini-columns may be a result of the exposure of a critical epitope on gelatin that is masked when the gelatin is bound to a microwell titre plate. The mutants F264Y and F324Y had an elution profile similar to the wild-type domain, thus reiterating our observations that the reduction of aromatic character decreases gelatin affinity and that introduction of a phenolic hydroxyl group does not alter gelatin affinity.

Using gelatin Sepharose chromatography the involvement of the wild-type module in the single Phe mutants was addressed. The mutant F264A/F322A eluted at a DMSO concentration greater than that of any single Ala mutants (DMSO %= 3.9). The seemingly high elution concentration of the double mutant F264A/F322A, which retained 83 % of its affinity for gelatin (Table 5), could be attributed to binding of the second module to gelatin binds gelatin at a capacity of 79% that of wild-type protein (Table 5). The second double mutant did not bind gelatin being solely in the flow through materials. This is in agreement with our ELISA results where F266A/F324A did not bind gelatin. From our

Table 5 ELISA and Elution Profile Data for mutants

Recombinant Protein	K_d x 10^{-6} (M) [n]	(K_d Mutant) / (K_d Wt)	Eluting DMSO Concentration (%) [n=2]	DMSO % Mutant / DMSO % Wt
rCBD123 Wt	0.028	0.3	nd	nd
rCBD23 Wt	0.084	1	4.2*	1.00
F264A	0.20	2.3	3.3*	0.79
F264Y	0.11	1.3	4.3*	1.02
F266A	0.49	5.8	2.4*	0.57
F266Y	0.093	1.1	4.2*	1.00
rCBD23 Wt	0.084	1	4.7#	1.00
F322A	0.24	2.8	2.8#	0.60
F322Y	0.19	2.2	4.7#	1.00
F324A	1.1	13	3.0#	0.64
F324Y	0.16	1.9	4.6#	0.98
F264A/F322A	0.46	5.4	3.9#	0.83
F266A/F324A	0	0	0#	0

*Column runs were performed by A. Connor, #Column runs were performed by T. Moore

characterisation of the hydrophobic interactions of the fibronectin type II modules of gelatinase A we have determined that the removal of aromatic character from this region causes a loss of gelatin affinity. We propose this may be through destabilisation of the Trp located at the bottom of the hydrophobic pocket. Evidence for Trp 40 interactions will be determined in the future by a Trp→Ala mutation. The present characterisation of interactions between the hydrophobic pocket and substrate has continued to extend our knowledge of gelatinase A substrate interactions. Further characterisation of the CBD is required in order to fully understand substrate interactions involving the contribution of polar and hydrogen bonding residues, binding modalities that are also believed to be present in fibronectin type II module/gelatin interactions.

Chapter 5

Conclusion

The aromatic side chains of residues F266 and F324 extend behind the Trp that lies at the bottom of the hydrophobic pocket (Figure 10). Therefore, these residues are in close enough proximity that removal of their aromatic character could disturb the Trp that is believed to form the base of a substrate binding pocket that is predicted to interact with substrate. This was best illustrated in our ELISA data, where F266A bound gelatin only 41 % as well as F264A and F324A bound gelatin only 22 % as well as F322A. The most profound illustration of the difference in gelatin binding between the two residues was the difference between F264A/F322A and F266A/F324A where gelatin binding was reduced and then completely abrogated.

The direction for future experiments should focus on directly implicating Trp 39 in gelatin binding. Mutating this residue to an alanine would remove the hydrophobic character at the bottom of the pocket which we hypothesise would eliminate gelatin binding. Upon complete mapping the hydrophobic pocket a more global view of possible CBD involvement in activation and collagenolysis/gelatinolysis could be investigated. This would be accomplished by introducing the above effective point mutations and/or the putative Trp 39 mutation into the full length enzyme. The first experiment would be to follow the process of activation since removal of the hydrophobic pocket with the third CBD will in all likelihood lead to a superactivatable gelatinase A. Upon determination

of the efficiency of gelatinase A binding to immobilized collagen through the use of ELISA and gelatin Sepharose binding/competition assays, an effective gelatin/collagen-minus binding mutant would allow for a determination of the mode of pericellular gelatinase A binding and sequestration. Activated gelatin/collagen-minus gelatinase A would demonstrate CBD-dependent/independent substrates of gelatinase A. This would either reveal new substrates since gelatinase A may be bound and quarantined by collagen or substrates that bind solely to the CBD. This CBD "minus" mutant would offer insight into the role of the CBD and a perspective into the mechanism of collagen triple helicase activity.

