The role of matrix metalloproteinases in cell-matrix interactions.

Thesis

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The role of matrix metalloproteinases in cell-matrix interactions

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A thesis submitted to the Open University for the degree of Doctor of Philosophy
Discipline: Cell Biology

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Abstract

Cellular interaction with the extracellular matrix influences basic processes such as proliferation, differentiation, migration and invasion. Matrix metalloproteinase (MMP) activity has been implicated in the remodelling of tissue associated with these events and in the migration of cells through tissue barriers. Evidence suggests that MMP expression and activation is influenced by the matrix components to which the cell is exposed.

The recognition sites of the integrins α1β1 and α2β1 within collagen IV are located in the cyanogen bromide fragment CB3[IV] region, close to the reported gelatinase A (GLA) cleavage site. The susceptibility of solubilised forms of collagen IV to GLA cleavage and the concomitant effects on cell adhesion were assessed. Preparations of collagen IV monomers with disrupted intramolecular disulphide bonds in the CB3[IV] region were cleaved by GLA into the two characteristic 100nm-300nm fragments at 30°C and were totally degraded at 37°C. This resulted in the partial or total inhibition of cell adhesion to the collagen via integrin receptors. Preparations of monomers with intact disulphide bonds in the CB3[IV] region were not susceptible to GLA cleavage. Furthermore, no effect on binding of cells to the GLA-treated collagen could be detected after treatment at 37°C. Dimeric collagen IV with intact disulphide bonds in the CB3[IV] region was not degraded by GLA at 37°C. Tetrameric collagen was highly susceptible to GLA degradation at 37°C, but this did not affect cell adhesion, indicating that the CB3[IV] region of the tetramers remained intact.

The effects of fibronectin and laminin-1 matrices on the activation of GLA was assessed. HT1080 fibrosarcoma cells cultured on fibronectin activated endogenous GLA to a level comparable with that elicited by phorbol ester treatment. In contrast, cells cultured on laminin-1 secreted mainly proGLA. Cells cultured on fragments of fibronectin derived from the central cell binding domain secreted levels of active GLA similar to those observed for full length fibronectin. A substrate of immobilised anti-α5 or anti-β1 integrin antibodies promoted GLA activation, whereas an anti-α6 antibody did not. The data demonstrate that that signals via the α5β1 integrin receptor may be involved in the fibronectin-induced up-regulation of GLA activation.

The effect of fibronectin on the components of the putative MT1-MMP/TIMP-2 'receptor' complex for GLA was assessed. Levels of TIMP-2 protein expressed by HT1080 cells did not vary detectably between cells cultured on fibronectin or laminin-1. However, a fibronectin substrate increased the processing of MT1-MMP to a truncated 45 kDa form, which was concomitant with GLA activation. Inhibitor studies showed that the truncation of MT1-MMP to 45 kDa is MMP mediated, although not inhibited by TIMP-1. This study raises the possibility that truncation of MT1-MMP to 45 kDa form represents a regulatory end-point in the activation pathway of GLA.
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Relevant publications

Material from this thesis has been published in the following reports:


Other reports related to this thesis to which the candidate has contributed during PhD candidature:


<table>
<thead>
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<th>Abbreviations</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>APMA</td>
<td>4-aminophenylmercuric acetate</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid assay</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAPS</td>
<td>3-[Cyclohexylamino]-1-propanesulfonic acid</td>
</tr>
<tr>
<td>CB3[IV]</td>
<td>cyanogen bromide fragment number three, prepared from collagen IV</td>
</tr>
<tr>
<td>CL</td>
<td>collagenase</td>
</tr>
<tr>
<td>CSPD</td>
<td>disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^2,4^7]decan}-4-yl) phenyl phosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagles medium</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E-64</td>
<td>trans-epoxysuccinyl-l-leucylamido (4-guanidino)-butane</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal related kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FCS</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>GLA</td>
<td>gelatinase A</td>
</tr>
<tr>
<td>GLB</td>
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<tr>
<td>GST</td>
<td>glutathione S transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>IFNβ</td>
<td>interferon beta</td>
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<tr>
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</tr>
<tr>
<td>JNK</td>
<td>Jun kinase</td>
</tr>
<tr>
<td>MAP kinase</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>membrane-type matrix metalloproteinase</td>
</tr>
<tr>
<td>ΔTM-MT1-MMP</td>
<td>recombinant trans-membrane deletion mutant of MT1-MMP</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NC1</td>
<td>non-collagenous domain number one</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PI3-kinase</td>
<td>phosphoinositide 3-kinase</td>
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<tr>
<td>PI5-kinase</td>
<td>phosphoinositide 5-kinase</td>
</tr>
<tr>
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<td>phosphatidylinositol 4,5-bisphosphate</td>
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<td>PKC</td>
<td>protein kinase C</td>
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<tr>
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<td>phospholipase C-γ</td>
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<td>PMA</td>
<td>phorbol 12 myristate 13-acetate</td>
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<td>PMSF</td>
<td>phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
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<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide-gel electrophoresis</td>
</tr>
<tr>
<td>SL</td>
<td>stromelysin</td>
</tr>
<tr>
<td>SBTI</td>
<td>soybean trypsin inhibitor</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor-α</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
</tr>
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Introduction
Chapter 1 Introduction

1.1 Overview
The extracellular matrix has dynamic roles not only in supporting cells and tissues, but also in regulating basic cellular functions such as proliferation, differentiation and survival. Cellular interactions with the extracellular matrix (ECM) are mediated in part by the cell surface integrin receptors, which promote cell adhesion and transmit signals to the cells regarding the surrounding environment. Cells can exert control over their environment by synthesising proteolytic enzymes that degrade the structural framework of the ECM. This affects cell anchorage, and processes associated with anchorage such as cell migration and differentiation. Proteolysis also releases matrix bound growth factors and cytokines, and bioactive matrix fragments (reviewed by Werb, 1997). The matrix metalloproteinases (MMPs) are a family of ECM degrading enzymes implicated in the pericellular proteolysis of the ECM. These enzymes are tightly controlled in normal tissue remodelling events such as embryonic development or wound healing. In pathological conditions such as arthritis, cardiovascular disease or cancer, the excessive or inappropriate expression of MMPs is believed to play a major role in tissue destruction. The aim of this study was to investigate aspects of cell-matrix interactions that involve MMP activity. The first part of this study questions whether the proteolytic degradation of basement membrane collagen IV affects cell adhesion to the collagen. The second part investigates the cellular interactions with the ECM that lead to increased MMP activity.

1.2 The matrix metalloproteinases
The MMPs are zinc and calcium-dependent enzymes that function at neutral pH. They are synthesised by a wide variety of cell types, including mesenchymal, hemopoietic and tumour cells. All MMPs are synthesised as inactive zymogens and most members of the family are secreted in zymogen form. The latency of MMPs is maintained by an N-terminal propeptide, which inserts into the catalytic site. Following the proteolytic removal of the propeptide, the active MMPs are subject to inhibition by a family of MMP specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). Currently there are sixteen members of the mammalian MMP family, which can be divided on the basis of
The collagenases (CL-1, CL-2, CL-3) have been named for their specificity for fibrillar collagens, however they do show some activity against other matrix molecules (see Table 1.1). The gelatinases (GLA, GLB) cleave non-fibrillar and denatured collagens (gelatins) and have been named the type IV collagenases, based on their ability to cleave solubilised preparations of collagen IV. The stromelysins (SL-1, SL-2, SL-3) have the broadest substrate specificities, cleaving proteoglycans, laminins, elastin, and a number of the collagens, including collagen IV. SL-3, however, shows only weak stromelysin-like activity and the substrates for this enzyme have not been identified. SL-3 is unique amongst the stromelysins as it is activated intracellularly by the furin family of enzymes. MMP-19 and MMP-20 have only recently been cloned, and to date there is no information on substrate specificity, however they have been included in the stromelysin family on the basis of structural similarities (Table 1.1). The MT-MMPs are grouped on the basis of structure rather than substrate specificity, as these MMPs possess a transmembrane domain that locates them to cellular membranes. Finally, metalloclastase and matrilysin have not been included in the major groupings, although they display strong stromelysin-like activity. Matrilysin is unique amongst the MMPs as it is lacking the C-terminal domain (MMP structure described below) and metalloclastase is unique as it undergoes both N-terminal and C-terminal processing to form an active 22 kDa enzyme.

1.2.1 The structure and function of MMP domains

Figure 1.1 shows a schematic representation of MMP domain-structure. All MMPs possess an N-terminal propeptide domain, a catalytic domain and a C-terminal domain (with the exception of matrilysin). The MT-MMPs have an additional transmembrane spanning region and a short cytoplasmic tail.

Details of the structure of the propeptide domain have been provided by X-ray crystallographic studies of SL-1. The propeptide consists of a separate folding unit of three
α-helices and an extended sequence, that protrudes into the active site cleft of the catalytic domain (Becker et al., 1995). The conserved amino acid sequence PRCGVPD in the propeptide region is a major determinant of enzyme latency, as the free cysteine residue coordinates the catalytic zinc. Propeptide removal is therefore an important step in the regulation of MMP activity. Details of the mechanism of MMP activation are given in Section 1.4.2.

The catalytic domain of the MMPs is characterised by the amino acid sequence **HEXXHXXGXXH**, which coordinates the catalytic zinc ion via the histidine residues. MMP catalytic domains have been studied in the presence or absence of inhibitors by X-ray crystallography (Bode et al., 1994; Dhanaraj et al., 1996; Fernandez-Catalan et al., 1998) and by NMR spectroscopy (Gooley et al., 1994). The catalytic domain has a characteristic spherical shape, notched by a shallow active site cleft close to the C-terminus of the domain. The main N-terminal region of the domain consists of a 5-stranded β sheet, flanked by 3 surface loops and 2 α-helices, the second helix termed the 'active-site helix'. The catalytic zinc ion is coordinated at the bottom of the active site cleft. Proximal to the active site cleft is the S1' pocket, which varies in depth between the MMPs, contributing to substrate specificity. Also coordinated in the catalytic domain is a second zinc ion, as well as 1-2 calcium ions, which most likely play a structural role. The gelatinases have an extra domain, comprising three fibronectin type II-like repeats, which are inserted into the catalytic domain N-terminally to the active site. Homology modelling has suggested that these repeats are inserted at the right hand end of the active site cleft, close to the S1' pocket of the enzyme, and they are therefore thought to be crucial for proper orientation of the substrate relative to the catalytic site (Banyai et al., 1996). Several groups have expressed these repeats in isolation and have shown that they bind to denatured type I collagen (Collier et al., 1992; Banyai et al., 1994), denatured type IV and V collagens, elastin and native type I collagen (Steffensen et al., 1995).

The catalytic domain of the MMPs is separated from the C-terminal domain by a hinge region, which varies in length across the MMP classes. In the case of the collagenases, the
The hinge region is rich in proline residues and may play a role in determining the specificity of these MMPs for fibrillar collagens.

The C-terminal domain of the MMPs bears strong sequence similarity with the hemopexin family of proteins. X-ray crystallographic studies of the C-terminal domain of porcine collagenase-1 (Li et al., 1995), human collagenase-3 (Gomis-Ruth et al., 1996) and human gelatinase A (Lisbon et al., 1995; Gohlke et al., 1996) have reported that this domain is comprised of four similar units that form a four-bladed β propellor structure. The blades of the propellor are formed from antiparallel four-stranded β sheets and are arranged around a tunnel that contains cations (calcium) and anions (chloride) at the core. A disulphide bridge between the first and fourth propellor, which is conserved across all the MMPs, acts to stabilise the structure. Studies have shown that the substrate specificity of the collagenases is determined by interactions between the fibrillar collagens and the C-terminal domain of the collagenases (Murphy et al., 1992a; Gomis-Ruth et al., 1996; Knäuper et al., 1997). Modelling experiments suggest that the relatively short hinge region of CL-1 allows the positioning of the C-terminal domain over the active site cleft, trapping triple helical collagen between the N- and C-terminii of CL-1 (De Souza et al., 1996). For the other classes of MMPs, the C-terminal domain does not appear to be important in determining substrate specificity. However, it does play an important role in the initiation of TIMP binding to the gelatinases, and consequently affects the activation of GLA (discussed in detail below).

The MT-MMPs are clearly a unique class of the MMPs, as they possess a transmembrane domain that locates them to the plasma membrane and a cytoplasmic tail. In addition, the MT-MMPs have an insert in the catalytic domain, the function of which is unknown, but crystallographic studies suggest that it forms an exposed loop (Fernandez-Catalan et al., 1998). The MT-MMPs also have a short basic peptide sequence RRKR in the propeptide that is a recognition sequence for the furin family of enzymes, which may contribute to the activation of MT-MMPs.
1.3 The TIMPs

The major inhibitors of the MMPs in vivo are the TIMPs (reviewed by Edwards et al., 1996). There are four members of the TIMP family, secreted by many cell types, including those of the connective tissue, and they play an important role in determining the effectiveness of tissue turnover by the MMPs. In normal tissue homeostasis the activity of MMPs is tightly controlled by the TIMPs, however in pathological conditions such as arthritis and cancer an imbalance between MMP and TIMP production has been linked with tissue destruction (Gomez et al., 1997).

TIMP-1 (Docherty et al., 1985; Carmichael et al., 1986) is a glycoprotein of 28.5 kDa and is the most ubiquitous of the TIMPs. The expression of TIMP-1 is influenced by many cytokines and growth factors. By comparison, TIMP-2 (Stetler-Stevenson et al., 1989; Boone et al., 1990) is a 21 kDa, non-glycosylated protein expressed constitutively by most cell types. TIMP-3 (21 kDa; may be glycosylated) is localised to the ECM (Pavloff et al., 1992; Leco et al., 1994), and thus differs from other TIMP family members which are freely diffusible. The recently cloned TIMP-4 (22 kDa; non-glycosylated) is more closely related to TIMPs -2 and -3 than to TIMP-1 (Greene et al., 1996; Leco et al., 1997). Some distinct tissue distributions have been observed for the TIMPs, suggesting separate and individual roles for the TIMPs in tissue remodelling (reviewed by Edwards et al., 1996).

All four TIMPs form high affinity, non-covalent bonds with active MMPs, at a 1:1 stoichiometry. MMP/TIMP complexes can be dissociated to give competent inhibitor (Murphy et al., 1989b), however complexes such as active GLA/TIMP-2 are very stable (Hutton et al., 1998) and binding is essentially irreversible. Overall, the TIMPs show relatively little selectivity in binding to the MMPs, however certain TIMPs do show specificity for some MMPs. GLA and GLB are more sensitive to inhibition by TIMP-2 than TIMP-1 (Howard et al., 1991) and GLB is very effectively inhibited by TIMP-3 (Edwards et al., 1996). MT1-MMP and MT2-MMP do not appear to be sensitive to TIMP-1 inhibition, but are inhibited by TIMPs-2 and -3 (Will et al., 1996; Butler et al., 1997). Interestingly, a recent study has shown that TNFα converting enzyme, a member of
the ADAM family of metalloproteinases, is inhibited by TIMP-3 but not by TIMPs -1, -2 and -4 (Amour et al., 1998). This enzyme processes membrane bound proTNFα to yield mature TNFα, a major inflammatory mediator. This study and others (Smith et al., 1997; Hargreaves et al., 1998) implicate TIMP-3 as a regulator of a broader class of metalloproteinases.

1.3.1 The structure and function of TIMP domains

The TIMPs are structurally similar, despite a low homology between their amino acid sequences. This is due to twelve conserved cysteine residues which form six disulphide bonded loops. These disulphide bonds confer resistance to protein denaturation by changes in pH, temperature or ionic strength. The six loops can be divided into two structurally distinct domains. The N-terminal domain (loops 1-3) is autonomously folding and is sufficient for MMP inhibition (Murphy et al., 1991b). Recent studies by NMR spectroscopy (Muskett et al., 1998) and X-ray crystallography (Gomis-Ruth et al., 1997; Fernandez-Catalan et al., 1998) of N-terminal or full length TIMPs complexed with catalytic domains of MMPs have confirmed that multiple regions on TIMP molecules are important for interaction with MMPs. A comparison between NMR studies of N-terminal TIMP-2 complexed with the catalytic domain of SL-1 (Muskett et al., 1998) and crystallographic studies of TIMP-1 complexed with catalytic domain of SL-1 (Gomis-Ruth et al., 1997) has revealed specific interactions between N-terminal TIMP-2 and SL-1 that do not occur between TIMP-1 and SL-1, which may contribute to TIMP specificities (Muskett et al., 1998).

Several activities have been mapped to the C-terminal domain (loops 4-6). Interactions between MMPs and TIMPs can be initiated by the 'docking' of the C-terminal domain of TIMPs with the C-terminal domain of MMPs (Willenbrock et al., 1993). These interactions are particularly important for the binding of TIMPs -1 -2 and -4 with active GLA (Willenbrock et al., 1993), TIMP-1 with active GLB (O'Connell et al., 1994), and TIMPs -1 and -3 with active CL-3 (Knäuper et al., 1997). However, for SL-1 the interactions between the C-terminal domain of TIMPs -1 and -2 and the C-terminal domain
of the enzyme are negligible (Nguyen et al., 1994). Significantly, the C-terminal domain of the TIMPs also bind the latent gelatinases. The C-terminal domain of TIMP-1 specifically binds the C-terminal domain of proGLB and the C-terminal domains of TIMPs -2 and -4 specifically bind the C-terminal domain of proGLA (Howard et al., 1991; Murphy et al., 1991b; Goldberg et al., 1992a, 1992b; Willenbrock et al., 1993; Bigg et al., 1997). ProGLA bound to TIMP-2 can be activated in vitro by organomercurial agents in the same way as 'free' proGLA is activated (activation mechanisms are discussed below), although the specific activity is reduced in comparison with 'free' enzyme (Goldberg et al., 1992b). TIMP-2 bound via its C-terminus retains its ability to inhibit active MMPs (Kolkenbrock et al., 1991). For GLA, the significance of these C-terminal domain interactions lies in the key role that they play in proGLA activation, which is discussed in more detail below.

TIMP-1 has also been described as erythroid-potentiating activity as it stimulates the growth of erythroid progenitor cells (Gasson et al., 1985). Several reports have demonstrated that TIMPs act as growth factors (reviewed by Gomez et al. 1997). Studies by Chesler et al. (1995) using N-terminal TIMP-1 point-mutants that lacked MMP inhibitory activity demonstrated that the effects of TIMP-1 on erythroid progenitor cell growth were independent of MMP inhibition.

1.4 Regulation of MMP activity

MMP activity is controlled at a number of levels. The transcription of MMP mRNA is regulated by a number of factors including cytokines, growth factors, and tumour promoters. The removal of the propeptide is a critical step in the control of MMP activity. Once they are activated MMPs are tightly controlled by the TIMPs and by α2-macroglobulin. Some aspects of MMP regulation are described below, with particular attention paid to the activation cascades that control MMP activity at the cell surface.
1.4.1 Regulation of transcription

Most cell types normally synthesise negligible amounts of MMPs, but production can be stimulated by cytokines such as IL-1 and TNF-α, growth factors (EGF, PDGF and bFGF), hormones, tumour promoters and agents which disrupt the cytoskeleton, such as cytochalasin D (reviewed by Birkedal-Hansen, 1993). Evidence suggests that cells in culture also synthesise MMPs in response to ECM molecules and matrix fragments. For example, fibronectin fragments induce CL-1, GLB and SL-1 expression by rabbit synovial fibroblasts (Werb et al., 1989) and vitronectin induces the secretion of GLA by melanoma cells (Bafetti et al., 1998). Collagen lattices induce MT1-MMP and GLA expression (Gilles et al., 1997; Haas et al., 1998); these are the first reports to show that MT-MMP expression is induced by ECM. It is likely that many of the effects of ECM on MMP expression are mediated by integrin receptors, and this is discussed later in the chapter. All of the MMP promoters studied to date contain a binding site for the Fos/Jun containing complex AP-1, with the exception of GLA and SL-3. Studies have shown that AP-1 activity is involved in the transcription of MMPs in response to IL-1, TNF-α, IFNβ and fibronectin induction (reviewed by Crawford and Matrisian, 1996). MMP promoters also contain multiple PEA3 sites (with the exception of GLA), which bind the Ets family of nuclear protooncogenes. NF-κB binding sites are common in MMP promoters, and cooperativity for MMP expression between AP-1 and PEA3 binding sites (Gutman and Wasylyk, 1990; Wasylyk et al., 1990) and between AP-1 and NF-κB binding sites has been documented (Gum et al., 1996). The similarity between the MMP promoters allows for coordinated transcription of the MMPs. However, differential transcription of the MMPs is also achieved and this most likely occurs as a result of the differing arrangements of binding elements between the promoters, as well as the number of Fos/Jun/Ets family members, each of which could have a different effect on MMP transcription (reviewed by Crawford and Matrisian, 1996).

1.4.2 MMP activation

MMP latency is maintained by the presence of the propeptide domain that extends into the active site of the enzyme. The free cysteine in the conserved PRCGVPD sequence
coordinates the catalytic zinc in the active site cleft. Activation of MMPs involves the disruption of this bond. This can be achieved in vitro by thiol reactive agents such as the organomercurial APMA, or by denaturants such as SDS and urea. Stepwise intramolecular and bimolecular cleavages then complete the removal of the propeptide (reviewed by Nagase, 1997). The 'cysteine switch hypothesis' predicts that in vivo, the first step in activation is the cleavage of a protease-sensitive 'bait region' between the first and second α-helices of the propeptide. This destabilises the interaction between the propeptide and the catalytic domain, and allows the coordination of a water molecule to the catalytic zinc. Further processing from the intermediate to the fully active form of the MMP is then completed by an MMP (reviewed by Nagase, 1997; Knäuper and Murphy, 1998). The bait sequence dictates which proteinases activate a particular MMP. The potential physiological activators of the MMPs include plasmin, plasma kallikrein, cathepsin B, cathepsin G, neutrophil elastase and the MMPs themselves (Murphy, 1995).

Most MMPs are secreted as latent zymogens and are activated extracellularly. SL-3 and the MT-MMPs are an exception, as they are activated intracellularly. These enzymes have the characteristic basic sequence motif RXK/RR, immediately N-terminal to the start of the catalytic domain. This sequence is recognised by the furin-type convertases, which are localised to the trans-Golgi network, and studies have implicated furin in the activation of SL-3 and MT1-MMP (Pei and Weiss, 1995; Pei and Weiss, 1996). The activation of MT1-MMP by furin is the subject of some controversy, however, as studies have shown that coexpression of recombinant full-length MT1-MMP and furin in COS cells has no effect on the molecular mass of the membrane-associated form, suggesting that proMT1-MMP is not a substrate for furin (Cao et al., 1996).

1.4.2a Cell surface activation cascades: the role of plasmin

The serine proteinase plasmin is considered to be one of the key enzymes in the activation of the MMPs. Active plasmin is generated by cleavage of plasminogen by the plasminogen activators urokinase-plasminogen activator (uPA) or tissue-type plasminogen activator (tPA). uPA is implicated in the generation of plasmin at the cell surface. Briefly,
the inactive pro-uPA is secreted by connective tissue cells in response to inflammatory mediators (such as IL-1) and binds to a specific cell-surface receptor (uPAR). Receptor bound pro-uPA is then cleaved to give uPA, which in turn cleaves plasminogen to plasmin. Plasmin can activate several MMPs, as shown in Figure 1.2. SL-1 is particularly susceptible to plasmin activation and once activated can potentiate CL-1 activity (reviewed by Murphy, 1995), thus contributing to the activation cascade (Fig. 1.2). Control of these activation cascades may be exerted by the plasminogen activator inhibitors (PAIs) and to a certain extent by the TIMPs, which can slow down or inhibit MMP autocatalytic cleavages (Murphy, 1995).

1.4.2b Cell surface activation cascades: the role of MT1-MMP

ProGLA is resistant to activation by a range of endopeptidases (Okada et al., 1990). Although CL-1 and matrilysin have been shown to activate proGLA, the rate of activation is very slow (Crabbe et al., 1994; Sang et al., 1996). Several recent studies have suggested that the uPA/plasmin system can activate GLA (Baramova et al., 1997; Mazzieri et al., 1997) although this remains the subject of controversy. Early studies with membranes isolated from concanavalin-A treated fibroblasts or phorbol ester treated fibrosarcoma cells showed that proGLA was activated by a membrane-dependent mechanism (Ward et al., 1991b; Strongin et al., 1993). Activation was prevented by the deletion of the C-terminal domain of GLA (Murphy et al., 1992b) and excess TIMP-2 inhibited proGLA activation, suggesting an MMP dependent activation process (Ward et al., 1991b). The first MT-MMP was cloned in 1994 (MT1-MMP) and was identified as a potential activator of proGLA (Sato et al., 1994). Studies with a recombinant transmembrane deletion mutant of MT1-MMP in solution showed that MT1-MMP performs the initial cleavage of the GLA propeptide, and activation is then completed by bimolecular autolysis (Atkinson et al., 1995). Strongin et al., (1995) were the first to suggest that TIMP-2 might aid in the location of GLA to the cell surface. These authors noted that proGLA activation is exquisitely sensitive to TIMP-2 concentrations, with high levels of TIMP-2 inhibiting GLA activation, whereas low levels facilitated activation. By cross-linking studies, Strongin and coworkers identified MT1-MMP/TIMP-2 complexes, and hypothesised that the N-terminal
domain of TIMP-2 binds the active site of MT1-MMP, leaving the C-terminal domain of TIMP-2 available for binding the C-terminal domain of proGLA. The concept of an MT1-MMP/TIMP-2 complex acting as a 'receptor' for proGLA has since been supported by several studies (Imai et al., 1996; Butler et al., 1997; Kinoshita et al., 1998). A hypothesis for GLA activation has emerged from these studies, that predicts that MT1-MMP/TIMP-2 complexes on the cell surface act as receptors for proGLA, and cleavage of the propeptide of GLA is initiated by an adjacent 'TIMP-2' free MT1-MMP molecule that is catalytically active. GLA activation is then completed by bimolecular autolysis (Butler et al., 1998). Figure 1.3 is a schematic representation of this hypothesis.

Studies in our laboratory have shown that CL-3 is also activated by a cell membrane-mediated process, involving MT1-MMP. Activation is enhanced by the presence of proGLA (Knäuper et al., 1996b), however it is unlikely that C-terminal interactions between CL-3 and TIMP-2 are involved (Knäuper et al., 1997). Studies with a chondrosarcoma cell line have shown that active MT1-MMP, GLA and CL-3 in turn activate proGLB (Cowell et al., 1998), implying an MT1-MMP initiated MMP activation cascade (see Fig. 1.2).

1.4.3 Inhibition of MMP activity

Specific inhibition of the MMPs by the TIMPs was discussed in section 1.3.1. The other physiological inhibitor of the MMPs is plasma α2-macroglobulin, which is a general proteinase inhibitor. This 780 kDa inhibitor has a proteinase sensitive bait region, and cleavage within this region initiates a conformational change that traps the enzyme and prevents access to large substrates (Starkey and Barrett, 1977). Inhibition by α2-macroglobulin is irreversible.

1.5 The extracellular matrix

The extracellular matrix components that are important to this study are reviewed in the following sections. Collagen IV and laminin create the structural framework that imparts
strength and flexibility to the basement membrane. Fibronectin is associated with the fibrillar collagens in the connective tissue stroma, where it promotes a wide range of cellular activities, including cell adhesion and migration.

1.5.1 Basement membrane

The basement membrane is a thin sheet of matrix proteins, in direct contact with cells, that provides both structural support and an interactive surface for cell adhesion, migration and differentiation (reviewed by Timpl and Brown, 1996; Yurchenco and O'Rear, 1993). Basement membranes divide tissue compartments and are found underlying epithelial and endothelial cell layers, and surrounding muscle cells and peripheral nerve axons. They are chiefly comprised of type IV collagen and laminin, both of which self assemble to form large irregular networks. These networks are linked by nidogen (entactin), and include heparan sulphate proteoglycans, SPARC (osteonectin, BM-40) and the fibulins. Some of the principle components of the basement membrane are described below.

1.5.1a Type IV collagen

Type IV collagen is a major constituent of basement membranes (reviewed by Timpl and Brown, 1996; Kühn, 1994). The major isoform consists of two α1(IV) chains and one α2(IV) chain that interact to form a 400 nm long flexible monomer with a globular, non collagenous (NC1) domain at the C-terminus (see Fig. 1.4). The triple helical domain of collagen IV is characterised by frequent non-triple helical interruptions. Collagen IV self-associates to form a dense meshwork, stabilised by covalent crosslinking. Insight into the molecular interactions that contribute to network formation was gained by partial proteolysis of basement-membranes derived from the murine Engelbreth-Holm-Swarm (EHS) tumour and human placenta. Electron microscopy revealed that dimers were formed by bonding between C-terminal NC1 domains, and tetramers were formed by parallel and antiparallel alignment of the N-terminal 7S domains. Studies of human amniotic membranes by electron microscopy also showed that lateral associations occur between the central triple helical portion of the molecule, and these lead to superhelical twisting between the laterally associated segments (Yurchenco and O'Rear, 1993). A schematic
representation of the network is shown in Figure 1.4B. Distinct kinks in the triple helical region of the molecule are caused by the non-triple helical interruptions, which vary in length between the α[IV] chains, and contribute to the flexibility of the molecule. These networks are stabilised by disulphide linkages and/or non-reducible lysine-aldehyde derived cross-links in the 7S and NC1 domains, and this renders the molecule highly insoluble.

Cells interact with type IV collagen via α1β1 and α2β1 integrin receptors. The integrin binding sites have been mapped to a triple helical region of the molecule CB3[IV], located 100 nm from the N-terminus (see Fig. 1.4A; Eble et al., 1993). Integrin recognition is dependent upon triple helical conformation, which is stabilised by intramolecular disulphide bonds in the CB3[IV] region. Further details on integrin recognition sites in collagen IV are given in Chapter 4.

1.5.1b Laminin

Laminin is a large glycoprotein comprising three chains α, β and γ (also designated A, B1 and B2 respectively) that associate to form a cross-shaped heterotrimer. The three short arms each have 2-3 globular domains that may be important for self-assembly (reviewed by Yurchenco and O'Rear, 1993). The chains of the long arm twist together to form an α-helical coiled-coil, and is terminated by a large C-terminal globular domain that is involved in cellular interaction (see Fig. 1.5). To date, 5 α chains, 3 β chains and 2 γ chains have been identified which combine to form 11 isoforms (reviewed by Aumailley and Smyth, 1998). Laminin-1 is the most extensively studied isoform, as it is easily purified from the murine EHS tumour, however it should be noted that in humans laminin-1 has a restricted expression. Although all isoforms possess a similar domain organisation, certain isoforms have a tissue-specific distribution, which suggests that the isoforms contribute to the functional diversity of basement membranes.

Laminin network formation has been studied by the in vitro polymerisation of laminin-1. Formation of oligomers is both concentration and divalent cation dependent. The network
is formed by end-to-end association of laminin molecules, in which each short arm binds to two or three neighbouring arms to form an hexagonal oligomer. The binding affinity between the laminin molecules is low, indicating that the laminin network lacks the strength of the collagen IV network. However, laminin networks are more dynamic structures and may be important in newly formed basement membranes that lack type IV collagen (reviewed by Yurchenco and O'Rear, 1993).

Cellular interaction with laminin has been mapped by proteolytic fragmentation of laminin, with most data derived from laminin-1 (see Fig. 1.5). Interactions with the short arms are mediated by α1β1 and α2β1 integrins (Pfaff et al., 1994) and recently the binding site has been mapped to the VI domain of the α-chain (Colognato et al., 1997). An integrin binding site has been mapped to the cross region of the three short arms, recognised by αvβ3 integrin, however this site is cryptic in murine laminin-1 and absent in human laminin-1 (Horton et al., 1994). The major cell binding site for laminin-1 is in the proteolytic fragment E8, in the C-terminal globular domain. The α6β1 integrin recognises this region (Sonnenberg et al., 1990) and is regarded as the classical laminin receptor. Other integrins that recognise this region are the α7β1, α6β4 and α3β1 integrins, although the binding of α6β4 integrin is controversial (Eble et al., 1998; reviewed by Aumailley and Smyth, 1998).

Interactions with laminins control cell adhesion and migration, differentiation and polarity, proliferation and cell fate, and affect gene expression. Laminins are crucial in mesenchymal-epithelial and mesenchymal-endothelial transitions that occur during embryonic development (reviewed by Dziadek, 1995). Diseases which are characterised by the absence or alteration of various laminin chains lead to skin blistering (epidermolysis bullosa) and muscular dystrophy (reviewed by Aumailley and Smyth, 1998).

1.5.1c Architecture of the basement membrane

There is no evidence to suggest that the laminin and collagen IV networks interact with each other directly, however other basement membrane components such as nidogen may
act as linkers. Nidogen (150 kDa) has a rod-like structure with three globular domains, two situated at the N- and C-terminii (G1 and G3) and one in the middle (G2). A high affinity interaction occurs between laminin and the G3 domain. The G2 domain can interact simultaneously with collagen IV or perlecan, the main heparan sulphate proteoglycan of basement membranes. These ternary complexes connect the two major networks, thus it is likely that nidogen plays a crucial role in the assembly of basement membranes (reviewed by Dziadek, 1995).

1.5.2 Fibronectin

Fibronectins are found in most body fluids, soft connective tissue matrices and granulation tissues. They are involved in a diverse range of cellular processes including cell adhesion, migration, differentiation, proliferation and survival and are implicated in oncogenic transformation (reviewed by Mohri, 1997). The importance of fibronectin in development is underscored by the observation that a transgenic mouse lacking fibronectin dies during embryonic development (Watt and Hodivala, 1994). There are two classes of fibronectin: plasma fibronectin and cellular fibronectin, which are very similar in structure and properties. Fibronectin is a disulphide bonded dimer of ~450 kDa, consisting of two similar subunits of ~225 kDa, composed of homologous repeating subunits designated type I, II and III repeats (Figure 1.6). It is encoded by a single gene, with variations in the structure arising from alternative splicing of the primary transcript. Three alternatively spliced regions have been identified, two of which consist of type III repeats ED-A and ED-B (also called EIII-A, EDI and EIIIB, EDII) which can be included or excluded by exon skipping (Figure 1.6). The third region, termed the connecting segment or IIICS region, is more complex and comprises a number of separate and independently spliced segments. Plasma fibronectin, which is secreted by hepatocytes and abundant in blood (300 μg/ml), does not contain the ED regions, and is smaller in size than cellular fibronectins. The cellular fibronectins may or may not contain the ED regions and exhibit various permutations of the IIICS region (Mohri, 1997).
Fibronectin has been shown to bind a number of matrix macromolecules, including collagen (and gelatin), heparin, heparan sulphate proteoglycans and fibrin (Figure 1.6). These interactions may play an important role in wound healing, for soluble fibronectin that is deposited on damaged collagen and/or fibrin serves as a guide for inwardly migrating fibroblasts (Greiling and Clark 1997). Other molecules that are reported to bind to fibronectin are DNA (although the physiological significance of this is unclear), IgG, C3 complement, thrombospondin, plasminogen and plasminogen activator (reviewed by Mohri, 1997).

Fibronectin interacts with cells via cell-surface receptors, controlling cell adhesion, cytoskeletal organisation and intracellular signalling. The most extensively studied cell attachment site maps to the type III repeats, known as the central cell binding domain (CCBD). A tetrapeptide of amino acid sequence RGDS in Fn III repeat no. 10 (Fn III 10) is essential for recognition by a number of integrin receptors (Figure 1.6) and a pentapeptide sequence in Fn III 9 synergises with the RGD site to promote full adhesion activity (Danen et al., 1995). Integrin dependent cell adhesion has also been mapped to the alternatively spliced IIICS region, which is recognised by integrins with α4 subunits. Studies using synthetic peptides spanning the entire IIICS region identified cell attachment sites in the peptides CS-1 and CS-5. Both sites have sequences based on the RGD-motif, with LDV acting as the active sequence in the CS-1 peptide and REDV the active sequence in the CS-5 peptide (Komorya et al., 1991; Mould et al., 1991). A third sequence IDAPS in the C-terminal heparin binding region of fibronectin is a homologue to the LDV sequence of the CS-1 peptide and interacts with the α4 integrin subunit (Mould and Humphries, 1991). Several sites in fibronectin promote RGD-independent cell adhesion. One has been mapped to the alternatively spliced ED-A region, (Xia and Culp, 1995) and another is located to the C-terminal heparin binding domain (Figure 1.5) which interacts with cell surface heparan sulphate proteoglycan to augment cell attachment and focal adhesion formation (McCarthy et al., 1990).
Fibronectin is a highly flexible molecule and its shape can be markedly affected by solution conditions such as pH and ionic strength. Electron microscopy has shown that plasma fibronectin adopts a range of shapes, from a compact structure to an elongated rod-like structure, and the deposition of fibronectin onto surfaces can result in irreversible unfolding to form an extended conformer (Erickson and Carrell, 1983; Tooney et al., 1983). In vivo, fibronectin forms a matrix of disulphide cross-linked fibrils. Fibril formation is initiated by the capturing of secreted fibronectin onto integrins α5β1 and αvβ3, and is completed by assembly and reorganisation of the cell-surface associated fibronectin into fibrils. Fibronectin self-association via FN III 1 is important in promoting this matrix assembly (reviewed by Magnusson and Mosher, 1998).

In vitro, it has been shown that certain functional domains of fibronectin are buried in the intact molecule and become fully exposed upon surface-adsorption, denaturation or binding to gelatin and heparin (Homandberg, 1987; Underwood et al., 1993; Ugarova et al., 1995). Fibronectin has a number of protease sensitive regions, and MMPs, cathepsins, plasminogen activators, plasmin and thrombin have all been shown to degrade fibronectin in vitro (Liotta et al., 1981; Fukai et al., 1995; Marchina and Barlati, 1996; Ugarova et al., 1996). Proteolytic cleavage of fibronectin also results in the release of functional domains (Homandberg, 1987; Ugarova et al., 1995). An interesting study by Fukai et al. (1995) showed that degradation of plasma fibronectin by MMPs resulted in the liberation of the CCBD, as well as the N-terminal and C-terminal fibrin-binding domains, the latter which stimulated RGD-independent fibroblast migration. It is well documented that fragments of fibronectin have biological activities distinct from full length fibronectin, with reports showing that various fragments increase cartilage aggrecan degradation and promote SL-1 expression by chondrocytes (Homandberg et al., 1992; Bewsey et al., 1996), control SL-1, CL-1 and GLB expression by rabbit synovial fibroblasts (Werb et al., 1989; Huhtala et al., 1995) and stimulate adipocyte differentiation and fibroblast migration (Fukai et al., 1995).
1.6 Integrin receptors

The integrins are a family of cell surface proteins that mediate cell adhesion, cytoskeletal organisation and the bidirectional transfer of information across the outer cell membrane. They are heterodimeric glycoproteins composed of non-covalently associated α and β subunits. To date 16 α and 8 β subunits have been described, which pair to form more than 22 receptors (see Table 1.2).

1.6.1 Integrin structure

Electron microscopy has shown that each integrin subunit has an N-terminal globular domain which projects from the cell by virtue of a rod-like 'stalk' (Carrell et al., 1985). Each subunit has a single transmembrane spanning region and a short cytoplasmic tail (see Fig. 1.7), with the exception of the β4 subunit, which has an extended tail (Tamura et al., 1990). A 7-fold repeat structure at the N-terminus of the integrin α subunit contributes to both ligand recognition and divalent cation binding, although the two activities probably map to different regions within the structure (reviewed by Schwartz et al., 1995; Humphries, 1996). Integrin-ligand recognition is cation dependent and it has been predicted that there are at least 3 cation binding sites, with each showing a preference for the type of cation (e.g. Mn^{2+}, Mg^{2+}, Ca^{2+}). Although it has yet to be determined how cation binding affects ligand binding, it is likely to be by effecting changes in integrin conformation (Humphries, 1996).

A subset of integrins (α1, α2, αD, αE, αL, αM, αX) have an insertion between repeats II and III of the seven-fold repeat structure, named the A-domain (or I-domain) for its homology with the A-domain of von-Willebrand factor (reviewed by Dickeson and Santoro, 1998). The A-domain is an autonomously folding unit, as evidenced by studies showing that isolated A-domain binds to ligand (Tuckwell et al., 1996; Messent et al., 1998). Data indicate that the A-domain is the major collagen I and collagen IV binding site of the α1β1 and α2β1 integrins (Calderwood et al., 1997). Furthermore, substituting the A-domain of the α1 integrin subunit with the A-domain of the α2 subunit results in an integrin with collagen specificities reflecting that of α2β1 integrin rather than α1β1
integrin (Kern and Marcantonio 1998), indicating the importance of this domain in determining the ligand specificities of these integrins.

The ligand binding site on the β integrin subunit has yet to be characterised, however a conserved region near the N-terminus of this subunit has been identified as a putative βA domain (Tuckwell and Humphries, 1997). The epitope for a function-blocking β antibody has been mapped to this site (Ryan et al., 1998) and mutagenesis studies indicate that this region bears some similarities to the αA domain (Lin et al., 1997).

1.6.2 Integrin function

1.6.2a Cell adhesion

Individual integrins often bind more than one ECM ligand and equally, individual ligands are recognised by more than one integrin (see Table 1.2). Most commonly the integrins recognise the amino acid sequence RGD, identified as the ligand recognition site in fibronectin and vitronectin. However, other sequences homologous to the RGD tripeptide are also recognised by integrins and some integrin-ligand interactions appear to be RGD independent (see Table 1.2). Some integrins also mediate cell-cell adhesion by binding to integral membrane proteins of the immunoglobulin superfamily (e.g. VCAM-1 and ICAMs).

1.6.2b Integrin signalling

Integrin receptors mediate bidirectional signals across the plasma membrane, from the 'outside-in' and from the 'inside-out'. Outside-in signalling is the subject of intense study and there is now a wealth of information regarding the signalling and cytoskeletal molecules that are involved in integrin-mediated signal transduction (reviewed by Howe et al., 1998; Vuori, 1998; Yamada and Geiger, 1997). Integrin transduced signals include Ca^{2+} influx, pH changes, protein-tyrosine phosphorylation, inositol lipid metabolism and activation of MAP kinases.
In cell culture, ligand binding induces integrins to cluster, forming a close apposition between the cell membrane and the underlying substrate, causing the formation of stress fibres within the cell. These contacts, known as 'focal adhesions', have provided a convenient model for analysing integrin signalling. However, focal adhesions are rare in vivo, where other adhesive structures occur, such as 'ECM-contacts'. In these contacts, integrins cluster along ECM fibrils, forming distinct cytoskeletal structures (Chen and Singer, 1982). Nevertheless most integrin signalling studies are based on observations of focal adhesions, and information from these studies is detailed here.

Integrin ligation drives the formation of complex protein aggregates, in which actin stress fibres are tethered to the cytoplasmic face of the membrane, via interactions with integrin cytoplasmic domains. Integrins are clustered within these structures (focal adhesions) and a variety of signal transduction molecules are induced to accumulate in the plane of the membrane. These can include focal adhesion kinase (FAK), Src family kinases, Ras, Raf and MAP kinases, each depending upon the integrin and the nature of the ligand to which it is bound. Tyrosine phosphorylation is a requirement for the aggregation of some signalling and cytoskeletal molecules (reviewed by Yamada and Geiger, 1997). In addition, the presence of receptors for the growth factors EGF, PDGF and bFGF in integrin induced transmembrane protein aggregates has been documented (Miyamoto et al., 1996). A number of the signalling mechanisms employed by integrin receptors are common to growth factor receptors and a growing number of reports describe synergism between integrin and growth factor signalling pathways (reviewed by Yamada and Geiger, 1997; Dedhar and Hannigan, 1996).

Integrins can adopt low affinity and high affinity states for ligand binding, and the regulation of integrin affinity is an important aspect of integrin function. Changes from low affinity to high affinity can be initiated by signals from other cellular receptors, such as chemokine, chemoattractant and antigen receptors (reviewed by Sugimori et al., 1997). Modulation of integrin affinity is then mediated by the binding of cytoplasmic proteins to the cytoplasmic domains of the integrin subunits. A conformational change is induced,
such that the integrin adopts an active, high affinity state, competent for ligand binding (termed inside-out signalling). Several amino acid sequences have been identified in integrin α and β subunits that are implicated in maintaining the integrin in an active or inactive state (reviewed by Dedhar and Hannigan, 1996). Perhaps the best documented example is the sequence KXGFFKR in the α2 subunit cytoplasmic tail. This holds the α2β1 integrin in an inactive state, probably via a salt-bridge with an adjacent region of the β1 subunit. The binding of calreticulin (a calcium binding protein) to the KXGFFKR apparently disrupts this salt bridge, such that α2β1 integrin is activated (Dedhar and Hannigan, 1996).

1.6.2c Cytoskeletal organisation

Integrins work with the Rho family of GTP-ases to regulate the structure of the actin cytoskeleton. Cdc42, Rac and Rho control the formation of filopodia, lamellipodia, and stress fibres and focal adhesions respectively (reviewed by Howe et al., 1998). The assembly of focal adhesions with well developed stress fibres requires both Rho GTPases and integrin ligation (for anchorage and counter tension). Rho-responsive serine/threonine kinase phosphorylation leads to the activation of myosin ATP-ase activity and actinomyosin contractility, which contribute to the tension of the stress fibres. In addition, Rho family members can stimulate signal transduction cascades leading to the activation of a number of transcription factors (Howe et al., 1998).

1.6.2d Matrix assembly

As mentioned earlier, integrin receptors play a role in fibronectin matrix assembly. It has been shown that anti-α5 and anti-β1 subunit antibodies block fibril formation (Akiyama et al., 1989) and transfection of α5 subunit into transformed Chinese hamster ovary cells results in a large increase in fibronectin matrix assembly (Giancotti and Ruoslahti 1990). Interestingly, in the latter study fibronectin matrix formation was associated with the loss of the transformed phenotype and a decrease in cell motility. Several observations suggest that only high affinity α5β1 integrin receptors participate in fibronectin fibrillogenesis (reviewed by Magnusson and Mosher, 1998; Schwartz et al., 1995). There is a close
correlation between actin stress fibre formation, cell contraction and fibronectin assembly indicating that fibrillogenesis may require tension transmitted by integrin binding, and the Rho family of GTPases have been implicated in this process (Magnusson and Mosher, 1998).

1.6.2e Integrin regulation of MMP expression

A growing body of evidence implicates integrin receptors in the regulation of MMP gene expression. Many studies have shown that MMPs are regulated by matrix components, and work with anti-integrin antibodies has confirmed that integrin receptors can mediate signals from the matrix that regulate MMP expression. It has emerged from these studies that the response to integrin ligation is cell type specific. For instance, ligation of the α3β1 integrin receptor by soluble anti-α3 and anti-β1 integrin antibodies induced GLB synthesis in human mucosal keratinocytes, but did not affect GLA expression (Larjava et al., 1993), whereas ligation of the α3 subunit by anti-α3 antibodies in human glioma cells led to the induction of GLA expression, but not GLB (Chintala et al., 1996). Treatment of the human melanoma cells A375M with anti-αvβ3 antibodies up-regulated the synthesis of GLA, but anti-α5β1 antibodies had no effect (Seftor et al., 1992). In contrast, however, the highly invasive human melanoma cell C8161, which express low levels of αvβ3 integrin, responded to anti-α5β1 antibodies with increased GLA synthesis (Seftor et al., 1993). These studies highlight the fact that cellular response to ECM is dependent both on integrin repertoire and on the intracellular signalling pathways induced in individual cell types. Furthermore, combinations of integrins may act together to regulate MMP expression, as demonstrated by Huhtala et al. (1995) who reported that α5β1 and α4β1 act antagonistically to regulate CL-1, SL-1 and GL-B expression in rabbit synovial fibroblasts. Ligation of the α5β1 integrin receptor with anti-α5 subunit antibodies or fibronectin fragments induced MMP expression, whereas the simultaneous ligation of α5β1 and α4β1 integrins down-regulated α5β1 mediated MMP induction.

Advances have been made using the well-characterised culture model of three-dimensional collagen lattices, to which cultured fibroblasts adhere and then contract the collagen
network. This process is accompanied by a fundamental change in fibroblast morphology, indicated by a down-regulation of collagen I synthesis and an up-regulation of MMP activity. Langholz et al (1995) demonstrated that the α2β1 integrin mediates signals from collagen I that up-regulate CL-1 mRNA expression, whereas signals from the α1β1 integrin cause the down-regulation of collagen I mRNA expression. Others have reported increases in MT1-MMP synthesis and GLA activation by endothelial cells cultured in collagen lattices (Haas et al., 1998). Tomasek et al. (1997) showed that the activation of GLA by cells cultured in collagen lattices may be linked to the level of mechanical stress placed on cells, implying that the mechanical properties of the ECM and cytoskeletal organisation act together to regulate MMP expression. These studies are of significance, as they show that signals from the matrix via integrin receptors can determine whether a cell enters matrix synthesis or matrix destruction mode.

Few studies have addressed the intracellular signalling pathways associated with integrin mediated regulation of MMP expression. However, Langholz et al., (1995) showed that the α2β1 mediated induction of CL-1 in collagen lattices was accompanied by tyrosine phosphorylation, and collagen-stimulated induction of keratinocyte CL-1 has been reported to be mediated by tyrosine kinase and PKC activities (Sudbeck et al., 1994). Most recently, a comprehensive study by Kheradmand et al. (1998) has dissected out the contribution of the Rho family of GTP-ases in the α5β1 mediated up-regulation of CL-1 by rabbit synovial fibroblasts. Briefly, Rac 1 activity was induced by soluble anti-α5 integrin antibodies, which in turn promoted the NF-κB-dependent transcription of IL-1, and IL-1 induced CL-1 expression. This study is discussed in more detail in later chapters.

1.6.2f Integrin gene ablation studies

Most of the integrin genes have been mutated in mice. Null mutations in the genes for the integrin subunits involved in fibronectin ligation (α5, αv, α4) all caused either embryonic or early post-natal death in mice. By comparison null mutations in the fibronectin gene resulted in abnormalities in gastrulation, thus none of the phenotypes from the fibronectin integrin receptor gene ablation studies reflected the range of defects associated with
fibronectin gene ablation (reviewed by Fässler et al. 1996). Based on the pairing of the β1 integrin subunit with 10 α subunits, it was predicted that the phenotype for the β1 gene null mutation would be severe, and indeed this mutation resulted in peri-implantation lethality (Fässler et al., 1995). Null mutations in the α6 gene (laminin receptor) produced viable mice, but with severe skin blistering similar to the human disease epidermolysis bullosa (Fässler et al., 1996). In contrast, null mutations in the α7 gene (another laminin receptor) were viable and fertile but displayed progressive muscular dystrophy, starting soon after birth (Mayer et al., 1997). This reflects the restricted expression of α7β1 integrin in skeletal and cardiac muscle. Interestingly, gene ablation of the α1 integrin subunit apparently conferred no phenotype, and these mice were both viable and fertile, despite the inability of embryonic fibroblasts derived from these mice to migrate on collagen IV (Gardner et al., 1996). This suggests that other integrins with overlapping substrate specificities may be compensating for α1β1 loss.

1.7 Cell migration

The movement of cells within the matrix provides an example of how matrix, integrins, growth factors and MMPs work together to coordinate cellular function. Cell migration involves a number of distinct events, including membrane protrusion to form a leading edge; formation of stable contacts between the cell and the ECM; cytoskeletal contraction, forward movement of the cell body; and release of contacts at the rear (Palecek et al., 1998). Integrin receptors are central to this process as they couple the cytoskeleton to the substratum and provide traction for the applied stress from the cytoskeleton (Lauffenburger, 1996). Forward movement involves the transfer of integrin receptors attached to the cytoskeleton from the leading edge to the trailing edge. This has been demonstrated clearly by Felsenfeld et al. (1996) who used videomicroscopy to track colloidal gold particles coated with the fibronectin fragment Fn III 7-10. These particles adhered to fibronectin integrin receptors on the surface of migrating fibroblasts, diffused randomly for less than a second, before being transported rapidly to the rear of the cell. Current models for cell migration predict that the same intracellular motile forces that send
integrins to the rear also control the recruitment of integrins to the leading edge, thus maintaining cell motility (reviewed by Lauffenburger, 1996; Huttenlocher et al. 1995). To allow locomotion, cell/substratum linkages at the rear of the cell are severed, by intracellular or extracellular fracture of the integrin/substratum bonds. The integrins are then recycled to the leading edge of the cell or deposited on the matrix at the rear of the cell (Palecek et al., 1998).