From the X-ray crystal structure of gelatinase A it was found that F37 of the propeptide bound back to the third CBD. Two experiments could be designed based on this observation: 1) synthesis of a propeptide analogue consisting 9-10 amino acids and 2) mutation of F37 to either an alanine or a tryptophan. Synthesis of a 9 to 10 residue peptide would allow for experiments into the activation of gelatinase A. Gelatinase A bound to the cell surface via CBD1 and CBD2 is maintained latent by the propeptide. Addition of the synthetic propeptide may dislodge the CBD3-bound propeptide indicating the importance of this interaction. If so, we propose that if the free propeptide liberated by MT1-MMP cleavage of gelatinase A may compete for binding of the propeptide on the CBD and thus may amplify the activation of gelatinase A. The F37 to A37 mutation would address this issue from another viewpoint. If there is no alteration in activation, we would conclude that they F37 interaction with CBD3 is an artifact of crystallization.

References

Abbey, R.S., Steffensen, B., and Overall, C.M. (2001). Module cooperation in gelatinase A collagen binding domain. *In Preparation*

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, Watson, J. (1994). Molecular Biology of the Cell (3rd ed.), pp 971 – 1000. New York: Garland Pulishing Inc.

Allan, J.A., Docherthy, A.J., Barker, P.J., Huskisson, N.S., Reynolds, J.J., and Murphy, G. (1994). Binding of Gelatinases A and B to Type-I Collagen and Other Matrix Components. *Biochem.J.* **309**, 299-306.

Aumailley, M. and Gayraud B., (1998). Structure and biological activity of the extracellular matrix. *J Mol Med* **76** 253-265

Bányai, L. and Patthy, L. (1991). Evidence for the involvement of type II domains in collagen binding by 72 kDatype IV procollagenase. *FEBS Lett* **282** 23-25

Bányai, L. and Patthy, L. (1994). The gelatin-binding site of human 72 kDa type IV collagenase (gelatinase A). *Biochem. J.* **298** 403-407

Bányai, L., Tordai. H., and Patthty, L. (1996) Structure and domain-domain interactions of the gelatin binding site of human 72-kilodalton type IV collagenase (gelatinase A, matrix metalloproteinase 2). *J. Biol. Chem.* **271** 12003-12008

Bigg, H. F., Shi, Y. E., Liu, Y. E., Steffensen, B., and Overall, C. M. (1997). Specific, high affinity binding of tissue inhibitor of metalloproteinases-4 (TIMP-4) to the COOH-terminal hemopexin domain of human gelatinase A. TIMP-4 binds progelatinase A and the COOH-terminal domain in a similar manner to TIMP-2. *J Biol Chem* **272** 15496-15500

Bode W., Fernandez-Catalan, C., Tschesche, H., Grams, F., Nagase H., and Maskos K. (1999). Structural properties of matrix metalloproteinases. *Cell Mol Life Sci* **55** 639-652

Boudreau, N. J. and Jones, P. L., (1999) Extracellular matrix and integrin signalling: the shape of things to come. *Biochem. J.* **339** 481-488

Bresnihan, B. (1999). Pathogenesis of joint damage in rheumatoid arthritis. *J Rheumatol* **26** 717-719

Briknarova, K., Grishaev, A., Banyai, L., Tordai, H., Patthy, L., and Llinas, M. (1999) The second type II module from human matrix metalloproteinase 2: structure, function and dynamics. *Structure Fold Des* **7** 1235-1245