Many studies have shown that maximum migration rates occur at intermediate levels of attachment (DiMilla et al., 1993; Keely et al., 1995; Palecek et al., 1998). At low adhesiveness, cells are unable to generate the traction needed for locomotion whereas at high adhesiveness the cytoskeletal forces are insufficient to break cell-substratum attachment. The strength of cell adhesion to the substratum is determined by multiple factors: by the number and type of integrin receptors expressed, by the affinity of the integrin for the ligand and by the concentration and type of the ECM ligand involved. To address the last point, the type of ECM presented to a cell can have a profound affect on migration. Some matrix proteins such as the tenascins and SPARC are described as anti-adhesive, and have surfaces which promote cell rounding and detachment from the substratum. Tenascin-C is spatially and temporally regulated during embryogenesis and is re-expressed in adult tissues in regions where ECM remodelling and cell migration occurs (reviewed by Chiquet-Ehrismann et al., 1995). Tenascin-C binds fibronectin specifically, and tenascin-C is often expressed in conjunction with fibronectin. Since it has been shown that tenascin-C bound to fibronectin disrupts integrin adhesion to fibronectin, it has been suggested that tenascin-C mediates cell migration during embryogenesis and remodelling by reducing cell adhesion to fibronectin (Chiquet-Ehrismann et al., 1995).

Interestingly, a combined substrate of fibronectin and tenascin-C induces CL-1, SL-1 and GLB synthesis by rabbit synovial fibroblasts cultured in vitro, whereas fibronectin alone has no effect (Tremble et al., 1994). Similarly the addition of SPARC to synovial fibroblasts cultured on fibrillar collagens induces CL-1 expression (Tremble et al., 1993). Many studies have shown that MMPs are expressed at sites of cell migration, but few have
provided clues as to how the MMPs might act to promote migration. Proteolysis of ECM by MMPs may lead to the exposure of cryptic sites, as reported by Gianelli et al. (1997) who showed that GLA treatment of laminin-5 leads to the exposure of a cryptic sequence that promotes the migration of breast epithelial cells. Pilcher et al. (1997) demonstrated that collagen I substrates induced CL-1 synthesis by human keratinocytes, and the addition of blocking anti-CL-1 antibodies in these studies revealed that CL-1 activity was a requirement for keratinocyte cell migration on collagen I. Furthermore, a substrate of collagen I with a mutated CL-1 cleavage site was not conducive to keratinocyte migration. The authors speculated that keratinocytes migrating on collagen leave behind a trail of CL-1-cleaved denatured collagen, which provides directionality to the cells, since sustained migration can only be maintained by moving forward onto intact collagen I. This has important implications for wound healing, where basal keratinocytes at the edge of the wound migrate in an organised manner over the viable dermis and wound bed during re-epithelialisation.

Cross-talk between growth factor receptors and integrin receptors also regulates cell migration. The integrin αvβ3 has been found to associate with activated insulin and PDGF receptors to potentiate PDGF dependent chemotaxis of NIH 3T3 fibroblasts (Schneller et al., 1997). Similarly, the migration and proliferation of endothelial cells is promoted by the coordinated activities of bFGF and αvβ3 integrin, in a process mediated by MAP kinase signalling (Eliceiri et al., 1998). Human pancreatic carcinoma cells readily migrate on collagen I using α2β1 integrin, but do not migrate on vitronectin unless the EGF receptor is ligated. PKC and tyrosine phosphorylation were implicated in this case (Klemke et al., 1994).

Clearly control of cell migration is a complex process involving integrin receptors, associated cytoskeletal proteins and signalling molecules, growth factors and proteolysis of matrix components. Cell migration is an integral step in tumour metastasis, and aspects of tumour biology are discussed in the last section of this chapter.
1.8 The role of MMPs in tumour invasion

Malignant transformation of tumour cells is accompanied by metastasis of the cells from the primary tumour to secondary sites within the body. The exact processes which govern the formation of metastases at distant sites are unknown, but some general properties apply (reviewed by Chambers and Matrisian, 1997; Ray and Stetler-Stevenson, 1994). Firstly, the transformed cell must be able to break free of existing ECM contacts, in order to invade the tissue stroma and gain access to the vascular or lymphatic system. This involves breaching the basement membranes that surround these tissues. Secondly, the invading cell must survive the circulation, arrest at a distant site and extravasate. Finally, the new environment must be suitable for tumour cell proliferation. Angiogenesis is critical for this last stage.

In many ways, tumour progression corresponds to normal tissue remodelling: the matrix environment controls cell migration and invasion, and protease activity is up-regulated. Many families of proteases have been linked with malignancy, including serine proteases and matrix metalloproteinases (reviewed by Edwards and Murphy, 1998; Chambers and Matrisian, 1997; Kramer et al., 1995). This discussion is restricted to the contribution of the MMPs. There is an extensive literature demonstrating a statistically significant association between MMP expression and tumour spread. Techniques such as immunolocalisation and in situ hybridisation of tissue samples, and ELISA studies of the body fluids of cancer patients have been used to show that all MMP classes are implicated in tumour progression (Ray and Stetler-Stevenson, 1994). MMPs are found associated with tumour cells as well as the surrounding stroma. It is postulated that MMPs produced by stromal cells are recruited to the surface of tumour cells to facilitate pericellular proteolysis (Werb, 1997). Evidence that MMP activity is important in malignant progression has been provided by inhibitor studies. Injection of human recombinant TIMP-1 into mice inhibited amnion invasion and lung colonisation by B16-F10 melanoma cells (Schultz et al., 1988). Furthermore, the transfection of antisense TIMP-1 encoding plasmids into NIH 3T3 cells resulted in cell lines that were more invasive than the parent line (Khokka et al., 1989). The synthetic MMP inhibitor marimastat is now undergoing...
clinical trials, and the preclinical data suggests that invasion, metastasis and angiogenesis are all reduced by marimastat treatment (Rasmussen and McCann, 1997).

Although there is abundant causal evidence to suggest that MMPs play a role in malignant progression, the precise contribution of MMPs needs to be clarified. Early hypotheses predicted that MMPs were involved in the proteolytic dissolution of basement membrane and stromal matrices during the initial stages of metastasis (Liotta and Stetler-Stevenson, 1991). However, studies have shown that the MMPs may have a more subtle effect on the surrounding matrix. Montgomery et al. (1994) showed that although human melanoma cells use $\alpha 2\beta 1$ integrin to bind collagen lattices, their survival is dependent upon $\alpha v\beta 3$ integrin ligation. MMP degradation of collagen I apparently exposed cryptic RGD sites, that were ligated by $\alpha v\beta 3$ integrin, thus promoting melanoma cell survival. In support of this, melanoma cells expressing $\alpha v\beta 3$ integrin are highly invasive compared with those that lack $\alpha v\beta 3$ (Marshall et al., 1991). As mentioned earlier, GLA cleavage of laminin-5 exposes a cryptic site that promotes cell migration (Gianelli et al., 1997), thus MMP activity may be important for remodelling of matrix molecules that would otherwise be refractory to tumour cell survival and migration.

Recent studies have indicated that the major contribution of MMPs to metastatic progression may not be in the early stages of extravasation. Evidence from intra-vital videomicroscopy studies suggests that MMPs may be involved in the growth of tumours at secondary sites (reviewed by Chambers and Matrisian, 1997). This technique monitors the microvasculature of living animals. Cells are injected into chick embryo chorioallantoic membrane and extravasation is monitored in real time. Studies investigating the metastasis of transformed NIH-3T3 cells gave the surprising results that the timing of extravasation for transformed cells was similar to the parent (untransformed) line, indicating that transformation conferred no advantage on cell migration from the primary tumour. However, the transformed cells proliferated more rapidly post-extravasation than the parent cells (Koop et al., 1996). Similarly, B16-F10 cells engineered to overexpress TIMP-1 extravasated at the same rate as parent cells, but the effects of TIMP-1 were manifest in the
reduction in size and numbers of tumours after 7 days (Koop et al., 1994). Studies such as these implicate MMPs in tumour growth, however the mechanisms by which MMPs regulate growth have yet to be characterised. Future studies should address whether degradation of the ECM frees cells from ECM-induced cell cycle arrest, or facilitates dedifferentiation, or promotes the release of growth factors and angiogenic factors (reviewed by Werb, 1997).

1.9 Aims and scope of this thesis

It is evident that a complex interplay occurs between matrix, integrin receptor and MMPs during cell migration and invasion. More information is needed to aid understanding of the precise roles of MMPs in these processes. Early hypotheses for tumour invasion predicted that MMPs were required during metastasis to degrade basement membrane barriers. However, new evidence suggests that the action of MMPs may be more subtle, and may involve the proteolytic modification of ECM molecules to expose new matrix fragments that promote cell migration, survival or proliferation.

Cell motility assays have shown that maximum migration is maintained on matrices which promote an intermediate level of cell adhesion. A tumour cell that successfully negotiates the basement membrane may be able to modulate adhesion to collagen IV. Studies for this thesis examined the novel hypothesis that MMPs cleave integrin recognition sites on collagen IV, thus affecting cell adhesion to the molecule. Solubilised collagen IV preparations were treated with GLA in vitro and cell adhesion via integrin receptors to the cleaved collagen was monitored. These studies are reported in Chapter 3.

Tumour cell invasion has been linked with the expression of MT1-MMP and active GLA, however little is known of the signals that regulate GLA activation. Tumour cells invading through the stroma encounter a range of ECM molecules, including collagen and fibronectin. Collagen matrices have been shown to up-regulate GLA activation, but the effects of fibronectin on activation have not been investigated. Studies for Chapter 4 and 5 of this thesis examined the effect of fibronectin on the expression of MT1-MMP and the
activation of GLA by a human fibrosarcoma cell line. The role of fibronectin integrin receptors in mediating the up-regulation of GLA was investigated.

These studies identified a processed form of MT1-MMP that appeared concomitantly with GLA activation. Studies for Chapters 5 and 6 were aimed at characterising this form of MT1-MMP, to shed some light on its role in GLA activation pathways.
Table 1.1 The substrate specificities of the matrix metalloproteinases
Compiled from Murphy and Knüper, 1997

<table>
<thead>
<tr>
<th>MMP no.</th>
<th>Enzyme</th>
<th>Molecular Mass (kDa)</th>
<th>Known Matrix Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Latent</td>
<td>Active</td>
</tr>
<tr>
<td>MMP-1</td>
<td>Interstitial collagenase</td>
<td>55</td>
<td>43</td>
</tr>
<tr>
<td>MMP-8</td>
<td>Neutrophil collagenase</td>
<td>75</td>
<td>58</td>
</tr>
<tr>
<td>MMP-13</td>
<td>Collagenase-3</td>
<td>60</td>
<td>48</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Stromelysin-1</td>
<td>57</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-10</td>
<td>Stromelysin-2</td>
<td>57</td>
<td>46</td>
</tr>
<tr>
<td>MMP-11</td>
<td>Stromelysin-3</td>
<td>51</td>
<td>44</td>
</tr>
<tr>
<td>MMP-19</td>
<td>no given name</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>MMP-20</td>
<td>Enamelysin</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Gelatinase A</td>
<td>72</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>Gelatinase B</td>
<td>92</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-7</td>
<td>Matrilysin (Pump)</td>
<td>28</td>
<td>20</td>
</tr>
<tr>
<td>MMP-12</td>
<td>Metalloelastase</td>
<td>54</td>
<td>45/22</td>
</tr>
<tr>
<td>MMP-14</td>
<td>MT1-MMP</td>
<td>66</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-15</td>
<td>MT2-MMP</td>
<td>72</td>
<td>?</td>
</tr>
<tr>
<td>MMP-16</td>
<td>MT3-MMP</td>
<td>64</td>
<td>52</td>
</tr>
<tr>
<td>MMP-17</td>
<td>MT4-MMP</td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>
MMPs have a propeptide domain (1) that maintains enzyme latency and a catalytic domain (2) with a coordinated zinc in the active site. All MMPs (with the exception of matrilysin) also have a C-terminal domain (3) that determines substrate specificity (collagenases), matrix binding properties (collagenases, stromelysin) or interaction with TIMPs (collagenase-3, gelatinases). The gelatinases have an insert of three fibronectin type II repeats in the catalytic domain (4) which determines their specificity for gelatins. The MT-MMPs have an extra insert in the catalytic domain (5) of unknown function and a transmembrane domain (6) which locates these enzymes to cell membranes.
Figure 1.2 Cell surface MMP activation cascades

Urokinase-plasminogen activator (uPA) bound to the uPA receptor generates active plasmin from the precursor plasminogen. Plasmin activates several MMPs, initiating an MMP activation cascade. MT1-MMP is activated intracellularly and locates to the cell surface, where it initiates the activation of progelatinase A and procollagenase-3. TIMP-2 bound to MT1-MMP is implicated in progelatinase A activation.
Figure 1.3 Cellular activation of progelatinase A

This figure represents a current hypothesis for the activation of progelatinase A at the cell surface. The N-terminal domain of TIMP-2 binds to the catalytic domain of MT1-MMP at the cell surface, leaving the C-terminal domain free for interaction with the C-terminal domain of progelatinase A. An adjacent, TIMP-2-free MT1-MMP which is catalytically active then cleaves the propeptide of progelatinase A and activation is completed by gelatinase A bimolecular autolysis. Excess TIMP-2 inhibits activation, by binding all MT1-MMP molecules at the cell surface.
Figure 1.4 Schematic representation of collagen IV and the collagen IV network
(adapted from Eble et al., 1996; Timpl and Brown 1995)

A. The collagen IV monomer, showing the N-terminal triple helical 7S region, the CB3[IV] region of the triple helix, and the non collagenous NC1 domain at the C-terminus.

B. Collagen IV network assembly. The single lines represent triple helices.

(1) The N-terminal 7S domains associate to form tetramers.

(2) Dimers form from the C-terminal NC1 globules.

(3) Lateral association between the triple helices form twisted superhelices.
Figure 1.5 Schematic representation of laminin-1

Laminin-1 is a heterotrimer of α, β, and γ chains which associate in an α-helical coiled-coil to form the long arm of the molecule (represented by straight lines). Short arms consist of the N-terminal portion of each chain. The recognition sites for integrin receptors have been marked.
Figure 1.6  A schematic representation of the structural and functional domains of human fibronectin

One arm of the disulphide bonded fibronectin dimer is represented. Fibronectin is comprised of homologous type I, type II and type III repeating units. The central cell binding domain comprises type III repeating units 1-11 and the integrin recognition sequence RGD is located in type III repeat no. 10. Domains that interact with other matrix molecules have been indicated. Extra domains may be inserted into one arm of the dimer: plasma fibronectin contains the alternatively spliced IIICS region and cellular fibronectin may contain the IIICS, ED-A and ED-B regions. The complex splicing of the IIICS region is indicated.
Figure 1.7 Schematic representation of integrin domain structure

The structures of the $\alpha$ and $\beta$ subunits are shown, with the extracellular domains to the left of the plasma membrane. The three sites implicated in ligand binding are ringed. The sites for binding of divalent cations are marked by a +.
Table 1.2 The integrin receptor family and their extracellular ligands

(Compiled from Sugimori et al., 1997; Mohri, 1996; Eble et al., 1998; and Knight et al., 1998)

<table>
<thead>
<tr>
<th>Integrin</th>
<th>Ligands</th>
<th>Binding site</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1β1</td>
<td>laminins, collagens I, IV, VI</td>
<td>-</td>
</tr>
<tr>
<td>α2β1</td>
<td>collagen I-IV, laminins, vitronectin, tenasin</td>
<td>GFP<em>GERGVEGPP</em>GPA where * is hydroxyproline</td>
</tr>
<tr>
<td>α3β1</td>
<td>laminins</td>
<td>-</td>
</tr>
<tr>
<td>α4β1</td>
<td>fibronectin, VCAM-1</td>
<td>LDV, IDSP</td>
</tr>
<tr>
<td>α5β1</td>
<td>fibronectin</td>
<td>RGD</td>
</tr>
<tr>
<td>α6β1</td>
<td>laminins</td>
<td>-</td>
</tr>
<tr>
<td>α7β1</td>
<td>laminins</td>
<td>-</td>
</tr>
<tr>
<td>α8β1</td>
<td>fibronectin, tenasin, vitronectin</td>
<td>-</td>
</tr>
<tr>
<td>α9β1</td>
<td>tenasin</td>
<td>-</td>
</tr>
<tr>
<td>αVβ1</td>
<td>fibronectin, vitronectin</td>
<td>RGD</td>
</tr>
<tr>
<td>αLβ2</td>
<td>ICAM-1, -2, -3</td>
<td>LDV</td>
</tr>
<tr>
<td>αMβ2</td>
<td>ICAM-1, -2, -3, fibrinogen, heparin, factor X, lipopolysaccharide</td>
<td>LDV</td>
</tr>
<tr>
<td>αXβ2</td>
<td>fibrinogen, lipopolysaccharide</td>
<td>-</td>
</tr>
<tr>
<td>αDβ2</td>
<td>ICAM-3</td>
<td>-</td>
</tr>
<tr>
<td>αIIβ3</td>
<td>fibronectin, fibrinogen, vitronectin, thrombospondin, collagens, denatured collagen, PECAM-1, von-Willebrand factor</td>
<td>RGD, KQAGDV</td>
</tr>
<tr>
<td>αVβ3</td>
<td>fibronectin, fibrinogen, vitronectin, thrombospondin, SPARC, tenasin, denatured collagen, von-Willebrand factor</td>
<td>RGD</td>
</tr>
<tr>
<td>α6β4</td>
<td>laminin</td>
<td>-</td>
</tr>
<tr>
<td>αVβ5</td>
<td>fibronectin, vitronectin</td>
<td>RGD</td>
</tr>
<tr>
<td>αVβ6</td>
<td>fibronectin, tenasin</td>
<td>RGD</td>
</tr>
<tr>
<td>α4β7</td>
<td>fibronectin, MadCAM-1</td>
<td>LDV</td>
</tr>
<tr>
<td>αEβ7</td>
<td>E-cadherin</td>
<td>-</td>
</tr>
<tr>
<td>αVβ8</td>
<td>vitronectin</td>
<td>-</td>
</tr>
</tbody>
</table>
Chapter 2

Materials and Methods
Chapter 2 Materials and Methods

2.1 Reagents

The majority of chemicals were obtained from Sigma Chemical Co. The manufacturers of specialist materials are referred to in the text below.

2.1.1 Recombinant human MMPs and MMP inhibitors

The following recombinant MMPs and TIMPs were kindly provided by Prof. G. Murphy (University of East Anglia, Norwich, UK): proGLA (Murphy et al., 1992b); proGLB (O'Connell et al., 1994); proSL-1 (Murphy et al., 1992a); TIMP-1 (Williamson et al., 1990); TIMP-2 (Willenbrock et al., 1993); Δ127-184 TIMP-1 (N-TIMP-1; Murphy et al., 1991b). proCL-3 (Knäuper et al., 1996a) was a kind gift from Dr V. Knäuper, University of East Anglia, Norwich, UK. The following recombinant MT-MMPs were kindly provided by Dr H. Will (InVitek GmbH, Berlin-Buch, Germany): the catalytic domain of MT1-MMP (Δ269-559 MT1-MMPcat; Will et al., 1996); the C-terminally truncated MT1-MMP, lacking the transmembrane domain and cytoplasmic domain (Δ502-559 TM-MT1-MMP; d'Ortho et al., 1997); and the catalytic domain of MT2-MMP (Δ270-628 TM-MT1-MMP; Butler et al., 1997). ΔTM-MT3-MMP was kindly provided by Dr M. Fox, University of East Anglia, Norwich, UK. The catalytic domain of MT4-MMP (ΔTM-MT4-MMP) was kindly provided by Dr M. Butler, University of East Anglia, Norwich, UK. The MMP inhibitor CT1746 (N1-(1-(S)-carbamoyl-2,2-dimethylpropyl)-N4-hydroxy-2-(R)-[3-(4-chlorophenyl)propyl]succinamide) was a gift from Dr A. Docherty, Celltech Research, Slough, UK.

2.1.2 Extracellular matrix proteins and peptides

The following preparations of type IV collagen were generously provided by Prof. K. Kühn (Max-Planck-Institut für Biochemie, Martinsried, Germany): murine monomeric type IV collagen isolated from EHS tumour (Kleinmann et al., 1982); human dimeric type IV collagen isolated from placenta (Timpl et al., 1981); human tetrameric type IV collagen
isolated from placenta (Timpl et al., 1981). Type I collagen prepared from rat skin (Cawston and Murphy, 1981) was kindly provided by Prof. G. Murphy, University of East Anglia, Norwich, UK. Murine laminin-1 was purchased from Sigma Chemical Company. Human plasma fibronectin was purchased from Bioproducts, Elstree, Hertfordshire, UK. Purified fibronectin (contaminating gelatinases removed; Smilenov et al., 1992) was generously provided by A.J. Messent, Strangeways Research Laboratory, Cambridge, UK. The fibronectin peptides Fn III 6-10 (wild type) and Fn III 6-10 SPSDN (synergy site mutant) were a kind gift from Dr S. Aota and Dr K.M. Yamada (National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland) and were prepared as described (Danen et al., 1995). The 120 kDa and 110 kDa fibronectin fragments were generously provided by Prof. L. Zardi (Instituto Nazionale per la Ricerca sul Cancro, Genoa, Italy) and were prepared by thermolysin digestion of human fibronectin as described (Borsi et al., 1986). The RGD peptide GRGDdSP was purchased from Gibco BRL and its inactive partner GRGESP was purchased from Sigma.

2.1.3 Antibodies

2.1.3a Anti-MMP and anti-TIMP antibodies

The polyclonal sheep anti-human TIMP-2 antibody (H225; Ward et al., 1991a) and the polyclonal sheep anti-human GLA antibody (X670; Hipps et al., 1991) were kindly provided by Prof. G. Murphy and Dr R. Hembry, University of East Anglia, Norwich, UK. The preparation of a new sheep anti-GLA antibody (K399) is described in detail in section 2.2. The mouse monoclonal antibody VB3, to the C-terminal domain of GLA was provided by Dr H. Birkedal-Hansen, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland. The polyclonal antibody N175 to human MT1-MMP was raised and characterised, also as described in section 2.2. The mouse monoclonal antibodies to rat MT1-MMP 3A10, 4G6, and 2G12 were gifts from Dr P. Basset, Université Louis Pasteur, Strasbourg, France. The antibodies 3A10 and 4G6 were raised to a recombinant fragment of rat MT1-MMP, residues 112-508, produced in E. coli and recognise the catalytic domain and the hemopexin domain human MT1-MMP respectively. 2G12 was raised to a recombinant fragment from the prodomain of rat MT1-
MMP, residues 47-66, but failed to react with proMT1-MMP and was used as a negative control for the mouse monoclonals.

The rabbit polyclonal antibody to MT2-MMP, raised to a recombinant fragment of human MT2-MMP (residues 91-305) was kindly provided by Dr. H. Will, InVitek GmbH, Berlin-Buch, Germany. The mouse monoclonal antibody against human MT3-MMP (raised to a peptide of residues 168-181) was a gift from Dr K. Iwata, Fuji Chemical Industries, Toyama, Japan. The following rabbit anti-MT4-MMP antibodies were gifts from Dr C. López-Otin (Departamento de Bioquímica y Biología Molecular, Facultad de Medecina, Universidad de Oviedo, Oviedo, Spain): anti-KLH-PEEP, raised to a peptide in the hinge region; anti-KLH RTLR, raised to a peptide in the C-terminal domain; and a rabbit polyclonal raised to a fragment of the C-terminal domain of MT4-MMP. The polyclonal antibody N33 was kindly provided by Dr M. Butler, University of East Anglia, Norwich, UK. This antibody was raised to the catalytic domain of human MT4-MMP, produced in E. coli, with N- and C-terminal histidine tags and a c-myc tag.

2.1.3b Antibodies to integrins

The mouse monoclonal to the α2β1 integrin Gi9 was purchased from Immunotech, Marseille, Cedex-9, France. The mouse monoclonal to α6 integrin subunit GoH3 was from TCS Biologicals, Botolph Claydon, Buckinghamshire, UK. The monoclonal antibody HP2/1 to the α4 subunit was purchased from Serotec, Oxford, UK. The rat monoclonal antibodies to the α5 integrin subunit, mAbs 16 and 11 (Akiyama et al., 1989; LaFlamme et al., 1992) and to the β1 subunit mAb 13 (Akiyama et al., 1989) were generous gifts from Dr K.M. Yamada, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland.

2.1.3c Secondary antibodies and conjugates

Peroxidase-conjugated sheep anti-mouse IgG was from Sigma; peroxidase-conjugated donkey anti-sheep and donkey anti-rabbit IgG antibodies were purchased from Jackson Immunoresearch Laboratories Inc. The FITC-conjugated sheep anti-mouse IgG was from
Sigma and the FITC-conjugated pig anti-sheep IgG was kindly provided by Dr R. Hembry, (University or East Anglia, Norwich, UK) prepared as described by Hembry et al. (1985).

2.2 Purification and characterisation of antibodies to MT1-MMP and GLA

2.2.1 Anti-MT1-MMP antibody N175

Sheep antiserum to recombinant human MT1-MMP was prepared by Dr M. P. d'Ortho (INSERM U492, Faculté de Médecine de Créteil, Créteil, France) and Dr R. Hembry (University of East Anglia, Norwich, UK). A GST-Δ511-582 TM-MT1-MMP fusion protein was prepared and expressed in E. coli as described (d'Ortho et al., 1998). GST-ΔTM-MT1-MMP was purified from inclusion bodies, subjected to reducing SDS-PAGE and electro-eluted, also as described (d'Ortho et al., 1998). GST-Δ511-582 TM-MT1-MMP (150 μg protein) was emulsified in 1 ml complete Freund's adjuvant and injected intramuscularly into Clun sheep. Booster injections of 100 μg were given on days 42 and 307 and blood was removed on days 7, 10 and 14 following each boost injection.

Immunoglobulins were purified from antiserum by triple ammonium sulphate precipitation from the bleed with the highest titre (bleed 6). A solution of 4 M (NH₄)₂SO₄ (6.7 ml) was added dropwise to 10 ml antisera and the mixture stirred for 10 minutes at room temperature. The precipitate was collected by centrifugation and resuspended in 10 ml distilled H₂O. Precipitation with 6.7 ml of 4 M (NH₄)₂SO₄ was repeated twice more as described above. The final precipitate was resuspended in 3 ml PBS and dialysed overnight against two changes of PBS. The optical density of the IgG solution was determined at 280 nm and the protein concentration calculated using the extinction coefficient of 1.4 (Harlow and Lane, 1988). IgG was then filter sterilised (0.2 μM) and stored at -20°C.

N175 antibody was further purified by adsorption to Protein-G Sepharose. One ml of Protein-G Sepharose (Sigma) was washed with 20 ml H₂O, followed by 20 ml wash buffer (50 mM Tris-HCl, pH 7.5). The Sepharose was packed into a disposable column, and
washed with a further 5 ml of wash buffer. IgG (20 mg protein from an ammonium sulphate precipitation) was diluted to 3 ml with the wash buffer and then applied to the column. Fractions of 0.5 ml were collected. The column was washed with 6 ml of wash buffer and then bound proteins were eluted with 0.1 M glycine, pH 2.7 containing 1 mM EGTA. Eluted proteins were immediately neutralised using a 2 M Tris-HCl solution, pH 8. Fractions were subjected to 10% SDS-PAGE followed by silver stain, to confirm the presence of purified IgG in the glycine eluates. Pooled IgG was dialysed against PBS, filter sterilised and the protein concentration determined as described above.

Affinity purified N175 was prepared by adsorption of the IgG to the catalytic domain of MT1-MMP as described in d'Ortho et al., (1998). Δ269-559 MT1-MMP_{cat} (300 μg) was adsorbed onto both sides of a 2 cm x 5 cm piece of nitrocellulose (Hybond ECL, Amersham Life Sciences). The nitrocellulose was then blocked for one hour with 5% (w/v) skimmed milk powder in wash buffer: 10 mM Tris-HCl, pH 7.4, 145 mM NaCl, 0.05% (v/v) Tween 20, 0.02% (w/v) sodium azide. 50 mg of ammonium sulphate purified N175 IgG was added to a solution of 2.5% (w/v) skimmed milk powder in wash buffer (50 ml) and incubated with the nitrocellulose overnight at 4°C. The nitrocellulose was then washed extensively with wash buffer, diced into 3 mm squares, and bound IgG was eluted from the nitrocellulose using 0.2 M glycine, pH 2.8 containing 1 mM EGTA and 0.02% (w/v) sodium azide. Elution fractions of 1 ml were immediately neutralised with a 2 M Tris-HCl solution, pH 8. The protein concentration of the eluted fractions was determined as described above. Purified N175 IgG was stored at 4°C for one month, or frozen at 5 μg/ml in a solution of 2.5% (w/v) skimmed milk powder in wash buffer, ready for use in Western blot analysis.

### 2.2.2 Anti-GLA antibody K399

Sheep antiserum to recombinant human GLA was prepared by Prof. G. Murphy and Dr R. Hembry (University of East Anglia, Norwich, UK) according to the method published previously for anti-GLA antibody X670 (Hipps et al., 1991). Immunoglobulins were purified from antiserum by triple ammonium sulphate precipitation from the bleed with the
highest titre as described above. K399 IgG was further purified by adsorption to Protein-G Sepharose as detailed above for N175.

2.3 Activation of MMPs
Recombinant MMPs were activated essentially as described by Murphy et al. (1991a). Briefly, proGLA and proGLB were activated by incubation with 2 mM APMA for 1 hour at 37°C. proCL-3 was also activated with 2 mM APMA for 1 hour at 37°C. proSL-1 was activated by incubation with trypsin (5.0 µg/ml) for 30 minutes at 25°C, the cleavage was stopped by addition of aprotinin or soybean trypsin inhibitor (SBTI), and the mixture was incubated on ice for 30 minutes. ΔTM-MT1-MMP was activated by incubation with active MT1-MMPcat (Butler et al., 1998). Briefly, proMT1-MMPcat (30 µg/ml) was incubated with 5 µg/ml trypsin at 25°C for 15 minutes. The trypsin was then inactivated with SBTI and the mixture was added to ΔTM-MT1-MMP at 1:20 w/w ratio; activation was completed by incubation of the mixture for 16 hours at 25°C. One batch of ΔTM-MT1-MMP was activated simply by incubation at 37°C for 4 hours.

2.4 Determination of binding affinity of gelatinase A to matrix proteins by ELISA
The ELISA method used to determine MMP binding affinity for matrix molecules is described by Murphy et al. (1994). Matrix components were air dried onto plastic wells at 100 µg/ml and cross-linked with glutaraldehyde. Pro- and active gelatinase A were added to the wells at 100 nM and incubated for 2 hours at 4°C. Bound enzyme was revealed by adding a polyclonal antibody to gelatinase A (X670) and incubating for 2 hours at 4°C, followed by a peroxidase-conjugated second antibody and 3,3', 5,5'-tetramethylbenzidine substrate (Kirkegaard and Perry, Maryland, USA). Optical density of the wells was read at 450 nm.
2.5 Cleavage of type IV collagen with matrix metalloproteinases

Type IV collagen preparations were dialysed into a buffer of 50 mM Tris-HCl pH 7.6, 150 mM NaCl and 0.02% azide prior to cleavage. Gelatinases A or B were added to type IV collagen at an enzyme:substrate ratio of 1:8. To activate the gelatinase, APMA was added to the collagen/gelatinase mixture to achieve a final concentration of 0.7 mM. The mixture was incubated for 20 hours at 30°C or 37°C, after which the cleavage was stopped by placing the tube on ice and adding TIMP-1 at a 3:1 molar ratio with gelatinase. The digestion of type IV collagen was assessed by SDS-PAGE using 6-10% polyacrylamide gels.

Type IV collagen was cleaved with collagenase-3 using the same protocol as for the gelatinases. Cleavage with stromelysin-1 also followed the same protocol, except that the stromelysin was pre-activated with trypsin as described above. APMA was not included in the stromelysin cleavages. Appropriate controls included incubation of collagen IV with APMA alone, or with trypsin/SBTI mixture.

2.6 Electrophoresis and silver stain

Proteins were analysed by SDS-PAGE (Laemmli and Favre, 1973) using IDEA Scientific mini gel electrophoresis equipment. Reagents were purchased from BioRad Laboratories, Hemel Hempstead, UK, with the exception of SDS, which was purchased from Serva.

Electrophoresed proteins were stained using silver nitrate. Briefly, gels were fixed in a solution of 40% (v/v) methanol, 10% (v/v) acetic acid for 30 minutes, followed by 2 x 15 minute incubations in 10% (v/v) ethanol, 5% (v/v) acetic acid. Gels were then incubated in potassium dichromate (1% w/v) for 5 minutes, followed by 2 x 5 minute rinses in water (250 ml/rinse). A solution of silver nitrate (2% w/v) was added to the gels for 20 minutes. To develop the stain, the silver nitrate solution was poured off, the gels rinsed briefly in water and then incubated in a solution of 2.9% (w/v) Na₂CO₃ containing 0.02% (v/v)
formaldehyde until protein bands were visible. The developing reaction was stopped by the addition of a 5% (v/v) acetic acid solution.

2.7 Cell Culture

The HT1080 human fibrosarcoma cell line was purchased from the European Collection of Animal Cell Cultures, Wiltshire, UK. These cells were used for the adhesion assays detailed in Chapter 3. A second HT1080 line that is N-ras transformed (Paterson et al., 1987) was a gift from Dr C. Marshall, Institute of Cancer Research, London, UK. These cells were used for the studies in Chapters 4-6 as their constitutive expression of the gelatinases was greater. Processing of GLA and MT1-MMP by the N-ras transformed HT1080 cells cultured on ECM substrates was compared with HT1080 cells from the European Collection. Both cell lines responded similarly. HT1080 cells were maintained in DMEM (Gibco BRL or Imperial Laboratories) supplemented with 10% FCS (Globepharm, Surrey, UK), 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco BRL). A third HT1080 cell line, stably transfected with wild-type MT1-MMP (Green et al., 1994) was a gift from Dr J. Clements, British Biotech Pharmaceuticals Ltd., Oxford, UK. These cells were maintained in a selection medium of DMEM supplemented with 10% FCS, 4 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, HT supplement (Gibco), 20 µM mycophenolic acid and 2 mM xanthine. The rat glioma cell line RuGLi was provided by Prof. K. Kühn, Max-Planck-Institut für Biochemie, Martinsried, Germany. The line was maintained in DMEM, 10% FCS, 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Human foreskin fibroblasts (HFFS) were prepared by S. Atkinson (University of East Anglia, Norwich, UK) using the method described in Heath et al. 1982. Cells were maintained in DMEM 10% FCS and used at passage 4-8. HL60 cells (human myeloid leukaemic cell line) were purchased from the European Collection of Animal Cell Cultures, Wiltshire, UK and maintained in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. All cell lines were maintained in an atmosphere of
5% CO₂. Adherent cells were passaged using trypsin/EDTA (Gibco BRL) according to standard procedures. Cells were routinely passaged 24-48 hours prior to experiment.

2.8 Immunolocalisation

For immunolocalisation studies, cells were seeded onto uncoated Labtek 8-well slides (Nunc), or onto slides coated with fibronectin or laminin-1 according to the procedure outlined in section 2.11 below. Cells were maintained for 24-48 hours in DMEM 10% FCS (uncoated slides) or DMEM 0.1% BSA (coated slides) prior to staining.

2.8.1 Surface staining

Cells were rinsed in serum-free DMEM and fixed in freshly prepared 4% (w/v) paraformaldehyde in PBS for 5 minutes. The fixative was rinsed from the cells using PBS (3 x 5 minute washes). Primary antibody (50 μg/ml for polyclonal antibodies, 5 μg/ml for monoclonal antibodies) was added in a solution of PBS with 5% serum added as a blocking agent. Blocking serum was chosen to match the species in which the secondary antibody was raised. Cells were incubated with the primary antibody for 30-60 minutes at room temperature, unbound antibody was aspirated and the cells washed three times with PBS. The FITC-conjugated secondary antibody was added in PBS with 5% blocking serum for 30 minutes at room temperature. Unbound antibody was aspirated, the cells washed three times with PBS and mounted in Citifluor (City University, London).

2.8.2 Intracellular staining

To increase the intracellular concentration of antigen, cells were incubated with the ionophore monensin. This reagent prevents protein secretion but not protein production, and causes an increase in the concentration of protein at the Golgi apparatus and secretory vesicles (Hembry et al., 1985). Monensin (5 μM; Sigma) was added to the cells for the last 3 hours of culture. Cells were fixed as described above and then permeabilised by incubation with a solution of 0.1% (v/v) Triton-X-100 in PBS, for 5 minutes at room
temperature. The cells were then washed with PBS three times, and treated with antibodies as described above.

Stained cells were viewed with a fluorescence microscope (Zeiss photomicroscope III; Carl Zeiss, Thornwood, New York). Photographs were taken using Kodak Panther 1600 ASA film. Staining was also analysed by Dr R. Hembry (Strangeways Research Laboratory, Cambridge, UK), using an MRC 600 confocal microscope with a krypton/argon laser. Data was collected by two methods: i) scanning the 488 nm FITC channel with a confocal aperture of 0.5 and Kalman averaging over 10 scans, at slow scan speed; ii) by collecting a Z-series of serial 1 μM sections through the cells and merging images with BioRad Comos software using false colour. Images were taken from the screen using Agfapan 25 film.

2.9 Flow cytometric analysis

HT1080 cells were removed from culture flasks using PBS containing 5 mM EDTA. HL60 cells were collected by centrifugation. Cells (1 x 10^6 cells) were washed with PBS containing 0.8% BSA (w/v) and incubated on ice in antibody buffer (PBS containing 0.8% BSA, 0.02% azide w/v), with or without the monoclonal antibody HP2/1 to the α4 integrin subunit at 10 μg/ml. After 30 minutes, the cells were collected by centrifugation, washed in ice-cold antibody buffer and resuspended in antibody buffer containing sheep anti-mouse-FITC IgG at 1:50 dilution. Cells were incubated for a further 30 minutes on ice and then collected by centrifugation, washed with ice-cold antibody buffer before fixing with PBS containing 2% (v/v) formaldehyde. Staining was assessed by a Becton Dickinson FACS-Calibur flow cytometer, using CellQuest version 3.1 software (Becton Dickinson).

2.10 Cell attachment assay

The method for the attachment assays is detailed in Vandenberg, et al. (1991). For the type IV collagen studies, untreated or GLA treated type IV collagen were adsorbed to 96 well tissue culture plates as a 1 μg/ml solution in PBS at 4°C overnight. Free binding sites were blocked with 1% (w/v) heat-denatured BSA in PBS for 2 hours at 4°C. Cells were seeded
onto coated wells at $6 \times 10^4$ cells/well in serum free medium and allowed to attach for 35 minutes at 37°C. Attached cells were washed twice with serum free medium and then fixed with 4% paraformaldehyde (v/v) in 0.15 M NaCl, pH 7.4. Cells were stained with 1% (w/v) methylene blue in 0.01 M borate buffer, pH 8.5. After washing extensively with distilled water the dye incorporated in the cells was solubilised with a solution of 0.05 M HCl, 50% (v/v) ethanol and the optical density was read at 630 nm.

For the studies with fibronectin, a coating concentration of 5 µg/ml fibronectin was used. Wells were washed and blocked as detailed above. HT1080 cells were pre-incubated with anti-integrin antibodies (10 µg/ml) for 30 minutes at 37°C prior to seeding onto the fibronectin coated wells (in the presence of the antibodies). Following incubation at 37°C for 35 minutes, the attached cells were fixed and stained as detailed above.

2.11 Preparation of extracellular matrix and antibody substrates

Culture plates (24 well; Costar) were coated with human plasma fibronectin, fragments of fibronectin, or laminin-1 following the method of Tremble et al. (1994). ECM was added to culture plates at 30 µg/ml in PBS and incubated overnight at 4°C. The solution was aspirated the following day, the wells washed with PBS and blocked with 1% (w/v) heat-denatured BSA in PBS for 1 hour at room temperature. Wells were then washed with PBS and used the same day. The following monoclonal antibodies to integrin subunits were coated to culture plastic at 100 µg/ml: anti-α5, mAbs 16 and 11; anti-β1, mAb 13; and anti-α6, GoH3 (sources of antibodies described above). The antibody-coated wells were washed with PBS and blocked with BSA according to the protocol above.

2.12 Culture of cells on substrates and preparation of conditioned media

Subconfluent cultures of cells were trypsinised and centrifuged, firstly in serum-containing medium, followed by two washes in serum-free medium. Cells were seeded at $1 \times 10^5$ cells/well onto uncoated 24 well tissue culture wells or substrate coated wells, and cultured for 48 hours in DMEM supplemented with 0.1% BSA (Fraction V, culture grade,
purchased from Sigma). This concentration of BSA was sufficient for cells to survive on ECM components in serum-free medium. Reagents (antibodies, MMP inhibitors) were added to the wells at cell seeding, or after the first 2 hours in culture (PMA, MMP inhibitors). At harvest, cells were trypsinised and the number of cells per well determined by counting the cells with a Neubauer haemocytometer.

2.13 Preparation of cell lysates

Cell monolayers were washed with cold PBS, and lysed according to the method of Lohi et al. (1996). The lysis buffer contained 50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1% (v/v) Triton-X-100, 0.02% (w/v) azide, with the protease inhibitors pepstatin A (1 µg/ml), E-64 (1 µg/ml), PMSF (100 µM), and EDTA (10 mM). A volume of 25 µl lysis buffer was used per well of a 24 well culture dish. Cells were scraped into the lysis buffer, aspirated through a Gilson 200 µl pipette tip and then centrifuged in a microfuge for 10 minutes at 4°C. The supernatant was collected and stored at -70°C until required. Total protein in the cell lysates was estimated using the bicinchoninic acid assay (Sigma), following the manufacturer's instructions.

2.14 Zymography

Gelatin degrading activity was assessed by zymography, according to the method of Heussen and Dowdle (1980). Samples of conditioned medium from tissue culture wells were loaded according to the number of cells/well at the end of the culture period. Samples were electrophoresed under non-reducing conditions on 7% SDS-PAGE gels containing 0.5 mg/ml type I calf skin collagen (Sigma) that had been heat-denatured (20 minutes, 60°C). Non-reduced samples were separated by electrophoresis, after which the gels were washed twice with 2% (v/v) Triton-X 100 at room temperature, 15 minutes per wash, to remove the SDS. Under these conditions, enzymes partially refold within the gel matrix, such that both pro- and active MMPs are capable of degrading gelatin. The gels were then rinsed in water and incubated overnight at room temperature in 100 mM Tris-HCl pH 7.9, 30 mM CaCl₂, 0.04% (v/v) Brij and 0.02% (w/v) sodium azide (TCB buffer).
The gels were stained with Coomassie brilliant blue to reveal white zones of lysis, which corresponded to gelatin degrading activity.

MMP-inhibitory activity was detected by adding an additional incubation step to the protocol, following the Triton-X-100 washes, as previously described (Herron et al., 1986). Briefly, gels were incubated in a preparation of active rabbit skin gelatinase (7 units/ml) for 40 min at 37°C, before incubation overnight in TCB buffer at room temperature. Coomassie blue staining revealed dark zones of metalloproteinase-inhibitory activity against a lighter background.

2.15 Western Blot

Lysate proteins (25 μg protein/lane), membrane proteins (10 μl/lane), column fractions (10-20 μl/lane) or cleavage reaction mixtures (10 μl/lane) were separated by 10% SDS-PAGE under reducing conditions. Proteins were transferred to nitrocellulose (Hybond ECL, Amersham Life Sciences) by electroblotting (Towbin et al., 1979) using a BioRad blotting apparatus. The transfer of protein was confirmed by Ponceau S staining, using a solution of 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid. Ponceau S stain was then removed by rinsing the membrane repeatedly in wash buffer (10 mM Tris-HCl, pH 7.4, 145 mM NaCl, 0.05% (v/v) Tween 20). The membrane was blocked in 5% (w/v) skimmed milk powder in wash buffer for 1 hour at 37°C. Specific antibodies (5-50 μg IgG/ml of 2.5% (w/v) skimmed milk powder in wash buffer) were incubated with the membrane for 3 hours at room temperature, or alternatively overnight at 4°C. Unbound antibody was removed by rinsing in wash buffer and the membrane blocked a second time as detailed above. The membrane was then incubated with a peroxidase conjugated secondary antibody in a solution of 2.5% (w/v) skimmed milk powder in wash buffer for 1 hour at room temperature. Following several rinses in wash buffer, bound antibodies were revealed by incubating the membrane with a chemiluminescent substrate (Supersignal™ CL-HRP Substrate System; Pierce). Luminescence was detected by exposure to autoradiographic film (Hyperfilm-ECL, Amersham Life Sciences), which was developed
using standard procedures. Densitometric scanning of Western blots was performed using a Stratagene Eagle-Eye II, and analysed using EagleSight software version 3.2.

2.16 Gelatin-agarose purification of GLA/TIMP-2 complexes
TIMP-2 complexed with GLA was purified from the conditioned medium by adsorption to gelatin-agarose (Butler et al., 1998). Gelatin-agarose (Sigma) was prepared by rinsing three times with wash buffer: 50 mM Tris-HCl, pH 7.5, 10 mM CaCl$_2$, 0.025% (v/v) Brij 35, 0.02% (w/v) sodium azide. Washed gelatin-agarose was added to conditioned medium at 10% v/v and the mixture rotated end-on-end for 2 hours at 4°C. The gelatin-agarose was then washed twice with the wash buffer, and bound material was eluted using Laemmli reducing sample buffer. Eluted material was analysed for TIMP-2 by Western blot.

2.17 Biosynthetic labelling and immunoprecipitation of MMPs and TIMPs
Cells were cultured on ECM substrates as detailed above. For labelling, the cells were washed twice with serum free, methionine/cysteine free DMEM (ICN Pharmaceuticals). Cells were incubated in this medium, containing 70 µCi/ml $[^{35}S]$-methionine/cysteine (Amersham Life Sciences) for 8 hours. The conditioned medium was harvested and the cells lysed with a 50 mM Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl, 1% (v/v) Triton-X-100, 0.02% (w/v) azide, 10 mM EDTA, 100 µM PMSF, 1 µg/ml pepstatin A and 1 µg/ml E-64. The conditioned medium or cell lysates were first pre-cleared with normal sheep IgG (50 µg IgG/ml sample) for 1 hour at 4°C. Protein-G Sepharose (Sigma) was washed with buffer A: 50 mM Tris-HCl, 300 mM NaCl, 0.02% (w/v) azide, 0.5% (v/v) Triton X-100 and then added to the mixtures at 10% (v/v). The solution was mixed end on end at 4°C for one hour, centrifuged and the supernatant retained for immunoprecipitation. A polyclonal antibody raised in sheep to recombinant human gelatinase A (X670) was added to the supernatants at 50 µg/ml and incubated for 3 hours at 4°C. Protein-G Sepharose was added for a further 1 hour incubation and the pellet collected by centrifugation. The pellet was washed three times with buffer A and then boiled in Laemmli reducing sample buffer. Solubilised proteins were separated by SDS-PAGE, the
gel stained with Coomassie brilliant blue and treated with a fluorographic reagent (Amplify, Amersham Life Sciences). The gel was dried under vacuum and exposed to Hyperfilm-MP (Amersham Life Sciences) at -70°C for 3-5 days. Film was developed using standard procedures.

2.18 Isolation of RNA and Northern blot analysis

HT1080 cells were cultured in DMEM 0.1% (w/v) BSA on plastic or ECM coated dishes. Cells were cultured for 24 hours prior to lysis and extraction of total cellular RNA by the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). RNA samples (5 μg/lane) were separated on a 1% (w/v) agarose, 2.2 M formaldehyde gel, transferred to a nylon membrane and cross-linked with UV light as described by Sambrook et al. (1989). The membrane was prehybridised for 1 hour at 68°C in hybridisation buffer: 0.75 M NaCl, 80 mM sodium citrate pH 7.0, 50% (v/v) formamide, 2% (w/v) Blocking Reagent (Boehringer Mannheim), 0.33% (v/v) Sarkosyl, 0.02% (w/v) SDS and 100 μg/ml denatured herring sperm DNA. Digoxygenin-labelled antisense riboprobes to MT1-MMP and MT4-MMP (Cowell, 1997) were kindly provided by Dr S. Cowell, Strangeways Research Laboratory, Cambridge, UK. Membranes were hybridised overnight at 68°C with the riboprobes, which were added to hybridisation buffer at a concentration of 50 ng/ml for the MT1-MMP probe, and 12.5 ng/ml for the GAPDH probe. The hybridisation solution was then removed, and the blots were washed at room temperature for 2 x 5 minutes in a buffer of 0.3 M NaCl, 32 mM sodium citrate, pH 7.0, containing 0.1% (w/v) SDS. Blots were then washed for 2 x 15 minute washes in 15 mM NaCl, 1.5 mM sodium citrate, pH 7.0 containing 0.1% (w/v) SDS at 68°C. The membrane was rinsed in maleate buffer (100 mM sodium maleate, pH 7.4, 150 mM NaCl) and blocked with a solution of 1% (w/v) Blocking Reagent in maleate buffer for 30 minutes at room temperature. To detect bound riboprobe, the membrane was incubated for 30 minutes at room temperature with an alkaline phosphatase conjugated anti-digoxigenin polyclonal antibody (Boehringer Mannheim) diluted 1/10 000 in blocking buffer. Excess antibody was removed by 2 x 15 minute washes in maleate buffer and the membrane equilibrated in a solution of 100 mM
Tris, pH 9.5, 100 mM NaCl, 50 mM MgCl₂ (TMN). The membrane was then soaked with
the chemiluminescent reagent CSPD (Boehringer Mannheim), diluted 1/100 in TMN.
Excess reagent was drained from the membrane, and the membrane was sealed in plastic
and incubated at 37°C for 15 minutes. Luminescence was detected by exposure to
autoradiographic film (Hyperfilm-ECL, Amersham Life Sciences). Film was developed
using standard procedures.

2.19 Preparation of cell membranes

Cell membranes were prepared according to the method of Ward et al. (1991b). Cell
monolayers were washed twice with ice-cold serum free DMEM and then scraped into
DMEM containing the protease inhibitors pepstatin A (1 μg/ml), E-64 (1 μg/ml) and
PMSF (100 μM). The mixture was centrifuged (2500 g, 10 minutes, 4°C), washed twice
with DMEM containing the inhibitors and the cell pellet frozen at -70°C. For the
preparation of membranes, thawed pellets were homogenised in a buffer of 5 mM Tris-
HCl, pH 7.6, 0.02% (w/v) sodium azide containing the inhibitors as indicated above. The
mixture was centrifuged at 10 000 g for 10 minutes at 4°C and the supernatant was
subjected to further ultra-centrifugation at 43 000 rpm (Sorvall rotor TFT 80.4) for 1 hour
at 4°C. The pellet was resuspended in 20 mM Tris-HCl, pH 7.8, 10 mM CaCl₂, 0.025%
(v/v) Brij 35, 0.02% (w/v) sodium azide containing the inhibitors. Protein concentration in
the membrane preparation was estimated by measuring optical density at 280 nm and
assuming an extinction coefficient of 1.0.

HT1080 cell membranes enriched in the 60 kDa form of MT1-MMP were prepared from
HT1080 cells stably transfected with wild-type MT1-MMP (Green et al., 1994).
Processing of MT1-MMP to the 45 kDa form was prevented by culturing the cells in the
presence of CT1746 inhibitor (1 μM) for 48 hours prior to harvest (Butler et al., 1998).
To remove excess CT1746 at harvest, the cells were washed with ice-cold DMEM (containing
inhibitors) with a pH adjusted to 9.0. After the cells were scraped, the pellet was washed
twice with this DMEM solution, using 25 ml solution/175 cm² cell monolayer/wash. The
amount of protein in the membrane preparation was estimated by the bicinchoninic acid assay to be 2.6 mg/ml and the amount of active MT1-MMP present in the membrane preparation was assessed by a quenched fluorescent peptide assay, kindly performed by Dr G. Butler, University of East Anglia, Norwich, UK. The concentration of active MT1-MMP was calculated using a $k_{cat}/K_m$ value of $1.9 \times 10^5$ M$^{-1}$ s$^{-1}$ obtained for soluble ΔTM MT1-MMP (Butler et al., 1998).

2.20 In vitro cleavage of recombinant MT1-MMP and native MT1-MMP

Recombinant MMPs were activated as detailed above. Active MMPs were added to either i) recombinant ΔTM-MT1-MMP (0.1-0.2 µg; d'Ortho et al., 1997) or ii) 10 µl of a 60 kDa MT1-MMP enriched cell membrane preparation. Mixtures were incubated at 37°C for the times indicated. The cleavage reaction was stopped by the addition of Laemmlli reducing sample buffer. The molecular mass of the resulting ΔTM-MT1-MMP fragments were estimated by SDS-PAGE, followed by i) silver stain or ii) Western blot analysis using anti-MT1-MMP antibody N175 as described above.