Brown, P. D., Levy, A. T., Margulies, I. M., Liotta, L. A., and Stetler-Stevenson, W. G., (1990). Independent expression and cellular processing of Mr 72,000 type IV collagenase and interstitial collagenase in human tumorigenic cell lines. *Cancer. Res.* **50** 6184-6191

Butler, G. S., Hutton, M., Wattam, B. A., Williamson, R. A., Knauper, V., Willenbrock, F., and Murphy, G., (1999) The specificity of TIMP-2 for matrix metalloproteinases can be modified by single amino acid mutations. *J. Bio.l Chem.* **274** 20391-20396

Calvete, J. J., Mann, K., Sanz, L., Raida, M., and Topfer-Petersen, E. (1996). The primary structure of BSP-30K, a major lipid-, gelatin-, and heparin-binding glycoprotein of bovine seminal plasma.*FEBS Lett.* **399** 147-152

Collier, I. E., Wilhelm, S. M., Eisen, A. Z., Marmer, B. L., Grant, G. A., Seltzer, J. L., Kronberger, A., He, C. S., Bauer, E. A., and Goldberg, G. I. (1998). H-ras oncogene-transformed human bronchial epithelial cells (TBE-1) secrete a

single metalloprotease capable of degrading basement membrane collagen. *J. Biol. Chem.* **263** 6579-6587

Collier, I. E., Krasnov, P. A.,. Strongin, A. Y, Birkedal-Hansen, H., and Goldberg, G. I., (1992). Alanine scanning mutagenesis and functional analysis of the fibronectin collagen-binding domain from human 92-kDa type IV collagenase. *J. Biol. Chem.* **267** 6776-6781

Constantine, K. L., Madrid, M., Banyai, L., Trexler, M., Patthy, L. and Llinas, M. (1992) Refined solution structure and ligand-binding properties of PDC-109 domain b. A collagen-binding type II domain. *J. Mol. Biol.* **223** 281-298

Costigan, M., Chambers, D. A. and Boot-Handford, R. P. (1995). Collagen turnover in renal disease. *Exp Nephrol* **3** 114-121

Davies B., Waxman J., Wasan H., Abel P., Williams G., Krausz T., Neal D., Thomas D., Hanby A. and Balkwill F. (1993) Levels of matrix metalloproteases in bladder cancer correlate with tumor grade and invasion. *Cancer Res.* **53** 5365-5369

Esch, F. S., Ling, N. C., Bohlen, P., Ying, S. Y., and Guillemin, R. (1983). Primary structure of PDC-109, a major protein constituent of bovine seminal plasma. *Biochem. Biophys. Res. Commun.* **113** 861-867

Fontana, A. and Delmas, P.D. (2000). Markers of bone turnover in bone metastases. *Cancer* **88** 2952-2960

Fukushima, D., Butzow, R., Hildebrand A., and Ruoslahti, E. (1993). Localization of transforming growth factor beta binding site in betaglycan. Comparison with small extracellular matrix proteoglycans. *J. Biol. Chem.* **268** 22710-22715

Hay, E.D. (1991) Cell Biology of Extracellular Matrix (2nd ed.), pp 124-127. New York: Plenium Press.

Ishizaki, J., Hanasaki, K., Higashino, K., Kishino, J., Kikuchi, N., Ohara, O., and Arita, H. (1994). Molecular cloning of pancreatic group I phospholipase A2 receptor. *J. Biol. Chem.* **269** 5897-5904

Itoh, T., Tanioka, M., Yoshida, H., Yoshioka, T., Nishimoto, H., and Itohara, S. (1998). Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. *Cancer Res.* **58** 1048-1051

Kleiner, D. E. and Stetler-Stevenson, W. G., (1999). Matrix metalloproteinases and metastasis. *Cancer Chemother Pharmacol* **43** S42-S51

McMullen, B. A. and Fujikawa, K., (1985). Amino acid sequence of the heavy chain of human alpha-factor XIIa (activated Hageman factor). *J. Biol. Chem.* **260** 5328-5341