For the cleavage of ΔTM-MT1-MMP complexed with TIMP-2, active ΔTM-MT1-MMP was pre-incubated with an equimolar concentration of recombinant TIMP-2 for one hour at 25°C. A three molar excess of active GLA was then added to the ΔTM-MT1-MMP/TIMP-2 mixture, and the solution incubated for a further five hours at 37°C. The molecular mass of the resulting ΔTM-MT1-MMP fragments was estimated by SDS-PAGE, followed by silver stain.

2.21 Immunoprecipitation of MT1-MMP

HT1080 cells were cultured in 175 cm$^2$ flasks in DMEM 10% FCS until nearly confluent. The cultures were then changed to serum free medium supplemented with PMA (10 ng/ml) and incubated for a further 24 hr. Cells were lysed according to the protocol described in section 2.13 above, using 800 µl lysis buffer/175 cm$^2$ cells. For N-terminal sequencing, the Triton-X-100 in the lysis buffer was replaced with Surfact-Amps X-100 (Pierce,
Pre-immune sheep IgG and anti-MT1-MMP antibody were purified by Protein-G Sepharose chromatography (section 2.2) prior to use in immunoprecipitation. Lysates were pre-cleared with pre-immune sheep IgG (20 μg/ml) for 1 hour at 4°C. Protein-G Sepharose was washed with buffer A: 50 mM Tris-HCl, 300 mM NaCl, 0.02% (w/v) azide, 0.5% (v/v) Triton X-100 (or Pierce Surfact-Amps X-100 for sequencing projects) and then added to the mixtures at 10% (v/v). The solution was mixed end on end for 1-2 hours at 4°C, centrifuged, and the supernatant retained for immunoprecipitation with the polyclonal antibody to MT1-MMP (N175; 20 μg/ml) overnight at 4°C. The mixture was loaded on a column of Protein-G Sepharose, pre-equilibrated in buffer A. Unbound proteins were washed from the column with buffer A, and bound proteins eluted with i) Laemmli reducing buffer (for Western blot) or ii) 25 mM Tris-HCl, pH 7.5, containing 8 M guanidinium hydrochloride (for reverse-phase HPLC).

2.22 Transfer of N175 immunoprecipitates to PVDF membrane

Lysates prepared from six 175 cm² flasks of PMA treated HT1080 cells were purified by immunoprecipitation, followed by Protein-G Sepharose chromatography, as described above. A 10% SDS polyacrylamide separating gel was poured and allowed to stand at room temperature overnight (to allow reactive species to dissipate). The stacking gel was then added, the gel set in the tank, and pre-electrophoresed for 20 minutes with the addition of reduced glutathione (50 μM) to the upper reservoir buffer. Following the pre-electrophoresis, the upper reservoir buffer was discarded and replaced with fresh reservoir buffer containing sodium thioglycollate (100 μM). Immunoprecipitates were then loaded on the gel and electrophoresed as normal.

Proteins in the gel were transferred to PVDF membrane (Immobilon, Millipore) by electroblotting at 100 mA, overnight at 4°C. A transfer buffer of 10 mM CAPS, pH 11, containing 10% (v/v) methanol was used. After transfer, the PVDF membrane was stained with Ponceau S (see section 2.15 above). A band at approximately 45 kDa was positively identified as MT1-MMP by concurrent Western blot analysis. The band was excised from
the membrane and subjected to automated sequencing using an Applied Biosystems 470A sequencer, performed by Dr Bryan Smith, Celltech Research, Slough, UK (Will et al., 1996).

2.23 Reverse-phase HPLC purification of N175 immunoprecipitates

Lysates prepared from six 175 cm² flasks of PMA treated HT1080 cells were immunoprecipitated as described above. Immunoprecipitates were applied to a column of Protein-G Sepharose, and eluted with a buffer of 25 mM Tris-HCl, pH 7.5, containing 8 M guanidinium hydrochloride. The proteins eluted were separated by reverse-phase HPLC (performed by Dr William English, University of East Anglia, Norwich, UK). A Dionex HPLC equipped with a diode array detector was used. 100 µl of eluted immunoprecipitate was applied to a C4 column (AllTec 24 x 0.46 cm), which had been pre-equilibrated with starting buffer (H₂O/0.1% trifluoroacetic acid). A continuous gradient from 0-100% A-B (where A= H₂O/0.1% trifluoroacetic acid and B= 60% isopropanol/40% acetonitrile/0.1% trifluoroacetic acid) was applied to the column over 50 minutes. Peak fractions were collected and dried down under vacuum. Proteins in the peak fractions were resuspended in buffer (50 mM Tris-HCl, pH 7.4 containing 1 mM EDTA) to 1/10 of the volume of the original fraction, and analysed by 10% SDS-PAGE followed by silver stain.
Chapter 3

Matrix metalloproteinase cleavage of collagen IV: implications for cell adhesion
Chapter 3 Matrix metalloproteinase cleavage of collagen IV: implications for cell adhesion

3.1 Introduction

Type IV collagen is a major structural component of basement membranes, and forms a network with other basement membrane constituents to provide a mechanically stable support for cells. Localised remodelling of basement membranes is an integral feature of wound repair and mammary gland involution, and is essential for the invasion of tumour cells to distant sites. One popular hypothesis for tumour invasion attributes the invading cell with a battery of enzymes capable of degrading type IV collagen, a requirement for moving through basement membranes (Liotta and Stetler-Stevenson, 1991). Fessler et al. (1984) reported that a mouse tumour MMP cleaved intact type IV collagen (derived from cell culture) into two fragments, 100 nm and 300 nm in length. Others, working with purified GLA and GLB, reported fragments of a similar size generated by proteolytic cleavage of type IV collagen preparations in vitro (Salo et al., 1983; Collier et al., 1988; Murphy et al., 1989a). This supported the concept that the gelatinases are 'type IV collagenases' (Liotta et al., 1981).

Cells interact with type IV collagen via the α1β1 and α2β1 integrin receptors. The integrin binding sites occur within a 40 nm long section of the molecule, which can be isolated as a triple helical cyanogen bromide fragment CB3[IV]. The CB3[IV] region is located 100 nm from the N-terminus of collagen IV (Vandenbberg et al., 1991), and the exact location for α1β1 binding has been mapped to an aspartate residue (Asp 461) of the α1(IV) and an arginine residue (Arg 461) of the two α2(IV) chains (Eble et al., 1993; Fig. 3.1). α1β1 integrin binding is entirely dependent upon triple helical conformation, requiring amino acid residues on adjacent α(IV) chains. The stability of the triple helix in this region is maintained by interchain disulphide bridges, with the α1β1 binding site occurring between two disulphide knots (Fig. 3.1). On either side of these two disulphide knots, in the relaxed triple helical end regions of CB3[IV], are the recognition sites for the α2β1 integrin (Kern et al., 1993).
GLA cleaves type IV collagen 100 nm from the N-terminus, in close proximity to the CB3[IV] region. This led to the question of whether GLA treatment alters the ability of collagen IV to interact with \( \alpha 1\beta 1 \) and \( \alpha 2\beta 1 \) integrins. Chapter 3 reports a test of the hypothesis that cell attachment to type IV collagen (via \( \alpha 1\beta 1 \) and \( \alpha 2\beta 1 \) integrins) is impaired by prior treatment of the collagen IV with GLA. Preparations of collagen IV isolated from the murine EHS tumour, or from human placenta, were incubated with GLA and the fragments generated assessed by SDS-PAGE. Cell adhesion to the collagen IV fragments generated by GLA treatment was compared with adhesion to intact collagen IV. As predicted, collagen IV preparations (in which the interchain disulphide bonds had been reduced during purification) were susceptible to cleavage in the CB3[IV] region, and cell attachment via \( \alpha 2\beta 1 \) and \( \alpha 1\beta 1 \) was greatly reduced. However, preparations of type IV collagen in which the interchain disulphide bonds remained intact, or reformed during purification, were not susceptible to GLA cleavage of integrin binding sites.

This work was done in collaboration with Prof. K. Kühn and his group (Max-Planck-Institut für Biochemie, Martinsried, Germany), who provided preparations of collagen IV; N-terminal sequence information for cleaved collagen IV; and worked with isolated integrins in a collagen IV binding assay to confirm the observations made here with the cell adhesion assays.

### 3.2 Extraction and purification of type IV collagen

Data from this chapter shows that cell adhesion to GLA cleaved type IV collagen is entirely dependent upon the methods used to extract and purify the collagen. All preparations of type IV collagen were supplied by Prof. K. Kühn. The different procedures used in his laboratory to isolate type IV collagen are discussed below.

#### 3.2.1 Preparation of type IV collagen monomers

Type IV collagen monomers were extracted from murine EHS tumour by treating the tissue with guanidine HCl containing dithiothreitol (DTT) to solubilise the collagen. Solubilised collagen IV was purified by DEAE cellulose chromatography, using 4M urea
and 2 mM DTT in the elution buffer (Kleinmann et al., 1982). Under the reducing conditions used for extraction, intermolecular disulphide bonds were broken to yield monomeric collagen IV. The intramolecular disulphide bonds in the CB3[IV] region and the N-terminal telopeptides were also susceptible to reduction, with variation between batch preparations. This resulted in preparations of type IV collagen in which the intramolecular disulphide bonds were either completely reduced (preparation A) or remained intact (preparation B; see Fig. 3.2) and some preparations which contained mixtures of both (Eble et al., 1996).

3.2.2 Preparation of type IV collagen dimers
Dimeric collagen IV was extracted from human placenta after a mild treatment with bacterial collagenase (Timpl et al., 1981). Bacterial collagenase (Worthington, Freehold, New Jersey) was added to minced tissue suspended in Tris-buffered saline and incubated for 24 hours at 4°C. Collagen IV was then precipitated from the mixture by adding NaCl (2-3 M) and further purified by DEAE-cellulose chromatography (Timpl et al., 1981). The purified collagen IV dimers still contained traces of collagenase, which continued to degrade the collagen IV during incubation at 37°C. This problem was addressed by removing the collagenase using molecular sieve chromatography, on an agarose 5.0m (BioRad) column (Eble et al., 1996).

Bacterial collagenase cleaves the collagen IV network 100 nm away from the N-terminus (Timpl et al., 1981). The resulting dimers are missing 100 nm of their N-termini, and are connected via the C-terminal NC1 domains (Fig. 3.2). Intramolecular disulphide bonds in the CB3[IV] remain intact (Eble et al., 1996).

3.2.3 Preparation of type IV collagen tetramers
Tetrameric collagen IV was extracted from human placenta by treatment with porcine pepsin (Vandenberg et al., 1991). Minced tissue was rinsed in saline, suspended in 0.5 M acetic acid and incubated with pepsin for 20 hours at 4°C. Type IV collagen was separated
from the protein mixture by precipitation with a buffer containing 1 M NaCl, followed by precipitation with a buffer containing 1.7 M NaCl (Vandenberg et al., 1991). The precipitated protein was further purified by DEAE-cellulose chromatography (Timpl et al., 1981).

Tetrameric collagen IV consists of four strands connected by covalent linkages at the 7S domain (Timpl et al., 1981; Fig. 3.2). The initial site for pepsin cleavage is located at the C-terminal end of the strands, just N-terminal to the NC1 globular domain. Rotary shadowing of the tetramers has shown that pepsin treatment destroys the NC1 globular domain, leaving the ends of the strands free (Timpl et al., 1981).

All collagen IV preparations were dialysed against acetic acid (0.2 M), lyophilised and stored at -70°C. Prior to experimentation, collagen preparations were redissolved in acetic acid, and dialysed against cleavage buffer as described in Chapter 2.

3.3 Results

3.3.1 MMP cleavage of collagen IV monomers

Monomeric type IV collagen was incubated with GLA for 20 hours, at 30°C and 37°C. Intact (untreated) collagen IV and collagen IV fragments were separated by SDS-PAGE and visualised by silver stain. Intact collagen IV (preparation A) migrated as a doublet of approximately 180 kDa (Fig. 3.3A lane 1), corresponding to the pro-α1(IV) and pro-α2(IV) chains respectively (Fessier et al., 1984). Considerable amounts of protein were retained in the stacking gel (Fig. 3.3A lane 1), most likely due to intermolecular lysine aldehyde-derived cross-linkages, causing the formation of multimers. Preparation A was cleaved by GLA at 30°C to generate two bands of 3/4 length of the monomer (Fig. 3.3A, lane 3), corresponding to the two α(IV) chains of the 300 nm long fragment (Fessier et al., 1984). Incubation of preparation A with GLA at 37°C led to complete degradation of the monomer (Fig. 3.3B, lane 3). Both 30°C and 37°C incubations with GLA resulted in a
clearance of protein from the stacking gel, indicating enzymatic degradation of the cross-linked multimers.

Surprisingly, preparation B was not susceptible to GLA action. SDS-PAGE analysis showed that GLA did not cleave preparation B at 30°C (data not shown), nor did it cleave at 37°C (Fig. 3.4). The molar ratio of GLA to preparation B was raised to 1:2, however even at this high ratio GLA failed to cleave preparation B (data not shown). Differences between preparations A and B were highlighted by electrophoresis under non-reducing conditions. The α1(IV) and α2(IV) chains of untreated preparation A entered the separating gel under non-reducing conditions (Fig. 3.5, lane 1), however, in contrast with preparation A, unincubated preparation B did not enter the separating gel (Fig. 3.5, lane 5). Incubation of preparation A at 30°C with GLA or GLB resulted in the generation of several lower molecular mass fragments (Fig. 3.5, lanes 3 and 4), and increasing the incubation temperature to 37°C caused a smearing of fragments upon separation by non-reducing SDS-PAGE (Fig. 3.6, lanes 3 and 4). Treatment of preparation B with GLA or GLB at 30°C failed to fragment the collagen (Fig. 3.5, lanes 7 and 8), however several fragments entered the separating gel when preparation B was treated with GLA or GLB at 37°C (Fig. 3.6, lanes 7 and 8). These data, and the data from the reducing SDS-PAGE (Figs. 3.3 and 3.4), indicated that despite apparently identical extraction conditions, there was a fundamental difference in the levels of disulphide bonding between preparation A and preparation B.

Experiments carried out in Prof. K. Kühn's laboratory confirmed the differences between the two preparations. Preparation B was treated with a reducing agent (DTT) and subjected to SDS-PAGE. The resulting profile on the gel was similar to preparation A. Likewise, treatment of preparation A with an oxidising agent (oxidised glutathione) led to an SDS-PAGE profile similar to preparation B (Eble et al., 1996). It was concluded that during some extractions the interchain disulphide bonds either remained intact, or reformed during purification, resulting in a preparation 'B'.
The commercial extraction of EHS type IV collagen is based on the same procedures used by Prof. Kühn's laboratory (Kleinmann et al., 1982). Preparations of type IV collagen from Sigma Chemical Company, and from Collaborative Biomedical Products (Becton Dickinson), were cleaved with GLA, and the fragments assessed by reducing and non-reducing SDS-PAGE. Sigma collagen IV was particularly susceptible to MMP action. Treatment with gelatinases at 30°C resulted in complete degradation of the α(IV) chains, as evidenced by reducing SDS-PAGE (Fig. 3.7). Collagen IV purchased from Collaborative Biomedical was not cleaved by gelatinases at 30°C (data not shown), but the α2(IV) chain was cleaved at 37°C by both GLA and GLB (Fig. 3.8). Clearly, the extent of intramolecular disulphide bonding also varied between commercial preparations.

3.3.2 Cell attachment to collagen IV monomers

Conditions for the attachment assay: Cell lines were chosen for attachment assays on the basis of integrin expression. The HT1080 human fibrosarcoma cell line expresses α2β1 integrin, but not α1β1 (Petermann et al., 1993) and binds to type IV collagen via α2β1 integrin (Vandenberg et al., 1991). This was confirmed in our laboratory by using an anti-α2β1 integrin antibody to inhibit cell adhesion to collagen IV (Fig. 3.9). In contrast, the mouse glioma cell line RuGLi expresses α1β1, but not α2β1 integrin (Petermann et al., 1993), and adheres to type IV collagen solely via the α1β1 integrin (Vandenberg et al., 1991). The conditions for the cell attachment assay were optimised by time course studies, and by serially diluting cell number and substrate concentrations (data not shown). Briefly, maximum attachment of cells to collagen IV occurred after 20 minutes incubation at 37°C; assays were routinely run for 35 minutes. A concentration of 6 x 10⁴ cells/well was chosen for both HT1080 and RuGLi cells, as it fell within the linear portion of the dose response. Half maximal binding of HT1080 cells was achieved at 0.1 μg/ml type IV collagen monomers, with a maximum at 1 μg/ml. Half maximal binding of RuGLi cells was at 0.1 μg/ml and maximum at 0.4 μg/ml type IV collagen monomers. A concentration of 1.0 μg/ml was chosen for the assay. Fragments of type IV collagen generated from treatment with MMPs were also plated at 1 μg/ml. The amount of intact or GLA-cleaved type IV collagen adsorbed to the plastic was determined by radiolabelling the collagen with ¹²⁵I-
iodine. Approximately 10-13% of the collagen added to the wells adsorbed to the plastic in both intact collagen IV controls as well as GLA cleaved collagen IV (data not shown). Finally, to control for MMPs and TIMP-1 present in cleaved collagen IV preparations, identical concentrations of MMPs and TIMP-1 were tested alone for adhesion promoting activity. Neither MMPs nor TIMP-1 promoted HT1080 or RuGLi cell adhesion (data not shown).

Type IV collagen preparation A was pre-incubated with GLA, and cell attachment to the GLA treated collagen compared with attachment to intact monomers. When collagen IV was pre-incubated with GLA at 30°C, neither HT1080 nor RuGLi cell adhesion to the collagen was affected (Fig. 3.10). When the temperature of the pre-incubation was raised to 37°C, both RuGLi and HT1080 cell attachment to GLA-cleaved-preparation A was severely affected (Fig. 3.10). RuGlLi cell adhesion was commonly reduced to 40-60% of the control (preparation A incubated in the absence of GLA), whereas HT1080 cell adhesion to preparation A pre-treated with GLA at 37°C was 0-10% of the control. The data suggested that the α2β1 integrin binding sites were degraded by GLA treatment at 37°C, but that some α1β1 integrin binding was still possible.

In contrast to the preparation A results, treatment of preparation B with GLA at 37°C did not affect HT1080 or RuGLi cell adhesion (Fig. 3.10). Cell adhesion to GLA treated preparation B could be inhibited by the addition of an anti-β1 integrin subunit antibody (mAb 13, added at 5 μg/ml; data not shown). The combined data indicated that the integrin binding sites on preparation B remained intact following treatment with GLA, a result consistent with the lack of fragmentation observed following GLA treatment (Fig. 3.4).

3.3.3 GLA treatment of collagen IV dimers and cell attachment to the GLA treated dimers

Dimeric collagen IV was incubated with GLA for 20 hours at 37°C. Analysis of the cleavage reaction mixture by reducing SDS-PAGE showed that the dimers were not
degraded by GLA (Fig. 3.11). However, when the dimers were stored at 4°C for periods longer than three weeks, they became particularly susceptible to GLA action, and some degradation was observed upon incubating the dimers alone (data not shown).

Freshly dialysed dimeric collagen IV was pre-incubated with GLA, and cell attachment to the GLA treated collagen compared with attachment to intact dimers. Figure 3.12 demonstrates that HT1080 and RuGLi cell attachment to collagen IV dimers was minimally affected by GLA pre-treatment of the collagen, indicating that \( \alpha 1 \beta 1 \) and \( \alpha 2 \beta 1 \) integrin binding sites were left mainly intact.

3.3.5 MMP cleavage of collagen IV tetramers and cell attachment to the cleaved tetramers

Tetrameric collagen IV migrated as several bands on SDS-PAGE, with apparent molecular mass between 130 and 190 kDa, as has been reported by others (Mackay et al., 1990). Incubation of tetramers with GLA at 30°C for 20 hours resulted in minor fragmentation (data not shown). Incubation at 37°C, however, induced marked degradation by GLA (Fig. 3.13). Furthermore, SL-1 and CL-3 also degraded the tetramers, and patterns were significantly different for each enzyme (Fig. 3.13). Surprisingly, despite the degradation of the tetramers induced by the MMPs, neither HT1080 nor RuGLi cell attachment was affected (Fig. 3.14).
3.4 Discussion

Many studies have shown that the expression of the gelatinases is up-regulated in tumour tissues, and that the appearance of active gelatinases is closely correlated with tumour metastasis (reviewed by Sato and Seiki, 1996). These enzymes are referred to as 'type IV collagenases', and it has been hypothesised that they aid cellular invasion by proteolytic dissolution of the basement membrane, allowing movement of the cell through the digested barrier (Stetler-Stevenson et al., 1993). However, clear evidence that the basement membrane is breached by invading cells has been difficult to obtain. It is possible that the action of the gelatinases is more subtle, and may involve destruction of points of contact between the cell and the substratum, allowing the migrating cell to move on.

Cleavage of collagen IV into two fragments by a gelatinase was first demonstrated by Salo et al. (1983) and Fessler et al. (1984). Similar patterns of type IV collagen fragmentation were reported by others, using gelatinases A and B purified from varied sources (Collier et al., 1988; Murphy et al., 1989a; Wilhelm et al., 1989). We speculated that the close proximity of the gelatinase cleavage site may be used by the invading tumour cell to penetrate basement membranes. Cells attached to basement membrane collagen IV via α1β1 and α2β1 integrins may secrete gelatinases that cleave the collagen IV network and degrade the integrin binding sites. This could then lead to cell detachment and penetration of the cell through the basement membrane at the site of proteolysis.

It was of concern to us that there are divergent reports concerning the ability of gelatinases to degrade type IV collagen. Several reports show little degradation of collagen IV by the gelatinases (Murphy et al., 1982; Rantala-Ryhanen et al., 1983; Hibbs et al., 1987; Mackay et al., 1990). Direct comparison of these studies with those reporting defined fragmentation is difficult, as studies were carried out using gelatinases purified, or partially purified, from a range of cell types. It was clear to us that the incubation conditions for GLA cleavage studies may be critical. Gelatinase activity on collagen IV appeared to be temperature-sensitive, ranging from little or no degradation at 30°C, to complete
degradation at 37°C (Murphy et al., 1991a; Okada et al., 1992). Indeed, Mackay et al.
(1990) were unable to reproduce the 1/4, 3/4 fragmentation of EHS collagen IV with GLA
or GLB at 25, 30 or 37°C, and suggested that the gelatinases were in fact not true type IV
collagenases.

Therefore, to begin this study, the action of GLA on collagen IV was reassessed using a
preparation of collagen IV monomers derived from the EHS tumour. This preparation was
termed 'preparation A' (in retrospect) to distinguish it from other preparations. At 30°C
GLA cleaved preparation A into the characteristic 1/4 and 3/4 fragments, as reported by
others (Salo et al., 1983; Fessler et al., 1984; Collier et al., 1988; Murphy et al., 1989a;
Wilhelm et al., 1989). At 37°C, preparation A was degraded by GLA action, resulting in a
number of low molecular mass fragments. In contrast, a second preparation of monomers
termed 'preparation B' was not susceptible to GLA action at 30°C. Even at 37°C, which is
close to the triple helical denaturation temperature for collagen IV (38°C; Kühn, 1994),
preparation B was resistant to GLA attack. In Prof. Kühn's group, treatment of preparation
B with GLA at 37°C did result in some cleavage N-terminal to the CB3[IV] region, as well
as degradation of the monomers from the C-terminal end (Eble et al., 1996). However in
our hands preparation B proved to be repeatedly resistant to GLA attack. The reason for
the differences between the studies is not known. Comparing preparation A and
preparation B by non-reducing SDS-PAGE confirmed that despite identical purification
procedures, preparation B retained a greater level of intramolecular disulphide bonding.
As the CB3[IV] region is the only segment within the triple-helical domain that is
stabilised by intramolecular disulphide bonds (Kühn, 1994), it was apparent that the
CB3[IV] region of preparation B was more like the native molecule.

To assess the effect of GLA action on integrin binding to collagen IV, cell attachment to
intact monomers was compared with attachment to cleaved monomers. Cleavage of
preparation A with GLA at 30°C to the 1/4 and 3/4 fragments did not affect HT1080 or
RuGLi cell adhesion, indicating that the α1β1 and α2β1 integrin binding sites remained
intact. However, fragmentation of preparation A with GLA at 37°C severely affected cell
adhesion to the monomers, with greater effect on α2β1 integrin interactions than α1β1 integrin interactions. This data was confirmed in Prof. Kühn's group, using isolated α2β1 and α1β1 integrins to study integrin binding to GLA cleaved preparation A (Eble et al., 1996). The reduced α2β1 integrin binding most likely reflects a loss of the N-terminal α2β1 integrin binding site (Fig. 3.1), which is close to the initial GLA cleavage site. The C-terminal α2β1 integrin binding site is of much lower affinity (Kern et al., 1993) and may be protected from proteolytic attack by the nearby disulphide stabilised region. Treatment of preparation B with GLA at 37°C did not affect cell adhesion to the molecule, or adhesion of isolated integrins (Eble et al., 1996), a result predicted due to the greater resistance of this preparation to GLA attack.

Attempts to determine the gelatinase cleavage site in preparation A did not generate identifiable sequences (Eble et al., 1996). The cleavage sites of GLA within denatured CB3[IV] and the triple helical segment of CB3[IV] (fragment F1) were mapped by Prof. Kühn's group. In denatured CB3[IV], sequences Gly-X-Y were cleaved between Gly and X, if position X was occupied by a hydrophobic or polar charged residue (Fig. 3.15). Hydroxyproline in position Y prevented cleavage. In the triple-helical fragment F1, GLA cleavage only occurred where the triple-helical structure appeared to be relaxed. No cleavage occurred in the region between the two disulphide knots (Fig. 3.15). Binding of α1β1 integrin to fragment F1 was unaltered by GLA treatment, whereas binding of α2β1 integrin was reduced slightly, due to the loss of the N-terminal α2β1 integrin binding site (Eble et al., 1996).