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

Miyazawa, K., Shimomura, T., Kitamura, A., Kondo, J., Morimoto, Y., and Kitamura, N. (1993). Molecular cloning and sequence analysis of the cDNA for a human serine protease reponsible for activation of hepatocyte growth factor. Structural similarity of the protease precursor to blood coagulation factor XII. *J. Biol. Chem.* **268** 10024-10028

Morgan, D. O., Edman, J. C., Standring, D. N., Fried, V. A., Smith, M. C., Roth, R. A., and Rutter W. J. (1987) Insulin growth factor II receptor as a multifunctional binding protein. *Nature* **329** 301-307

Morgunova, E., Tuuttila, A., Bergmann, U., Isupov, M., Lindqvist, Y., Schneider, G., and Tryggvason K. (1999). Structure of human pro-matrix metalloproteinase-2: activation mechanism revealed. *Science* **284** 1667-1670

Moscatelli, D (1987). High and low affinity binding sites for basic fibroblast growth factor on cultured cells: absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. *J. Cell. Physiol.* **131** 123-130

Murphy, G., Nguyen, Q., Cockett, M. I., Atkinson, S. J., Allan, J. A., Knight, C. G., Willenbrock, F., and Docherty, A. J. (1994). Assessment of the role of the fibronectin domain of gelatinase A by analysis of a deletion mutant. *J. Biol. Chem.* **269** 6632-6636

Murphy, G. and Knauper, V. (1997). Relating matrix metalloproteinase structure to function: why the "hemopexin" domain? *Matrix Biol.* **15** 511-518

Nagase, H and Woessner J. F., (1999) Matrix Metalloproteinases and TIPMs, pp 100-101. New York: Oxford University Press.

Nomura, H., Fujimoto, N., Seiki, M., Mai, M. and Okada, Y. (1996). Enhanced production of matrix metalloproteinases and activation of matrix metalloproteinase 2 (gelatinase A) in human gastric carcinomas. *Int. J. Cancer.* **6**: 9-16

Overall, C. M. and Sodek, J., (1987). Initial characterization of a neutral metalloproteinase, active on native 3/4-collagen fragments, synthesized by ROS 17/2.8 osteoblastic cells, periodontal fibroblasts, and identified in gingival crevicular fluid. *J. Dent. Res.* **6**: 1271-1282

Overall, C. M. and Sodek, J., (1990). Concanavalin A produces a matrix-degradative phenotype in human fibroblasts. Induction and endogenous activation of collagenase, 72-kDa gelatinase, and Pump-1 is accompanied by the suppression of the tissue inhibitor of matrix metalloproteinases. *J. Biol. Chem.,* **265** 21141-21151

Overall, C. M., Wrana, J. L. and Sodek, J., (1991). Induction of formative and resorptive cellular phenotypes in human gingival fibroblasts by TGF-beta 1 and concanavalin A: regulation of matrix metalloproteinases and TIMP. *J. Periodontal Res.* **26** 279-282

Overall, C. M., Wrana, J. L. and Sodek, J. (1991) Transcriptional and post-transcriptional regulation of 72-kDa gelatinase/type IV collagenase by transforming growth factor-beta 1 in human fibroblasts. Comparisons with collagenase and tissue inhibitor of matrix metalloproteinase gene expression. *J. Biol. Chem.* **266** 14064-14071

Overall, C. M., King, A. E., Sam, D. K., Ong, A. D., Lau, T. T., Wallon, U. M., DeClerck, Y. A., and Atherstone J. (1999) Identification of the tissue inhibitor of metalloproteinases-2 (TIMP-2) binding site on the hemopexin carboxyl domain of human gelatinase A by site-directed mutagenesis. The hierarchical role in binding TIMP-2 of the unique cationic clusters of hemopexin modules III and IV. *J. Biol. Chem.* **274** 4421-4429

Overall, C. M., King, A. E., Bigg, H. F., McQuibban, G. A., Atherstone, J., Sam, D. K., Ong, A. D., Lau, T. T.,. Wallon, U. M, DeClerck, Y. A., and E. Tam (1999) Identification of the TIMP-2 binding site on the gelatinase A hemopexin C-domain

by site-directed mutagenesis and the yeast two-hybrid system. *Ann. N. Y. Acad. Sci.* **878** 747-753

Owens, R. J. & Baralle, F. E. (1986) Mapping the collagen-binding site of human fibronectin by expression in Escherichia coli. EMBO J 5: 2825-2830

Parks, W. C. and Mecham, R. P (1998) Matrix Metalloproteinases, pp 85-105. New York: Academic Press.