Several commercial preparations of EHS-derived collagen IV monomers were compared with preparations A and B. From non-reducing SDS-PAGE, it was apparent that the commercial preparations varied in the extent of disulphide bonding. GLA incubation studies showed that the preparations which resembled the native molecule (with interchain disulphide bridging intact) required a higher temperature for cleavage to occur. It is likely therefore, that the choice of commercial preparation of collagen IV monomers will have significant bearing on experimental results, if gelatinase degradation of the collagen is a
requirement of the experiment. For future studies, it would be interesting to compare preparations of Matrigel (the commercial preparation of basement membrane from the EHS tumour) to see whether variations in the level of collagen IV intramolecular disulphide bonding occur between preparations. One would predict that cells expressing active GLA would show an altered rate of invasion through preparations of Matrigel containing mostly reduced collagen IV. This may not necessarily reflect the in vivo situation.

Collagen IV preparations extracted by several methods were compared in this study. Dimeric collagen IV was purified from human placenta by treatment of the tissue with bacterial collagenase at 25°C. Bacterial collagenase preferentially cleaves the collagen IV network at an N-terminal site, close to the CB3[IV] region (Timpl et al., 1981). An attempt was made by Prof. Kühn's group to obtain an N-terminal sequence for bacterial collagenase-cleaved dimers, however a unique sequence could not be identified. Other studies with preparation A showed that soluble collagen IV was cleaved by bacterial collagenase between residues Pro-393 and Gly-394 (α1(IV)) and between Pro-390 and Gly-391 (α2(IV); Eble et al., 1996), 20 residues away from the first α2β1 integrin recognition site (Fig. 3.15). Dimers in solution were susceptible to further attack by bacterial collagenase, particularly at 37°C (A. Ries and Prof. K. Kühn, personal communication). However, studies here showed that GLA, in contrast to bacterial collagenase, was unable cleave the dimers, even at 37°C. At this temperature the non-triple helical interruptions in the vicinity of CB3[IV] should be relaxed. Lack of GLA action in comparison with bacterial collagenase may reflect the different specificities of the two enzymes. Bacterial collagenase will attack all Pro-Gly and Hyp-Gly bonds in the relaxed regions, thus bacterial collagenase may cleave at many more sites in the relaxed regions than GLA (Eble et al., 1996).

Cell attachment sites in the CB3[IV] region of the dimers were unaffected by GLA treatment, as evidenced by HT1080 and RuGLI cell adhesion studies. Additionally, studies with isolated α1β1 and α2β1 integrins showed that GLA treatment did not affect integrin
binding to collagen IV (Eble et al., 1996), supporting the cell adhesion results. It is likely that intramolecular disulphide bonds within the CB3[IV] region protected this region from GLA attack and that the C-terminal ends of the dimers were protected by the interactions between adjacent NCI domains.

In contrast to collagen IV dimers, tetramers prepared by pepsin extraction of human placenta were particularly susceptible to MMP action. Mackay et al. (1990) also reported gelatinase-induced fragmentation of pepsin-extracted type IV collagen, and noted that tetramers were more susceptible to gelatinase action than monomers. In the current study, despite extensive fragmentation of tetramers by MMPs at 37°C, the attachment of HT1080 cells and RuGLi cells was not affected. This indicates that the CB3[IV] region remained intact, and as the N-terminal end is sealed by interactions in the 7S domain, it can be assumed that fragmentation occurred from the C-terminal end.

Several MMPs were used in the studies with tetramers, which highlighted the different cleavage specificities of the MMPs, and confirmed that enzymes other than the gelatinases are capable of cleaving collagen IV. SL-1 is a potent type IV collagen-degrading enzyme (Collier et al., 1988). In addition to its action on the tetramers, SL-1 also cleaved preparation B monomers at 37°C, fragmenting the α2(IV) chain (data not shown). Okada et al. (1990) also reported cleavage of EHS-derived, monomeric α2(IV) chain with SL-1 (at 32°C); interestingly, GLA failed to cleave the collagen under the same conditions. Studies in our laboratory with CL-3 have shown that it is more efficient at cleaving type IV collagen preparation B than the gelatinases (Knäuper et al., 1996a). In combination, these observations question the validity of referring to the gelatinases as 'type IV collagenases', in agreement with Mackay et al (1990).

It is an unfortunate consequence of these studies that the methods used to extract soluble collagen IV from the basement membrane network compromised the structural integrity of the molecule. For future studies, it would be interesting to compare the extracted and solubilised collagen IV preparations used here with soluble collagen IV expressed by PF-
HR9 mouse teratocarcinoma cells. These cells secrete collagen IV monomers into the culture medium, and can be induced to secrete disulphide-linked collagen IV tetramers by culturing the cells in medium devoid of cysteine (Fessler et al., 1984). Monomers and tetramers can be purified from PF-HR9 conditioned medium using DEAE-chromatography and velocity sedimentation. Interestingly, 1/4 and 3/4 collagen IV fragments were obtained from digestion of these monomers and tetramers with a 'type IV collagenase' isolated from the culture media of mouse metastatic PMT sarcoma (Fessler et al., 1984). As the type IV collagenase in these studies was only partially purified, this raises the possibility that several MMPs may act in concert to cleave collagen IV. Thus future studies designed to compare extracted collagen IV with soluble secreted collagen IV should also investigate the degradative action of combinations of MMPs.

In summary, this chapter was designed to test the hypothesis that cell attachment to type IV collagen (via $\alpha_1\beta_1$ and $\alpha_2\beta_2$ integrins) is impaired by the prior action of gelatinases on the collagen. The data collected from studies with solubilised collagen IV suggests that the integrin recognition sites are resistant to degradation by the gelatinases. However, it is an open question whether the recognition sites are accessible to proteolytic attack when incorporated into the macromolecular network, and whether several proteases act in concert on the collagen IV network. The discovery that the CB3[IV] region is particularly resistant to MMP degradation raises the intriguing possibility that $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrin receptors on migrating cells may remain occupied by protease resistant recognition sites, even when the surrounding collagen IV network is largely degraded. This may prevent the cell from interacting with intact molecules of the macromolecular membrane and may in turn modify the migration of the cell throughout the basement membrane.
Figure 3.1  Amino acid sequence of the CB3[IV] region of human collagen IV
(adapted from Eble et al., 1996)

The triple helical area of the cyanogen bromide derived fragment CB3[IV] extends from position 408-571. The N-terminal protruding ends of the α1(IV) chains (positions 291-407) of CB3[IV] are non-triple-helical. The cysteine residues involved in intramolecular disulphide bonds are located in two non-triple-helical areas numbered I (positions 449-456) and II (positions 486-496). The two areas containing the N- and C-terminal recognition sites of α2β1 integrin, as well as the essential residues of the α1β1 integrin recognition site are shaded: Dots over P and K in Y positions indicate hydroxyproline and hydroxylysine, respectively. Position numbers of the aligned α1(IV) and α2(IV) chains do not coincide with the residue numbers of the single chains (Brazel et al., 1988).
\[ \begin{align*}
\alpha_1 & \ 341 \quad KG \text{EA} \text{GP} \text{PG} \text{PP} \text{GIVGT} \quad \text{GPLGE} \text{K} \text{GERGY} \text{P} \text{GTPGPRGE} \text{PGPKGFPGLPGQPGPP} \quad \text{GLPVPGQ} \\
\alpha_2 & \ 341 \quad \text{GPPGLS} \quad \text{IGDGDQ}_{R} \\
\alpha_1 & \ 401 \quad AGAPGPFP \text{GER} \text{GEK} \text{GDRGFP} \text{GTSLP} \quad \text{GPSGRD} \quad \text{GLPG} \text{PPGSPGPQPGQPGQGYT} \quad \text{NGIVECQP} \quad \text{GPPG} \\
\alpha_2 & \ 401 \quad \text{RGLPGEMGP} \text{KG} \text{F} \text{I} \quad \text{GDPGP} \text{P} \text{ALYG} \quad \text{GPPGPD} \quad \text{GKRGPPGPPGLPFPGPD} \\
\alpha_1 & \ 461 \quad \text{S} \text{C} \text{L} \text{ICD} \text{IDGYR} \quad \text{GPPPGPQPGBE} \text{IGFPQGPQGAK} \text{GDR} \\
\alpha_2 & \ 461 \quad \text{RARFPGLPGSPGARGPKGWKGDAGE} \quad \text{CRCTEGDEAIK} \quad \text{GLPG} \text{GLPGKFGAGPNGRKGDK} \\
\alpha_1 & \ 521 \quad \text{GLPGRD} \quad \text{GVAGVPGPQGTPLILGQPGAKEGPEGF} \quad \text{YFDLRLK} \quad \text{GDKGDPPFGPQPQPGM} \\
\alpha_2 & \ 521 \quad \text{GDPGQH} \quad \text{GLPGFPGLKGPQPGNIGAPGPKGAKGDSS} \quad \text{RTITTKGER} \quad \text{GQPGPVPGVPGM} \\
\end{align*} \]

\text{non triple helical interruption}
Figure 3.2 Schematic representation of the collagen IV preparations used in this study (adapted from Eble et al., 1996)

Collagen IV monomers were isolated under reducing conditions from the murine EHS tumour. In preparation A the intramolecular disulphide bonds of the CB3[IV] region and the N-terminal telopeptides (7S domain) are split by reduction, whereas the interchain disulphide bonds in the NC1 domains remain intact. In preparation B the three α(IV) chains are connected by disulphide bonds in the NC1 domains, the CB3[IV] region, and possibly also in the area of the telopeptides.

Collagen IV dimers were isolated from human placenta after treatment with bacterial collagenase, which removed the 100-nm-long N-terminal region of the triple helical domain. The intramolecular disulphide bonds of the CB3[IV] region are intact and the C-terminal end of the triple-helical domain is sealed by the hexameric NC1 complex.

Tetrameric collagen IV was isolated from human placenta by treatment with porcine pepsin, which removed the NC1 globular domain of each molecule. Tetramer formation is a result of interactions between the four molecules in the area of the telopeptides.

N-terminal telopeptides and the CB3[IV] region with intact intramolecular disulphide bonds are shaded.
Murine collagen IV monomers:

Preparation A

7S  CB3

\[ \text{NC1} \]

\[ \alpha_1 \]

\[ \alpha_2 \]

Preparation B

7S  CB3

\[ \alpha_1 \]

\[ \alpha_2 \]

\[ \alpha_3 \]

Human collagen IV dimer:

CB3

[NC1]

bacterial collagenase

Human collagen IV tetramer:

\[ \text{pepsin} \]

\[ \text{pepsin} \]
Figure 3.3 Preparation A collagen IV monomers treated with GLA at 30°C and 37°C

Monomers were incubated with or without added GLA for 20 hours at 30°C or 37°C. Cleavage products were separated by SDS-PAGE under reducing conditions, and analysed by silver stain.

A. Preparation A incubated at 30°C, followed by 6% SDS-PAGE. Monomers incubated alone (lane 2), or in the presence of GLA (lane 3). Control monomers, unincubated (lane 1). The $\alpha_1$(IV) and $\alpha_2$(IV) chains are marked with black arrows. The blue arrows indicate the 3/4 and 1/4 fragments generated by GLA cleavage (lane 3).

B. Preparation A incubated at 37°C, followed by 8% SDS-PAGE. Monomers incubated alone (lane 2), or in the presence of GLA (lane 3). Control monomers, unincubated (lane 1). The arrowheads indicate the boundary between the stacking gel and the separating gel. The migration positions of type I collagen $\beta$ and $\alpha$ chains and molecular mass markers are indicated on the right.
Figure 3.4 Preparation B collagen IV monomers treated with GLA at 37°C

Monomers were incubated with or without added GLA for 20 hours at 37°C. Cleavage products were separated by 8% SDS-PAGE under reducing conditions, and analysed by silver stain. Preparation B incubated alone (lane 3), or in the presence of GLA (lane 4). Unincubated preparation B (lane 2), unincubated preparation A (lane 1). The migration positions of type I collagen β and α chains and molecular mass markers are indicated on the right.
Figure 3.5 Collagen IV monomers (preparations A and B) treated with gelatinases at 30°C. Separation of fragments by non-reducing SDS-PAGE

Monomers were incubated with or without added GLA or GLB for 20 hours at 30°C. Cleavage products were separated by 6% SDS-PAGE under non-reducing conditions, and analysed by silver stain.

Preparation A incubated alone (lane 2); or in the presence of GLA (lane 3), or GLB (lane 4). Preparation A, unincubated (lane 1). Preparation B incubated alone (lane 6); or in the presence of GLA (lane 7), or GLB (lane 8). Preparation B, unincubated (lane 5).

The arrowhead indicates the boundary between the stacking gel and the separating gel. The migration positions of type I collagen β and α chains and molecular mass markers are indicated on the right.
Figure 3.6 Collagen IV monomers (preparations A and B) treated with gelatinases at 37°C. Separation of fragments by non-reducing SDS-PAGE

Monomers were incubated with or without added GLA or GLB for 20 hours at 37°C. Cleavage products were separated by 6% SDS-PAGE under non-reducing conditions, and analysed by silver stain. Preparation A incubated alone (lane 2); or in the presence of GLA (lane 3), or GLB (lane 4). Preparation A, unincubated (lane 1). Preparation B incubated alone (lane 6); or in the presence of GLA (lane 7), or GLB (lane 8). Preparation B, unincubated (lane 5).

The arrowhead indicates the boundary between the stacking gel and the separating gel. The migration positions of type I collagen β and α chains and molecular mass markers are indicated on the right.
Figure 3.7 Treatment of collagen IV monomers purchased from Sigma with gelatinases at 30°C
Monomers were incubated with or without GLA or GLB for 20 hours at 30°C. Cleavage products were separated by 6% SDS-PAGE under reducing conditions, and analysed by silver stain. Monomers incubated alone (lane 2); or in the presence of GLA (lane 3), or GLB (lane 4). Unincubated monomers (lane 1).
The migration positions of type I collagen $\beta$ and $\alpha$ chains and molecular mass markers are indicated on the right.
Figure 3.8 Treatment of collagen IV monomers purchased from Collaborative Biomedical Products with gelatinases at 37°C

Monomers were incubated with or without GLA or GLB for 20 hours at 37°C. Cleavage products were separated by 6% SDS-PAGE under reducing conditions, and analysed by silver stain. Monomers incubated alone (lane 2); or in the presence of GLA (lane 3), or GLB (lane 4). Unincubated monomers (lane 1).

The migration positions of type I collagen β and α chains and molecular mass markers are indicated on the right.
Figure 3.9 HT1080 adhesion to collagen IV is α2β1 integrin mediated

HT1080 cells were incubated for 30 minutes alone, or in the presence of 10 µg/ml mouse anti-α2β1 integrin antibody Gi9. Cells were then seeded onto a substrate of collagen IV dimers and incubated at 37°C for 35 minutes. Adherent cells were stained with methylene blue and the absorbance of the wells was read at 630 nm. Values are the mean +/- standard deviation of triplicate wells.
Figure 3.10 Attachment of HT1080 and RuGLi cells to immobilised preparation A and preparation B monomers after treatment with GLA at 30°C and 37°C

Collagen IV monomers were incubated with or without GLA for 20 hours at 30°C or 37°C. Culture plates were coated with collagen IV overnight at 4°C. Cells were allowed to attach to collagen IV substrates for 35 minutes at 37°C. Adherent cells were stained with methylene blue and the absorbance of the wells read at 630 nm. Attachment of cells is expressed as a percentage of the control (monomers incubated in the absence of GLA). HT1080 cells are represented by the grey bars and RuGLi cells by the black bars. Values are the mean +/- standard deviation for a representative experiment.
Figure 3.11 Collagen IV dimers treated with GLA at 37°C

Dimers were incubated with or without added GLA for 20 hours at 37°C. Cleavage products were separated by SDS-PAGE under reducing conditions, and analysed by silver stain. Dimers incubated alone (lane 3), or in the presence of GLA (lane 4). Control dimers, unincubated (lane 2) and monomers (preparation A, lane 1). The migration positions of type I collagen β and α chains and molecular mass markers are indicated on the right.
Figure 3.12 Attachment of HT1080 and RuGLi cells to immobilised collagen IV dimers after treatment with GLA at 37°C

Collagen IV dimers were incubated with or without GLA for 20 hours at 37°C. Culture plates were coated with collagen IV dimers overnight at 4°C. Cells were allowed to attach to the dimers for 35 minutes at 37°C. Adherent cells were stained with methylene blue and the absorbance of the wells read at 630 nm. Attachment of cells is expressed as a percentage of the control (dimers incubated in the absence of GLA). HT1080 cells are represented by the grey bars and RuGLi cells by the black bars. Values are the mean +/- standard deviation for a representative experiment.
Figure 3.13 Collagen IV tetramers treated with MMPs at 37°C

Tetramers were incubated with or without added GLA, SL-1 or CL-3 for 20 hours at 37°C. Cleavage products were separated by 6% SDS-PAGE under reducing conditions, and analysed by silver stain. Tetramers incubated alone (lane 2,3); or in the presence of GLA (lane 4,5), SL-1 (lane 6,7), or CL-3 (lane 8,9). Control tetramers, unincubated (lane 1,2). The migration positions of type I collagen β and α chains and molecular mass markers are indicated on the right.
Figure 3.14 Attachment of HT1080 and RuGLi cells to immobilised collagen IV tetramers after treatment with MMPs at 37°C

Collagen IV tetramers were incubated with or without GLA, SL-1, CL-3 for 20 hours at 37°C. Culture plates were coated with collagen IV tetramers overnight at 4°C. Cells were allowed to attach to collagen IV tetramer substrates for 35 minutes at 37°C. Adherent cells were stained with methylene blue and the absorbance of the wells read at 630 nm. Attachment of cells is expressed as a percentage of the control (tetramers incubated in the absence of MMPs). HT1080 cells are represented by the grey bars and RuGLi cells by the black bars. Values are the mean +/- standard deviation for a representative experiment.
Figure 3.15 Gelatinase A cleavage sites within the CB3[IV] region of human collagen IV  
(adapted from Eble et al., 1996)

Many of the features of this figure were described in the figure legend for Fig. 3.1. Shown below is additional information regarding gelatinase A cleavage sites in the triple helical region of collagen IV.

The largest trypsin derived fragment of CB3[IV], F1, comprises almost the entire triple helical region (positions 408-533) of the CB3[IV], depicted by horizontal arrows. Arrows with blue heads designate cleavage sites of gelatinase A within the denatured α(IV) chains. Arrows with black heads designate peptide bonds cleaved by gelatinase A within the triple-helical F1 fragment. Arrowheads show the cleavage sites of bacterial collagenases determined after incubation of murine preparation A with purified collagenase matched to the human sequences. Integrin recognition sites are shaded.
Chapter 4

Extracellular matrix regulation of gelatinase A activation
Chapter 4 Extracellular matrix regulation of gelatinase A activation

4.1 Introduction

Despite evidence to suggest that gelatinases are important in processes of cell migration and invasion, the precise role of these enzymes has yet to be defined. It may be that these enzymes destroy existing adhesion sites (as discussed in Chapter 3) or expose cryptic adhesion sites, or promote the release of matrix fragments, matrix-bound growth factors and cytokines (reviewed by Werb, 1997). GLA may be particularly important in pericellular degradation events, by virtue of its activation at the cell surface.

Processes governing the cell surface activation of proGLA have been a focus of studies in our group. Most of these studies have required the use of agents such as concanavalin A or phorbol esters to induce activation. There is little known of the factors that influence GLA activation in vivo, however, there is increasing evidence to suggest that signals from the ECM may influence both GLA expression and activation. Increases in GLA activation have been reported following culture of several cell types on or within collagen type I gels (Azzam and Thompson, 1992; Gilles et al., 1997; Haas et al., 1998). The increased GLA activation in collagen gels probably reflects the level of mechanical stress, as cells in stressed collagen lattices show a marked reduction in GLA activation in comparison with mechanically relaxed lattices (Tomasek et al., 1997). These authors showed that activation of GLA may be linked to changes in the actin cytoskeleton, for the treatment of fibroblasts with cytochalasin D, (which disrupts stress fibres) caused an increase in GLA activation. Other matrix components also affect GLA activation. Ligation of the vitronectin receptor is reported to stimulate GLA expression by melanoma cells and to stimulate invasion of these cells through Matrigel (Seftor et al., 1992). Recently, soluble vitronectin has been shown to stimulate increases in GLA and TIMP-2 secretion by B16-F1 and B16-F10 melanoma cells (Bafetti et al., 1998).

The aim of this thesis was to further investigate ECM molecules that regulate the cellular activation of GLA. The fibrosarcoma cell line HT1080 was chosen as a model, as this cell line was used previously for studies of collagen I induced activation of GLA (Azzam and
In this chapter the ECM molecules fibronectin and laminin-1 were compared in GLA activation studies. The data collected showed that culture of HT1080 cells on fibronectin up-regulated processing of proGLA to the active form. Furthermore, activation was induced by fragments of fibronectin encompassing the RGD integrin binding site. In marked contrast, culture of HT1080 cells on laminin-1 did not promote GLA processing. Anti-integrin antibodies were used in this study to determine whether integrin receptors binding fibronectin can elicit signals leading to proGLA activation.

4.2 Results

4.2.1 A comparison of the effects of fibronectin and laminin-1 substrates on HT1080 expression and activation of gelatinases

HT1080 cells cultured on plastic constitutively express GLA and GLB as detected by gelatin zymography (Fig. 4.1). Cells cultured on laminin-1 expressed mainly latent, proGLA, molecular mass 66 kDa (Fig. 4.1). In contrast, cells cultured on fibronectin displayed an apparent increase in processing of proGLA from the 66 kDa latent form, via an intermediate at 62 kDa, to the fully active 59 kDa protein. This increase in GLA processing induced by fibronectin was similar to the levels of processing induced by PMA (Fig. 4.1), a known stimulator of GLA activation for these cells (Foda et al., 1996; Lohi et al., 1996). By phase contrast microscopy, it was apparent that GLA activation was not linked to changes in cell shape, for the cells spread well on fibronectin and laminin-1, but rounded up when treated with PMA.

To confirm the increases in GLA processing observed using zymography, secreted GLA was biosynthetically labelled with [35S]-methionine. Labelled GLA was then immunoprecipitated from the conditioned medium and the precipitates analysed by SDS-PAGE and fluorography. Time course studies indicated that levels of fully processed GLA were highest after 28 hours in culture (Fig. 4.2). Several attempts were made to compare GLA processing by HT1080 cells cultured on fibronectin or laminin-1 using this technique. However the levels of GLA synthesised proved to be at the limit of detection. Although processing of GLA appeared greater when cells were cultured on fibronectin in
comparison with laminin-1, the quality of the data was poor (not shown) and so zymography was used routinely to monitor GLA processing.

The purification of fibronectin from plasma involves gelatin-Sepharose chromatography, which results in the copurification of plasma gelatinases. Analysis of the fibronectin preparation by zymogram (developed overnight at 37°C) showed low levels of contaminating gelatinases (data not shown). This raised the possibility that the increase in GLA processing observed when cells were cultured on fibronectin was due to the addition of small amounts of exogenous active GLA, associated with the fibronectin substrate. A fibronectin preparation with the contaminating gelatinases removed was kindly provided by A. Messent (Strangeways Research Laboratory, Cambridge, UK) who used a zinc iminodiacetic acid Sepharose column to separate out the gelatinases (d'Ortho et al., 1997). This purified fibronectin also promoted increases in GLA processing, with levels of active GLA apparently identical to that induced by unpurified fibronectin (data not shown).

GLB secretion was up-regulated by PMA treatment (Fig. 4.1), however, neither fibronectin nor laminin-1 substrates affected GLB expression. Activated GLB was not detected in this system; activation of GLB is known to proceed via different mechanisms to GLA (Murphy et al., 1992c).

When laminin-1 and fibronectin were added in solution to HT1080 cells cultured on plastic, no increases in GLA processing were noted in comparison with the plastic control (Fig. 4.3). Reich et al. (1995) reported that soluble laminin added to HT1080 cultures for 6 hours increased GLA mRNA and protein expression, but no comment was made as to whether GLA activation was affected. In the current study, soluble laminin-1 added to cultures of HT1080 cells for 24 or 48 hours did not appear to alter GLA protein expression, or GLA activation.

Studies have shown that at high concentrations GLA self-activates, in a process that is concentration dependent and is enhanced by the presence of heparin (Crabbe et al., 1993).
One way in which immobilised fibronectin might induce GLA processing is by binding GLA, thereby increasing pericellular concentration of the enzyme. Solid phase studies have demonstrated the binding of a recombinant C-terminal domain fragment of GLA to fibronectin (Wallon and Overall, 1997). In the current study, the binding of full length pro- and active GLA to fibronectin was measured using the ELISA method of Allan et al. (1995). GLA bound to collagen type I as described (Allan et al., 1995), but binding of pro- or active GLA to fibronectin was not significant (Fig. 4.4). Therefore, it was considered unlikely that pericellular fibronectin locates GLA to the cell surface.

4.2.2 Fibronectin fragments from the CCBD promote proGLA activation by HT1080 cells

To identify whether specific regions of fibronectin have the potential to affect GLA activation, defined peptide fragments of fibronectin were coated to culture plastic. HT1080 cells were cultured on the 120 kDa fragment comprising the fibronectin type III repeats 2-11 (Fn III 2-11) of the central cell binding domain (CCBD; Fig. 4.5). This fragment supported HT1080 cell adhesion and spreading. Analysis of the conditioned media by zymography showed that GLA was processed to the fully active form, in contrast to cells cultured on laminin-1 (Fig. 4.6). A second fragment of 110 kDa, which lacked the alternatively spliced ED-B domain (Fig. 4.5) also induced GLA activation (data not shown), indicating that regions in the CCBD other than the ED-B domain effect changes in GLA activation.

It is interesting to note that the effect of the 120 kDa fragment of fibronectin on GLA activation was equivalent to that observed for full length fibronectin (Fig. 4.6). Werb et al. (1989) reported that rabbit synovial fibroblasts respond to the 120 kDa fragment by up-regulating the synthesis of CL-1 SL-1 and GLB, but do not respond to full length fibronectin. A later study by the same group (Huhtala et al., 1995) demonstrated that the reason full length fibronectin did not signal MMP synthesis was due to additional interactions between α4β1 integrins and the alternatively spliced CS-1 region of fibronectin (Fig. 4.5). They showed that α4β1 integrin/CS-1 interactions signalled a
down-regulation in MMP expression, opposing the signals generated in response to α5β1 integrin/CCBD binding (Huhtala et al., 1995). In view of this report, it was considered possible that HT1080 cells were not receiving information transduced by α4β1 integrin/CS-1 interactions. FACS analysis of HT1080 cells with an anti-α4 integrin antibody (HP2/1) showed that whilst these cells expressed detectable levels of the α4 subunit (Fig. 4.7), levels were less than that expressed by the human myeloid leukaemic cell line HL60, which is considered to express relatively low levels of the α4 subunit (Hemler et al., 1987). The antibody HP2/1 has been shown to inhibit α4 subunit mediated adhesion of osteosarcoma cell lines to VCAM-1 (Mattila et al., 1992). In the current study this antibody failed to inhibit HT1080 adhesion to fibronectin (Fig. 4.8), whereas an antibody to the α5 subunit (mAb 16) completely inhibited HT1080 cell adhesion to fibronectin (Fig. 4.8). The latter observation that HT1080 adhesion to fibronectin is mediated by the α5β1 integrin is in agreement with the studies by Yamada et al. (1990). From this data it was considered unlikely that interactions between HT1080 cells and the CS-1 region of fibronectin occur.

To further identify regions in the CCBD that may be important in the processing of GLA, HT1080 cells were cultured on the fibronectin fragment Fn III 6-10 (Fig. 4.5), which comprises the RGD sequence, and subregions in Fn III 8 and 9 that act synergistically with the RGD sequence for full adhesion activity (Danen et al., 1995). Unfortunately, cell adhesion and spreading on this fragment 6-10 was poor, and the reason for this was unclear. It is possible that other regions in the 120 kDa fragment of fibronectin are vital for HT1080 cell adhesion and spreading, and also possible that Fn III 6-10 adsorption to plastic was poor. It was interesting to note, however, that despite the lack of cell adhesion and spreading, the activation of GLA by cells cultured on this fragment appeared greater than activation by cells cultured on a laminin-1 (Fig. 4.9).
4.2.3 Antibodies to the α5 and β1 integrin subunits promote proGLA activation by HT1080 cells

To assess the importance of CCBD integrin receptors in signalling GLA activation, HT1080 cells were cultured directly on a substrate of anti-integrin antibodies. As HT1080 cell adhesion to fibronectin is α5β1 mediated, monoclonal antibodies to the α5-integrin subunit (mAb 16 and mAb 11) and β1-integrin subunit (mAb 13) were chosen for this study. HT1080 cells spread well on a mAh 16 substrate, and GLA processing was up-regulated, with a processing profile comparable to cells cultured on fibronectin (Fig. 4.10). Interestingly, mAb 11 substrate failed to promote GLA activation (Fig. 4.10). Both mAb 16 and mAb 11 have been shown to stimulate integrin receptor clustering, but mAb 16 (in contrast to mAb 11) also mimics the action of the fibronectin ligand (Miyamoto et al., 1995b). Fibronectin receptor occupancy would therefore appear to be important in the signalling of GLA activation by HT1080 cells. Consistent with this hypothesis, mAb 13 to the β1 integrin subunit also promoted GLA activation (Fig. 4.10); this antibody also mimics receptor occupancy (Chen et al., 1995).

HT1080 cells adhere to laminin-1 via α6β1 integrin (von der Mark et al., 1991; confirmed in our laboratory by Dr J. Gavrilovic). An antibody to the α6 subunit (GoH3; Sonnenberg et al., 1988) supported cell adhesion and spreading, but failed to promote the processing of GLA to the levels seen when fibronectin or mAb 16 were used as a substrate (Fig. 4.10). The GLA processing profile on a GoH3 substrate was similar to that observed for a laminin-1 substrate (Fig. 4.10), suggesting that the α6 integrin subunit is unlikely to be involved in signalling GLA activation.

The combined data from the CCBD fragment study and the anti-integrin antibody study suggest that α5β1 integrin receptor occupancy signals GLA activation in HT1080 cells. It is presumed that mAb 11 failed to signal GLA activation because this antibody does not mimic the action of the fibronectin ligand. It follows that the addition of a fibronectin ligand to cells cultured on mAb 11 should restore signals to increase GLA activation. To test this hypothesis, soluble RGD peptides were added to HT1080 cells cultured on mAb
11. The addition of GRGDdSP peptide (based on the fibronectin sequence) to cells cultured on mAb 11 failed to restore GLA activation to the levels observed for mAb 16 or fibronectin substrates (Fig. 4.11). Using this approach, larger peptide fragments of fibronectin could have been tested, for it is well documented that soluble RGD peptides do not always mimic the action of ECM ligands. However, the stocks of mAb 11 available did not permit such studies.

4.3 Discussion

Fibronectin matrices influence cellular functions including adhesion, migration, and differentiation via interactions with cell surface integrin receptors. Following tissue injury, fibronectin is expressed locally and deposited in the provisional matrix where it forms a conduit for the inward migration of cells involved in early wound repair (Greiling and Clark 1997). Fibronectin is also re-expressed in tumour tissues, and several peptide and antibody inhibitors of fibronectin/integrin function effectively inhibit metastasis (Akiyama et al., 1995).

This chapter demonstrates that fibronectin up-regulates the activation of GLA by HT1080 fibrosarcoma cells, and that fibronectin induces a change in the levels of processed GLA which is similar to that induced by phorbol ester. In comparison, the processing of GLA by cells on laminin-1 appeared to be quite low. Both fibronectin and laminin-1 supported cell spreading, which suggests that changes in the level of GLA activation were independent of major changes in cell shape.

Steffensen et al. (1995) showed by ELISA studies that the fibronectin type II-like modules of GLA bind fibronectin. Unfortunately, the interaction of full-length GLA with fibronectin was not addressed in this report. We hypothesised that binding of GLA to cell surface-bound fibronectin could potentially increase GLA activation, by providing a pool of GLA at the cell surface for activation by MT1-MMP. Hence the binding of pro- and active recombinant GLA to fibronectin was assessed by ELISA studies, but no interaction was detected. Subsequent to the completion of practical work for this thesis, a very
interesting report was published by Steffensen et al. (1998), demonstrating interaction of the GLA fibronectin type II-like modules with cell surface-bound collagen I. These interactions promoted proGLA binding to the cell surface, but contrary to our original hypothesis, the pool of proGLA bound to cell surface collagen proved less likely to be activated than GLA bound to MT1-MMP via TIMP-2.

Several reports have demonstrated that adsorbing fibronectin to a solid surface causes the molecule to adopt an unfolded and extended shape (Fukai et al., 1995; Ugarova et al., 1995). This exposes sites important for cellular interaction, including both the RGD sequence and the CS-1 peptide region. In the current study, soluble fibronectin added to the culture medium had no effect on GLA activation, indicating that exposure of specific sites was required for GLA activation. This prompted a closer examination of specific regions of fibronectin in these studies.

The 120 kDa fragment of fibronectin, comprising most of the CCBD region, induced a level of GLA processing comparable to levels induced by full length fibronectin. This contrasted with the study by Werb et al. (1989) who demonstrated induction of MMP synthesis (CL-1, SL-1, GLB) by rabbit synovial fibroblasts in response to the 120 kDa fragment, but not full length fibronectin. Results from the current study differed on several points from the study by Werb et al. Firstly, HT1080 synthesis of GLB did not appear to be induced by fibronectin substrates and secondly, no activation of rabbit synovial fibroblast GLA was noted when these cells were cultured on fibronectin or fibronectin fragments (Werb et al., 1989). The differing responses of the two cell types to fibronectin may reflect different integrin expression patterns. Rabbit synovial fibroblasts interact with fibronectin via α5β1 and α4β1 integrins (Huhtala et al., 1995), whereas the HT1080 cells used in this study expressed only low levels of α4β1 integrin and adhered to fibronectin solely via α5β1 integrin. It was considered unlikely that the CS-1 region of fibronectin affects GLA activation by HT1080 cells and so attention was focussed on other regions of the fibronectin molecule.
Smaller fragments of the CCBD, encompassing the RGD integrin recognition site were tested. Problems were encountered with impaired cell adhesion and spreading on fragments such as Fn III 6-10, possibly as a result of poor adsorption of these molecules to plastic (Dr K. Yamada, personal communication). Interestingly, despite a low level of adhesion to Fn III 6-10, the processing of GLA indicated that this molecule contained regions capable of promoting GLA processing by HT1080 cells. A second Fn III 6-10 fragment was available for these studies, in which the pentapeptide sequence in Fn III 9 (PHSRN), that synergistically enhances the cell adhesion promoting activity of the RGD in FN III 10, was mutated to an inactive SPSDN (Fn III 6-10 SPSDN; Danen et al., 1995). It was hoped that this fragment would further define whether the synergy site is necessary for signalling GLA activation, but HT1080 cells failed to attach to this fragment in adhesion studies (data not shown).

To circumvent the problems encountered working with small peptide fragments of fibronectin, integrin receptors were perturbed directly by culturing the cells on a substrate of anti-integrin antibodies. Previous studies have shown that anti-integrin antibodies support cell adhesion, cause integrin receptor clustering, and in some cases mimic the action of the ligand, inducing transmembrane accumulation of signal transduction molecules at the cell surface (Miyamoto et al., 1995b). HT1080 cells spread well on antibodies to the α5, α6 and β1 integrin subunits (mAb 16, GoH3 and mAbl3, respectively). Adhesion to mAb 16 and mAb 13 promoted GLA processing, whereas adhesion to GoH3 (anti-α6) did not, consistent with induction of GLA activation by fibronectin substrates but not laminin-1. Further information regarding integrin signalling of GLA activation was gained from a comparison of two anti-α5 integrin antibodies. mAb 16 and mAb 11 to the α5 integrin subunit both induce integrin receptor clustering, but mAb 16 additionally mimics the action of the fibronectin ligand (Miyamoto et al., 1995b). The lack of GLA activation on a mAb 11 substrate in comparison with mAb 16 indicated the need for fibronectin receptor occupancy in signalling GLA activation by HT1080 cells. Miyamoto et al. (1995b) described an interesting study in which mAb 11 and mAb 16 coated latex beads were added to foreskin fibroblasts in culture. mAb 16 caused integrin
receptor clustering and the aggregation of actin associated cytoskeletal proteins, whereas mAb 11 promoted only receptor clustering. When a soluble RGD peptide was added to the mAb 11 cultures, an aggregation of six actin associated cytoskeletal proteins was observed, implying that fibronectin receptor occupancy was achieved. Although RGD peptides added to HT1080 cells cultured on plastic did not induce GLA activation (data not shown), it was predicted that addition of the peptide GRGDdSP to cells cultured on mAb11 would restore GLA activation. However this was not observed. It is possible that the RGD peptide chosen did not have sufficient secondary structure to mimic fibronectin action, and that larger CCBD fragments were needed (Dr K. Yamada, personal communication). It is also possible that the approach of coating mAb 11 to latex beads is a more successful technique for these studies. Unfortunately there was not sufficient mAb 11 to pursue these studies.

Several other reports have described the regulation of gelatinase expression in response to treatment with anti-integrin antibodies (Seftor et al., 1992; Larjava et al., 1993; Seftor et al., 1993). In a study with human gingival keratinocytes, GLB expression was up-regulated by the addition of soluble anti-β1 antibody (mAb 13) and an anti-α3 antibody, but not by anti-α5 or α2 integrin antibodies (Larjava et al., 1993). No differences in the expression of GLA were observed in this study. Kubota et al. (1997) demonstrated that soluble anti-α2 and anti-α3 integrin antibodies induced proGLA secretion and activation by human rhabdomyosarcoma cells. Studies with two glioblastoma cell lines showed that GLA expression was increased by treatment with anti-α3β1 or anti-α5β1 integrin antibodies (Chintala et al., 1996). Clearly, increases in GLA expression and activation may be signalled by several of the integrin receptors and the response to individual integrins is cell type specific. In our laboratory, human foreskin fibroblasts (HFF) are commonly used to investigate GLA activation systems, and so a comparison was made between HT1080 cells and HFF. Although HFF express α5β1 integrin, neither full length fibronectin nor the 120 kDa fragment of fibronectin induced GLA activation (data not shown). This differential response may indicate diverging intracellular signalling
pathways following integrin ligation in these two cell types and further work is needed to elucidate the mechanisms involved.

As the experiments described in this chapter were conducted over 24-48 hours, there is the possibility that fibronectin induces the expression of an endogenous cytokine or growth factor, that in turn signals changes to GLA activation. Preliminary experiments indicated that the protein synthesis inhibitor cycloheximide inhibits fibronectin induced GLA activation, implying that protein synthesis is required (data not shown). It has been reported that several soluble fibronectin fragments induce SL-1 expression by chondrocytes in cartilage explant culture and that this involves an IL-1 autocrine loop (Homandberg et al., 1997). Furthermore, IL-1 has been shown to induce GLA activation by smooth muscle cells in culture (Galis et al., 1994). IL-1 synergises with signals via the fibronectin receptor, as substrate bound fibronectin is permissive for IL-1 induced up-regulation of IL-6 mRNA by fibroblasts (Ostberg et al., 1995). In an extensive study published recently, Kheradmand et al (1998) showed that soluble ligands for the fibronectin receptor induce CL-1 expression by rabbit synovial fibroblasts via the GTP-binding protein Rac1. Signalling via Rac1 was accompanied by cell rounding and induced the expression of IL-1, which was in turn responsible for the induction of CL-1 expression. Future studies should investigate whether an IL-1 autocrine loop is involved in fibronectin up-regulation of GLA activation. De novo synthesis of growth factors should also be considered. Reports have shown that growth factors synergise with extracellular matrix/integrin mediated signalling pathways (Schwartz et al., 1995). Ligand-mediated integrin clustering leads to the accumulation of growth factor receptors and synergy between growth factors and integrins leads to a marked increase in growth factor receptor tyrosine phosphorylation (Plopper et al., 1995; Miyamoto et al., 1996). β1 integrin receptor occupancy results in the enhancement of EGF and PDGF growth factor receptor tyrosine phosphorylation, which in turn leads to the transient activation of MAP kinases (Miyamoto et al., 1996). Others have shown that the fibronectin receptor synergises with PDGF to promote inositol lipid metabolism (McNamee et al., 1993). Clearly, the possibility that a cytokine or growth factor is involved in the fibronectin up-regulation of
GLA activation should be addressed in future studies. A discussion of the signalling mechanisms involved following fibronectin receptor ligation is included in Chapter 7.

In summary, HT1080 cells cultured on fibronectin activated endogenous GLA, to a level comparable with that elicited by treatment with phorbol ester. In contrast, cells cultured on laminin-1 secreted mainly proGLA. Work with a 120 kDa fragment of fibronectin encompassing the CCBD indicated that this region of fibronectin alone is sufficient to signal the up-regulation of GLA activation. Studies with anti-integrin antibodies implicated the α5β1 fibronectin receptor as a mediator of GLA activation in HT1080 cells and suggested that α5β1 integrin receptor occupancy is essential to signal activation. The following chapter describes a study of the effect of fibronectin on other molecules involved in the GLA activation cascade.
Figure 4.1 Increased processing of proGLA to the active form by HT1080 cells cultured on fibronectin in comparison with laminin-1

HT1080 cells were cultured under serum free conditions on substrates of fibronectin or laminin-1, or on culture plastic with or without PMA for 48 hours. Samples of conditioned media were analysed for gelatin degrading activity by zymography. Cells cultured on fibronectin (lane 1), cells cultured on laminin-1 (lane 2); cells cultured on plastic in the presence of PMA (lane 3) or in the absence of PMA (lane 4). Arrows indicate the electrophoretic mobility of recombinant pro-GLB and recombinant pro and active GLA. Molecular mass markers are indicated on the right.
Figure 4.2 Biosynthetic labelling and immunoprecipitation of GLA secreted by HT1080 cells

HT1080 cells were cultured under serum free conditions on a substrate of fibronectin. Cells were biosynthetically labelled with \(^{35}\text{S}\)-methionine and the conditioned media was immunoprecipitated with a polyclonal antibody to GLA. Immunoprecipitates were separated by SDS-PAGE and analysed by fluorography. Cells were labelled for 0-14 hours (lane 1), 14-28 hours (lane 2), 28-42 hours (lane 3) or 38-52 hours (lane 4) after cell seeding.

Molecular mass markers are indicated on the right.
**Figure 4.3 Soluble fibronectin and laminin-1 do not affect proGLA processing by HT1080 cells**

HT1080 cells were cultured under serum free conditions on substrates of fibronectin or laminin-1, or on culture plastic with or without soluble fibronectin (30 µg/well) or soluble laminin-1 (30 µg/well). Samples of conditioned media were analysed for gelatin degrading activity by zymography. Cells cultured on fibronectin (lane 1), cells cultured on laminin-1 (lane 2); cells cultured on plastic alone (lane 3) or in the presence of soluble fibronectin (lane 4) or soluble laminin-1 (lane 5). Molecular mass markers are indicated on the right.
Figure 4.4 An ELISA study to determine whether MMP-2 binds to a substrate of fibronectin

Fibronectin or collagen type I were coated onto plastic wells at 100 μg/ml. GLA was added to the wells at 100 nM and incubated for 2 hours at 4°C. Bound enzyme was revealed using a polyclonal antibody to gelatinase A, followed by a horse-radish peroxidase conjugated second antibody and 3,3', 5,5'-tetramethylbenzidine substrate. Absorbance of the wells was read at 450nm. ProGLA is represented by grey bars and active GLA by black bars. Values are the mean +/- the standard deviation of triplicate wells.
Figure 4.5 Schematic diagram of plasma fibronectin and the fibronectin fragments used in this study
Figure 4.6 Levels of proGLA processing by HT1080 cells cultured on the 120 kDa fragment of fibronectin are similar to the levels effected by full length fibronectin

HT1080 cells were cultured under serum free conditions on substrates of fibronectin, 120 kDa fibronectin or laminin-1 for 48 hours. Samples of conditioned media were analysed for gelatin degrading activity by zymography. Cells cultured on fibronectin (lane 1), cells cultured on laminin-1 (lane 2) cells cultured on 120 kDa fibronectin (lanes 3). Molecular mass markers are indicated on the right.
Figure 4.7 HT1080 cells express low levels of the α4 integrin subunit.
The expression of α4 integrin subunit on the surface of HT1080 and HL60 cells was analysed by flow cytometry. The cells were incubated with the anti-α4 monoclonal antibody HP2/1, followed by anti-mouse-FITC conjugated secondary antibody (penned in green). Negative controls were incubated without the first antibody (penned in purple). Log fluorescence was obtained using a Becton Dickinson FACS-Calibur flow cytometer.

(ii) A dot-plot of log fluorescence (FL1-H) versus side scatter.
(iii) A histogram of log fluorescence. The data show that HL60 cells express greater levels of α4β1 integrin than HT1080 cells.
HT1080 adhesion to fibronectin is α5β1 integrin mediated

HT1080 cells were incubated for 30 minutes alone, or in the presence of 10 μg/ml of the following: rat anti-α5 integrin antibody mAb 16 (anti-α5); non-immune rat IgG control (NRlG); mouse anti-α4 integrin antibody HP2/1 (anti-α4); or non-immune mouse IgG control (NMlG). Cells were then seeded onto fibronectin coated plastic and incubated at 37°C for 35 minutes. Adherent cells were fixed and stained with methylene blue and the absorbance of the wells was read at 630 nm. Values are the mean +/- standard deviation of triplicate wells.
Figure 4.9 ProGLA processing by HT1080 cells cultured on the Fn III 6-10 fragment of fibronectin

HT1080 cells were cultured under serum free conditions on substrates of fibronectin, 120 kDa fibronectin, Fn III 6-10 or laminin-1 for 48 hours. The number of cells/well was determined at the end of the incubation. Samples of conditioned media were analysed for gelatin degrading activity by zymography. The volume of conditioned medium loaded per lane was adjusted to reflect the number of cells/tissue culture well at the end of the culture period. Cells cultured on fibronectin (lanes 1-2), cells cultured on laminin-1 (lane 3-4) cells cultured on 120 kDa fibronectin (lanes 5-6). Molecular mass markers are indicated on the right.
Figure 4.10  Processing of proGLA to the active form by HT1080 cells is up-regulated by culture of the cells on antibodies to the α5 and β1 integrin subunits

HT1080 cells were cultured under serum free conditions for 48 hours on substrates of fibronectin, laminin-1, or on immobilised monoclonal antibodies to integrin subunits. Samples of conditioned media were analysed for gelatin degrading activity by zymography. Cells cultured on fibronectin (lane 1), cells cultured on anti-α5 antibody mAb 16 (lane 2), on anti-β1 antibody mAb 13 (lane 3), on anti-α5 antibody mAb 11 (lane 4), on anti-α6 antibody GoH3 (lane 5). Cells cultured on laminin-1 (lane 6). Molecular mass markers are indicated on the right.
Figure 4.11 Addition of RGD peptides to HT1080 cells cultured on the monoclonal anti-\(\alpha_5\) integrin antibody mAb11 does not restore processing of proGLA to the levels effected by anti-\(\alpha_5\) integrin antibody mAb 16

HT1080 cells were cultured under serum free conditions for 48 hours on substrates of fibronectin, laminin-1, or on immobilised monoclonal antibodies to integrin subunits. Soluble RGD peptides (200 \(\mu\)g/ml) were added to the culture media at the start of incubation. Samples of conditioned media were analysed for gelatin degrading activity by zymography. Cells cultured on fibronectin (lane 1), or on anti-\(\alpha_5\) antibody mAb 16 (lane 2). Cells cultured on anti-\(\alpha_5\) antibody mAb 11 alone (lane 3), or in the presence of GRGDDSP (lane 4) or GRGESP (lane 5). Cells cultured on laminin-1 (lane 6). Molecular mass markers are indicated on the right.
Chapter 5

Extracellular matrix regulation of MT1-MMP processing
Chapter 5 Extracellular matrix regulation of MT1-MMP processing

5.1 Introduction

The physiological mechanism by which proGLA is activated has been the subject of intense study by many laboratories. Evidence from early studies with transformed or concanavalin A treated cells implicated cell membrane-bound molecules in the activation process (Overall and Sodek, 1990; Ward et al., 1991b). Several laboratories demonstrated that proGLA activation was initiated by binding of GLA to the cell surface via its C-terminus (Murphy et al., 1992b; Strongin et al., 1993; Ward et al., 1994). Work was then directed at identifying the cell surface molecule(s) that bind the C-terminal domain of GLA and the enzyme responsible for activating surface-bound GLA.

The discovery of membrane-bound MT-MMPs (Sato et al., 1994; Takino et al., 1995; Will and Hinzmann, 1995; Puente et al., 1996) led to the hypothesis that MT-MMPs participate in the activation of proGLA. MT1-MMP is the best characterised, and is located at the surface of activated cells (Sato et al., 1994; Strongin et al., 1995) where it may be associated with TIMP-2 (Strongin et al., 1995; Imai et al., 1996). ProGLA binds to TIMP-2 via its C-terminus, which has led to the hypothesis that MT1-MMP and TIMP-2 form a 'receptor' complex, that binds proGLA (Strongin et al., 1995; Imai et al., 1996; Butler et al., 1998). Evidence for this trimolecular complex has been provided by cross-linking experiments (Strongin et al., 1995) and by incubation of proGLA and TIMP-2 with MT1-MMP immobilised on agarose beads (Kinoshita et al., 1998). A model for GLA activation has been described in which proteolysis of GLA bound in the complex requires an adjacent MT1-MMP molecule that is TIMP-2 free and therefore catalytically active (Butler et al., 1998; Kinoshita et al., 1998; see Fig. 1.3).

In this chapter, the effects of fibronectin on HT1080 MT1-MMP and TIMP-2 expression were assessed. A new polyclonal antibody to MT1-MMP was characterised for these studies. By Western blot, this antibody recognised the 63 and 60 kDa forms of MT1-MMP commonly reported in the literature, but also detected a processed form of MT1-MMP at
45 kDa. This processed form of MT1-MMP was up-regulated when HT1080 cells were cultured on fibronectin, implying that fibronectin stimulates the action of an (unknown) protease which cleaves MT1-MMP to a 45 kDa form. Attempts were made to identify this protease.

5.2 Results

5.2.1 Expression of TIMP-2 protein by HT1080 cells cultured on fibronectin or laminin-1

The effect of culture substrate on HT1080 TIMP-2 expression was investigated using several methods. Reverse zymography was used to measure secreted TIMP-2, however the levels detected were too low to allow an accurate comparison of the effects of fibronectin and laminin-1 substrates (data not shown). Newly synthesised TIMP-2 was radiolabelled with $^{35}$S-methionine and immunoprecipitated with the sheep anti-TIMP-2 antibody H225 (Ward et al., 1991a). This technique also did not prove sensitive enough to allow an accurate comparison (data not shown). TIMP-2 bound to GLA in the conditioned medium was concentrated by gelatin-Sepharose chromatography and analysed by Western blot using H225. The TIMP-2 was easily detected using this technique and no difference was noted in the levels of TIMP-2 secreted when cells were cultured on fibronectin or laminin-1 (Fig. 5.1A). Cell lysates were then prepared and analysed by Western blot; no obvious differences in the levels of cell-associated TIMP-2 were noted between the substrates (Fig. 5.1B).

5.2.2 MT-MMP expression by HT1080 cells

5.2.2a Western blot

Western blot analysis was used to characterise the expression of MT-MMPs by HT1080 cells. To detect MT1-MMP, a polyclonal antibody raised in a sheep to GST-ΔMT1-MMP (N175) was affinity purified and characterised as described (d'Ortho et al., 1998; also detailed in Chapter 2). By Western blot, this antibody recognised recombinant MT1-MMP (Δ502-559) recognising pro-enzyme at 60 kDa and major processed forms at approximately 55 and 31 kDa (Fig. 5.2, lane 1), but did not cross react with MT2-MMP, GLA, GLB; CL -
1, -2 and -3; SL -1 and -2; TIMPs -1, -2, -3 or GST (data not shown). N175 recognised wild-type MT1-MMP present in membranes prepared from HT1080 cells, detecting a major band at 60 kDa, a doublet at 63 kDa and a minor band at 45 kDa (Fig. 5.2, lane 2). This profile is very similar to that reported by Lohi et al. (1996), who described two polyclonal antibodies to MT1-MMP that detected the doublet at 63 kDa and bands at 60 and 43 kDa in HT1080 cell lysates.