Pickford, A. R., Potts , J. R., Bright, J. R., Phan, I., and Campbell, I. D. (1997). Solution structure of a type 2 module from fibronectin: implications for the structure and function of the gelatin-binding domain. *Structure* **5** 359-370

Prockop, D. J. and Kivirikko, K. I. (1995). Collagens: molecular biology, diseases, and potentials for therapy. *Annu. Rev. Biochem.* **64** 403-434

Rooprai, H. K., Van Meter, T., Rucklidge, G. J., Hudson, L., Everall, I. P. and Pilkington, G. J. (1998). Comparative analysis of matrix metalloproteinases by immunocytochemistry, immunohistochemistry and zymography in human primary brain tumours. *Int. J. Oncol.* **13** 1153-1157

Sato, H., Takino, T., Kinoshita, T., Imai, K., Okada, Y., Stetler-Stevenson, W. G., and Seiki, M. (1996) Cell surface binding and activation of gelatinase A induced by expression of membrane-type-1-matrix metalloproteinase (MT1-MMP). *FEBS Lett.* **385** 238-240

Schwarzbauer, J. E. and Sechler, J. L. (1999). Fibronectin fibrillogenesis: a paradigm for extracellular matrix assembly. *Curr. Opin. Cell. Biol.* **11**: 622-627

Shipley, J. M., Doyle, G. A., Fliszar, C. J., Ye, Q. Z., Johnson, L. L., Shapiro, S. D., Welgus, H. G. and Senior, R. M. (1996) The structural basis for the elastolytic

activity of the 92-kDa and 72-kDa gelatinases. Role of the fibronectin type II repeats. *J. Biol. Chem.* **271** 4335-4341

Steffensen, B., Wallon, U. M., and Overall, C. M. (1995). Extracellular matrix binding properties of recombinant fibronectin type II modules of human 72-kDa gelatinase/type IV collagenase. High affinity binding to native type I collagen but not native type IV collagen. *J. Biol. Chem.* **270** 11555-11566

Steffensen, B., Bigg, H. F., and Overall, C. M. (1998). The involvement of the fibronectin type II modules of human gelatinase A in cell surface localization and activation. *J. Biol. Chem.* **273** 20622-20628

Steffensen, B., Maurus, R., Ryberg, E., and Overall, C. M. (2001), *in preparation.*

Sticht, H., Pickford, A. R., Potts, J. R., and Campbell, I. D. (1998). Solution structure of the glycosylated second type 2 module of fibronectin. *J. Mol. Biol.* **276** 177-187

Still, K., Robson, C. N., Autzen, P., Robinson, M. C., and Hamdy, F. C. (2000). Localization and quantification of mRNA for matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) in human benign and malignant prostatic tissue. *Prostate* **42** 18-25

Streuli, C. (1999). Extracellular matrix remodelling and cellular differentiation *Curr. Opin. Cell Biol.* **11** 634-640

Taylor, M. E., Conary, J. T., Lennartz, M. R., Stahl, P. D., and Drickamer, K. (1990) Primary structure of the mannose receptor contains multiple motifs resembling carbohydrate-recognition domains. *J. Biol. Chem.* **265** 12156-12162

Tordai, H. and Patthy, L. (1999). The gelatin-binding site of the second type-II domain of gelatinase A/MMP-2. *Eur. J. Biochem.* **259** 513-518

Zubay, G., Parson, W., and Vance, D. (1995) Principles of Biochemistry, pp 79-81. Dubuque, IA: Wm C. Brown Publishers.