The monoclonal antibody to MT2-MMP detected the recombinant catalytic domain of MT2-MMP at 30 kDa, but did not detect MT2-MMP in HT1080 cell membranes (Fig. 5.2, lanes 3 and 4). Similarly, the monoclonal antibody to MT3-MMP failed to detect MT3-MMP in the HT1080 membranes, but reacted with a band of 66 kDa corresponding to recombinant proMT3-MMP (Fig. 5.2, lanes 5 and 6). For both MT2- and MT3-MMP the chemiluminescent detection reaction was allowed to develop to completion overnight, but no MT2- or MT3- protein was detected in HT1080 cell membranes (data not shown). To test for MT4-MMP expression, a new polyclonal antibody to MT4-MMP was used. This antibody (N33) was developed and characterised in our laboratory by Dr M. Butler. It recognises MT4-MMP, does not cross react with MT1-MMP, gelatinases, collagenases or TIMPs; but does cross react with SL-1 and MT3-MMP (Dr M. Butler, personal communication). N33 recognised the recombinant catalytic domain of MT4-MMP at 33 kDa (Fig. 5.2, lane 7) and reacted with protein at 62 kDa in HT1080 cell lysates, as well as several bands between 30 and 45 kDa (Fig. 5.2, lane 8). The identity of the bands could not be confirmed as MT4-MMP however, as there was no recombinant MT4-MMP available for affinity purification of the antibody, and to date there is no cell type available in our laboratory that is known to express MT4-MMP.

**5.2.2b Northern blot**

Based on the data from Western analysis, HT1080 cells were tested for MT1-MMP and MT4-MMP mRNA expression. Cells were cultured on plastic for 24 hours prior to RNA preparation. Northern blots of the prepared total RNA were probed with a digoxygenin-labelled MT1-MMP riboprobe. A single band of MT1-MMP was detected (Fig. 5.3),
consistent with the 4.5 kilobase transcript for HT1080 cells reported by Sato et al. (1994). Northern blots were probed with a digoxygenin-labelled MT4-MMP riboprobe, but MT4-MMP, if present, was below the level of detection (data not shown).

5.2.2c Immunolocalisation

MT1-MMP: Immunolocalisation studies were used to determine the cellular distribution of MT1-MMP in HT1080 cells. To detect intracellular MT1-MMP, the cells were treated with the ionophore monensin, which prevents secretion and results in the intracellular accumulation of MMPs (Hembry et al., 1985). Cells were fixed and permeabilised after 3 hours monensin treatment and incubated with N175. Weak perinuclear immunofluorescence was apparent (Fig. 5.4A). To study cell surface expression of MT1-MMP, cells cultured in the absence of monensin were fixed and stained with N175. In some experiments, cell surface staining of MT1-MMP was detected (Fig. 5.4B). Confocal microscopy was used to collect sections through the cells, and staining was seen to cover the entire cell surface (Fig. 5.5). However, over several experiments the results were variable, and often the level of MT1-MMP was below, or at the limits of detection.

MT4-MMP: Although Northern blot analyses failed to detect MT4-MMP mRNA in HT1080 cells, the Western blot analyses did suggest that the protein might be present. An attempt was made to immunolocalise MT4-MMP in these cells. Several antibodies were tested: two rabbit anti-peptide antibodies (anti-KLH-PEEP and anti KLH-RTLKR); a rabbit polyclonal antibody to a C-terminal fragment of MT4-MMP; and the sheep polyclonal antibody N33. The two peptide antibodies stained the perinuclear region of monensin treated HT1080 cells very brightly and also gave bright cell surface staining, however, these antibodies failed to react to HT1080 lysate proteins on Western blot (data not shown). Both the rabbit polyclonal antibody to MT4-MMP and N33 failed to stain HT1080 cell surfaces, and did not stain intracellularly (data not shown). It is possible that the peptide antibody staining was non-specific, and so no conclusion could be drawn regarding HT1080 expression of MT4-MMP protein.
In summary, the expression of MT1-MMP by HT1080 cells was verified by Northern and Western blot analyses, and by immunolocalisation. The presence of other MT-MMPs in HT1080 cells could not be confirmed. Therefore, further studies were designed to determine whether the ECM substrates fibronectin and laminin-1 regulate MT1-MMP expression.

5.2.3 Messenger RNA levels for MT1-MMP expressed by HT1080 cells are not altered by culture on fibronectin or laminin-1 substrates

Fibronectin and laminin-1 were examined for their potential to regulate MT1-MMP expression at the mRNA level. RNA was prepared from HT1080 cells cultured on fibronectin or laminin-1 for 24 hours, and analysed by Northern Blot. No differences in the steady state levels of MT1-MMP mRNA were noted between the culture substrates (Fig. 5.6, lanes 2 and 3).

5.2.4 Immunolocalisation of MT1-MMP expressed by HT1080 cells cultured on fibronectin or laminin-1 substrates

HT1080 cells were cultured on fibronectin and laminin-1 substrates and the cell surface expression of MT1-MMP protein was compared using the immunolocalisation techniques described above. Cell surface staining of MT1-MMP was low on both substrates, and did not photograph successfully (data not shown). However, it appeared that the levels of MT1-MMP expressed on the cell surface were similar when the cells were cultured on either fibronectin or laminin-1.

5.2.5 Processing of MT1-MMP protein on fibronectin substrates

Western blot analysis was used to compare the total MT1-MMP protein levels present in HT1080 cells cultured on fibronectin or laminin-1. Using the anti-MT1-MMP antibody N175, it was apparent that the levels of MT1-MMP protein were increased when the cells were cultured on fibronectin in comparison with laminin-1. More specifically, the levels of the 45 kDa form were increased, whereas levels of the 63 kDa doublet and the 60 kDa band...
were unaffected by the different culture conditions (Fig. 5.7A). Scanning densitometry of the bands in Figure 5.7A revealed that the overall MT1-MMP protein was 1.6 times greater when cells were cultured on fibronectin versus laminin-1 substrates, and the levels of 45 kDa MT1-MMP were 3 times greater. In subsequent experiments, the levels of the 45kDa form were up to 8 times greater on the fibronectin substrates (data not shown). PMA treatment also induced the expression of 45 kDa MT1-MMP (Fig. 5.7A, lane 3). Strikingly, where increases in the 45 kDa band were detected, there was a concomitant increase in the processing of GLA to the active form as detected by zymography (Fig. 5.7B).

The remainder of this chapter describes a) studies to identify the protease(s) involved in MT1-MMP processing to 45 kDa and b) investigations to determine whether GLA activation and MT1-MMP processing to 45 kDa are interdependent events, or the result of separate effects of fibronectin.

5.2.6 Inhibition of MT1-MMP processing to 45 kDa by MMP inhibitors
To investigate which protease(s) are involved in the processing of MT1-MMP to 45 kDa, HT1080 cells were cultured on fibronectin in the presence of protease inhibitors for 46 hours prior to harvest. Inclusion of the hydroxamate inhibitor CT1746 at 1 µM abolished the 45 kDa band (Fig. 5.8A, lane 2). This inhibitor is a general metalloproteinase inhibitor when used at micromolar concentrations (Dr J. O'Connell, personal communication), thus indicating that a metalloproteinase is involved in the processing of MT1-MMP to 45 kDa. Exogenously added TIMP-2 also inhibited processing of MT1-MMP to 45 kDa, further defining the processing enzyme as an MMP (Fig. 5.8A, lane 3).

A zymogram of the conditioned media from this experiment showed that the activation of GLA paralleled increases in the levels of the 45 kDa band, and was inhibited by CT1746 and TIMP-2 (Fig. 5.8B, lanes 1-3). Based on this observation, it was considered possible that active GLA at the cell surface was involved in processing MT1-MMP to 45 kDa. However, MT1-MMP self processing to 45 kDa was also considered a possibility. Our
laboratory has demonstrated that MT1-MMP activity is not susceptible to inhibition by TIMP-1 (Will et al., 1996), which distinguishes MT1-MMP from other MMPs (including GLA). Therefore, to indicate whether MT1-MMP self-processing was occurring, TIMP-1 was included in the cultures. Even at high concentrations (150 nM), TIMP-1 was ineffective as an inhibitor of MT1-MMP processing to 45 kDa (Figure 5.8A, lane 4), implicating MT1-MMP self-processing to the 45 kDa form.

Notably, the result from this experiment was not clear-cut, for when the conditioned medium was examined by zymography it was apparent that TIMP-1 also failed to inhibit GLA activation (Fig. 5.8B, lane 4). Although it was expected that the processing of proGLA to the intermediate form would not be inhibited by TIMP-1 (this cleavage is performed by MT1-MMP), the processing from the intermediate to fully active form of GLA should have been susceptible to TIMP-1 inhibition, as this cleavage is due to GLA autolysis (Atkinson et al., 1995).

It has been shown previously that a mutant of GLA, with the C-terminal hemopexin domain removed (NGL) is poorly inhibited by TIMP-1, but is inhibited by a C-terminally truncated form of TIMP-1 (N-TIMP-1; Nguyen et al., 1994). It is possible in the current study that GLA bound by its C-terminus to the MT1-MMP:TIMP-2 complex behaved similarly to NGL and was therefore not susceptible to inhibition by TIMP-1. Therefore, it was predicted that N-TIMP-1 should inhibit GLA processing from the intermediate to the fully active form. When N-TIMP-1 was added to HT1080 cells cultured on fibronectin the processing of GLA to the fully active form was indeed inhibited (Fig. 5.9A, lane 4), in contrast to full length TIMP-1 which failed to inhibit (Fig. 5.9A, lane 3).

The processing of MT1-MMP to 45 kDa was examined when N-TIMP-1 was included in the culture and it was observed that whilst N-TIMP-1 prevented the production of fully active GLA (Fig. 5.9A, lane 4), it did not inhibit the processing of MT1-MMP to 45 kDa (Fig. 5.9B, lane 4). This suggested that active GLA is not required for MT1-MMP processing to 45 kDa.
5.2.7 Addition of antibodies to MT1-MMP and GLA to HT1080 cells on fibronectin: is MT1-MMP processing to 45 kDa inhibited?

Blocking antibodies would help to determine whether MT1-MMP or GLA are involved in MT1-MMP processing to 45 kDa. Our laboratory has not yet identified blocking antibodies to MT1-MMP, and so several MT1-MMP antibodies of unknown activity were added to HT1080 cells cultured on fibronectin. The antibody N175 (purified by Protein-G Sepharose chromatography) failed to prevent the activation of GLA, and did not affect the processing of MT1-MMP to the 45 kDa form (Fig. 5.10A and B respectively, lane 4). MT1-MMP monoclonals kindly provided by Dr P. Basset (Université Louis Pasteur, Strasbourg, France) were tested. These antibodies (3A10 and 4G6, to the catalytic and hemopexin domain of rat MT1-MMP respectively) reacted with human MT1-MMP on Western blot, recognising bands of 63 and 60 and 45 kDa as well as additional bands at 50 and 68 kDa in HT1080 cell lysates (data not shown). As 3A10 and 4G6 were provided as culture supernatants, a third culture supernatant (2G12) was chosen as a control, on the basis that it did not cross react with MT1-MMP on Western blot (data not shown) or stain MT1-MMP in cells (Dr R. Hembry, personal communication). Neither 3A10 (data not shown), nor 4G6 (Fig. 5.10A and B, lane 2), nor the control antibody inhibited the activation of GLA, or the processing of MT1-MMP to the 45 kDa form. Thus none of the antibodies tested inhibited MT1-MMP activity, and the hypothesis that MT1-MMP autolytically processes to 45 kDa could not be verified using the antibodies available.

Anti-GLA antibodies were tested for their ability to block GLA activation and the processing of MT1-MMP to 45 kDa. We have recently raised a new polyclonal antibody to GLA (K399) using the same procedure as reported for the anti-GLA antibody X670, that was raised in our laboratory (Hipps et al., 1991). This antibody was characterised by Western blot analysis, and recognised recombinant human GLA, but did not cross react with GLB, CL -1, -2, -3, SL-1, -2 or TIMPs -1, -2, -3 (data not shown). NS0 mouse myeloma cells stably transfected with GLA cDNA stained brightly with this antibody using immunolocalisation techniques (data not shown). The antibody was purified by adsorption to Protein-G Sepharose, and added to HT1080 cells cultured on fibronectin.
Analysis of the conditioned medium by zymography showed that this antibody sequestered all GLA secreted, such that none was detected (Fig. 5.11A), but failed to inhibit the processing of MT1-MMP to the 45 kDa form (Fig. 5.11B). Preliminary data also suggested that a monoclonal antibody to the C-terminal domain of GLA (VB3) inhibited GLA activation, but did not affect the processing of MT1-MMP to 45 kDa (data not shown). Thus the data from these studies did not support the hypothesis that GLA processes MT1-MMP to the 45 kDa form.
5.3 Discussion

The aim of this chapter was to determine whether fibronectin up-regulates the expression of molecules important for the cell-surface activation of GLA in HT1080 cells. Studies focussed on TIMP-2 and the MT-MMPs, based on the hypothesis that TIMP-2 and MT1-MMP together form a 'receptor' complex that binds and initiates GLA activation. However, other hypotheses for GLA binding to the cell surface were also considered when designing and interpreting these studies, and these deserve mention.

Interactions between the integrin αvβ3 and C-terminus of GLA have been demonstrated in human melanoma cells (Brooks et al., 1996; Brooks et al., 1998), although the enzyme involved in the cleavage of the GLA propeptide was not identified in these reports. Certainly, αvβ3 integrin is widely expressed, however there are many examples of cell types which activate GLA but do not express αvβ3 integrin. Therefore, whether integrin/proGLA binding is a general mechanism for concentrating GLA at the cell surface has yet to be determined. In the current study it was considered unlikely that GLA/αvβ3 integrin interactions were important, since immunolocalisation studies of our HT1080 cells with several anti-αv or αvβ3 integrin antibodies indicated that these cells apparently do not express αvβ3 integrin (A. Messent and J. Gavrilovic, unpublished observations). Another mechanism for GLA binding to the cell surface was described recently. Steffensen et al. (1998) showed GLA binding to cell surface-bound collagen via GLA fibronectin type II domains. However GLA bound via this mechanism was less susceptible to activation than GLA bound to the surface via TIMP-2. As fibronectin acts to increase GLA activation in HT1080 cells, the collagen-GLA interactions were considered unlikely to be relevant to this study.

Therefore, to begin the studies, fibronectin was examined for its potential to affect TIMP-2 expression or distribution. Many reports have demonstrated that the level of TIMP-2 present is critical for the activation of GLA. An excess of TIMP-2 surrounding the cell leads to inhibition of GLA activation, whereas cell surface-bound TIMP-2 is a requirement for activation. In the current study, it was considered possible that fibronectin matrices
might i) alter the levels of secreted TIMP-2 or ii) alter the proportion of cell surface bound TIMP-2 such that GLA activation is favoured. Unfortunately, it proved difficult to detect TIMP-2 in the conditioned medium from HT1080 cells. Gelatin-agarose was used to concentrate GLA-bound-TIMP-2 in the media from fibronectin and laminin-1 cultures; no difference in TIMP-2 levels between the cultures was noted. To give an indication of total TIMP-2 in the conditioned medium, reverse zymography was attempted, however the levels of TIMP-2 were at the detection limit of this technique. The media were concentrated and analysed by reverse zymography by S. Atkinson (University of East Anglia, Norwich, UK); the results indicated that the levels of TIMP-2 were similar in fibronectin and laminin-1 substrates (S. Atkinson, personal communication). Cell surface TIMP-2 was analysed by Western blot, but again no variation in TIMP-2 levels were detected in the lysates of cells cultured on fibronectin or laminin-1 matrices. Finally, fibronectin was examined for its potential to bind TIMP-2, as an interaction with the matrix may help to bind TIMP-2 near the cell surface. The ELISA technique detailed in Chapter 4 was used to study fibronectin/TIMP-2 interaction, but no binding was observed (data not shown).

The next possibility examined was whether fibronectin substrates alter the expression of MT-MMPs by HT1080 cells. Firstly, HT1080 cells were analysed to determine which MT-MMPs they express. MT1-MMP expression by HT1080 cells was described by Sato et al. (1994) and confirmed in the current study using Northern and Western blot analyses, and immunolocalisation. MT2- and MT3-MMP protein was not detected by Western blot of HT1080 cell lysates. Following completion of studies for this thesis, a study published by Lehti et al (1998) confirmed that these cells do not express MT2- or MT3-MMP. The polyclonal antibody to MT4-MMP detected a band that approximated to the molecular mass of MT4-MMP in the lysates of HT1080 cells, but the presence of MT4-MMP was not confirmed by immunolocalisation, and MT4-MMP mRNA was below the level of detection. To continue these studies, a cell type that is known to express MT4-MMP is needed as a positive control, and the MT4-MMP antibodies require further characterisation. However, with respect to GLA activation studies, it is known that MT1-, MT-2- and MT3-
MMP can initiate GLA activation (Sato et al., 1994; Takino et al., 1995; Butler et al., 1997), whereas it has yet to be shown that MT4-MMP can activate GLA. Therefore MT1-MMP was targeted for the current study.

According to the hypothesis of Butler et al. (1998), MT1-MMP/TIMP-2 binds GLA to the cell surface, and an adjacent TIMP-2-free MT1-MMP molecule is needed to initiate the activation of GLA. Since fibronectin apparently did not regulate TIMP-2 expression, it was hypothesised that MT1-MMP expression might be up-regulated, resulting in a greater level of 'free' MT1-MMP. ECM regulation of MT1-MMP expression was recently described by Gilles et al., (1997) and Haas et al., (1998) who showed that the culture of several cell types on collagen type I gels or within collagen lattices induces MT1-MMP mRNA expression and a corresponding increase in GLA activation. However, in the current study MT1-MMP mRNA levels were similar when cells were cultured on fibronectin or laminin-1 substrates.

To determine whether post-translational regulation of MT1-MMP is affected by fibronectin, MT1-MMP protein was analysed by Western blot using a newly described antibody to MT1-MMP. Two major immunoreactive bands were detected at 60 kDa and 45 kDa, and a faint doublet at 63 kDa. Only one other laboratory has described a band of similar size (43 kDa) detected in lysates prepared from HT1080 fibrosarcoma cells and stimulated lung fibroblasts (Lohi et al., 1996). Two polyclonal antibodies to MT1-MMP were detailed in this study, raised to the hemopexin and cytoplasmic domains of MT1-MMP, and these antibodies detected a truncated form of MT1-MMP at 43 kDa. In most studies, however, antibodies raised to a peptide in the hinge region of MT1-MMP have been used in Western blot analysis (peptide CDGNFDTVAMLGEM, residues 310-333). In our laboratory, we have noted that a monoclonal antibody to this peptide (113-5B7; Sato et al., 1994) has a very low affinity for the 45 kDa band, such that in most analyses it fails to detect the band (data not shown).
The 60 kDa band recognised by N175 probably corresponds to the active form of MT1-MMP (S. Atkinson, G. Butler, G. Murphy, unpublished results; Strongin et al., 1995). Although the level of the 60 kDa form was not affected by the various culture conditions employed, it was clear that the overall MT1-MMP protein levels increased when the cells were cultured on fibronectin, as the 45 kDa band was up-regulated. Similarly, PMA treatment up-regulated the expression of the 45 kDa band, as has been reported by Lohi et al., (1996). Increases in the 45 kDa band were noted to be concomitant with the activation of GLA, an observation that agrees with the report by Lohi et al. (1996).

Following this initial observation of a truncated 45 kDa form of MT1-MMP, a number of cell types were examined in our laboratory to determine whether similar processing events occurred. Although HT1080 cells were the only cell type of those analysed to respond to culture on fibronectin by increased processing of MT1-MMP to 45 kDa, other cell types processed MT1-MMP to 45 kDa in response to concanavalin A (SW1353 human chondrosarcoma cells, Cowell, et al. (1998); human foreskin fibroblasts, S. Atkinson, H. Stanton, G. Murphy, unpublished results; G361 mouse myeloma cells; H Stanton, G. Murphy, unpublished results) or processed MT1-MMP to 45 kDa in response to PMA (endothelial cells, C. Lindsay, G. Murphy, personal communication). Transfection of MT1-MMP into Chinese hamster ovary cells (d'Ortho et al., 1998) or HT1080 fibrosarcoma cells (J. Gavrilovic, H. Stanton, J. Clements, G. Murphy, unpublished results) also resulted in a greatly up-regulated 45 kDa band. The combined data indicate that processing of MT1-MMP to a 45 kDa form occurs in response to various stimuli and is common to many cell types. With the exception of the PMA-treated endothelial cells, the processing of MT1-MMP to the 45 kDa form was always mirrored by an increase in the activation of GLA.

Studies with the protease inhibitors CT1746 and TIMP-2 indicated that the culture of HT1080 cells on fibronectin up-regulated the activity of an (unknown) MMP, responsible for the processing of MT1-MMP to 45 kDa. GLA was considered a likely candidate, as the activation of GLA was up-regulated by fibronectin and concomitant with MT1-MMP
processing to 45 kDa. However, the lack of inhibition of MT1-MMP processing to 45 kDa by TIMP-1 implied MT1-MMP autolysis. Studies with N-TIMP-1 further supported this hypothesis, for N-TIMP-1 prevented the processing of GLA to the fully active form, but did not inhibit MT1-MMP processing to 45 kDa. As an interesting aside, the final step in the activation of GLA was susceptible to N-TIMP-1 but not TIMP-1, possibly because GLA bound to the cell surface by its C-terminus behaves more like N-terminally truncated GLA, and is therefore not susceptible to TIMP-1 inhibition (Nguyen et al., 1994).

Antibodies to MT1-MMP and GLA were added to HT1080 cells cultured on fibronectin, with the intention of blocking enzymatic activity and unravelling the sequence of events involved in fibronectin up-regulation of MMP processing. None of the MT1-MMP antibodies tested inhibited MT1-MMP or GLA processing, implying i) that the antibodies were not blocking antibodies or ii) that antibody access to the MT1-MMP/GLA complex was impeded in some way, or iii) that GLA activation by HT1080 cells was not dependant upon MT1-MMP activity. Unfortunately, with the antibodies available, the hypothesis that MT1-MMP processing to 45 kDa is autolytic could not be proved or disproved. The new anti-GLA polyclonal antibody K399 bound all secreted GLA, such that none was detected in the conditioned medium. This was probably due to endocytosis of the antibody/GLA complex during the course of the experiment. Interestingly, K399 did not prevent MT1-MMP processing to 45 kDa. The combination of the TIMP inhibition studies and the anti-GLA antibody studies indicated that active GLA is not involved in processing MT1-MMP to 45 kDa and implied that a culture substrate of fibronectin stimulates two separate events: the increased activation of GLA and an up-regulation of an unknown protease that cleaves MT1-MMP to 45 kDa.

In summary, HT1080 TIMP-2 levels were not up-regulated by a substrate of fibronectin. The overall expression of MT1-MMP protein was increased by fibronectin, but more specifically, the proteolytic processing of active 60 kDa MT1-MMP to a 45 kDa product was promoted. This processing was MMP-mediated and concomitant with GLA activation, but TIMP and antibody inhibition studies indicated that active GLA was not
involved in the processing of MT1-MMP to 45 kDa.

It is apparent from these studies, and from the studies by Lohi et al. (1996) that the 45 kDa form MT1-MMP is still membrane-associated. Hence MT1-MMP must be cleaved within the catalytic domain to yield a band of 45 kDa. It is likely that this would render the molecule inactive, which would represent an important regulatory step in MT1-MMP activation cascades. An N-terminal sequence of the 45 kDa form would shed more light on its role, and Chapter 6 details strategies to purify sufficient 45 kDa MT1-MMP for sequencing.
Figure 5.1 TIMP-2 protein expression by HT1080 cells cultured on fibronectin or laminin-1
HT1080 cells were cultured under serum free conditions on substrates of fibronectin or laminin-1 for 48 hours.
A. Western blot analysis of secreted TIMP-2. TIMP-2/GLA complexes were purified from the conditioned medium by adsorption to gelatin-Sepharose. Bound proteins were eluted using reducing sample buffer and separated by SDS-PAGE. Proteins were transferred to nitrocellulose by electroblotting and probed with a polyclonal antibody to TIMP-2. Two separate experiments are depicted. Cells cultured on fibronectin (lanes 1, 3), cells cultured on laminin-1 (lanes 2, 4). Recombinant TIMP-2 (2 ng; lane 5).
B. Western blot analysis of HT1080 cell lysates. Cells were lysed in a buffer containing Triton-X-100. Lysate proteins from three separate experiments were separated by 10% SDS-PAGE, transferred to nitrocellulose and probed with a polyclonal antibody to TIMP-2. Two separate experiments are depicted. Cells cultured on fibronectin (lane 1, 3), cells cultured on laminin-1 (lane 2, 4); TIMP-2 standard (2 ng; lane 5).
Molecular mass markers are indicated on the right.
Figure 5.2 Western blot analysis of MT-MMP protein expression

Cell membranes were prepared from HT1080 cells cultured in serum. Membrane proteins (10 µl) were separated by 10% SDS-PAGE, electroblotted to nitrocellulose and probed with antibodies to MT1-MMPs. Recombinant proteins: MT1-MMP (lane 1), MT2-MMP (lane 4), MT3-MMP (lane 5) and MT4-MMP (lane 7). HT1080 cell membranes (lanes 2, 3, 6, 8). Lanes 1 and 2 were developed with sheep anti-human MT1-MMP antibody; lanes 3 and 4 with a rabbit anti-human MT2-MMP antibody; lanes 5 and 6 with a mouse monoclonal antibody to human MT3-MMP; lanes 7 and 8 with a sheep anti-human MT4-MMP antibody.
Figure 5.3 Expression of MT1-MMP mRNA by HT1080 fibrosarcoma cells

Northern blot of RNA from HT1080 cells cultured on plastic. Cells were cultured for 24 hours prior to extraction of RNA. Total RNA (5 μg) was separated on an agarose gel, transferred to a nylon membrane and MT1-MMP mRNA detected by hybridisation with a digoxigenin-labelled riboprobe. The position of the 28S and 18S ribosomal RNA is shown. A GAPDH riboprobe was used as an internal standard.
Figure 5.4 Immunolocalisation of MT1-MMP in HT1080 cells

HT1080 cells were cultured on glass slides in the presence of fetal calf serum.

A. Intracellular staining: Monensin was added for the last 3 hours of culture. Cells were fixed, permeabilised and stained by indirect immunofluorescence with sheep anti-MT1-MMP antibody, followed by pig anti-sheep-Fab’-FITC.

B. Cell surface staining: cells were fixed and stained with sheep anti-MT1-MMP antibody, followed by pig anti-sheep Fab’-FITC.

Bars = 25 μm
Figure 5.5 MT1-MMP covers the entire surface of HT1080 cells

HT1080 cells were cultured on glass slides in the presence of fetal calf serum. Cells were fixed and stained by indirect immunofluorescence with sheep anti-MT1-MMP antibody, followed by pig anti-sheep Fab'-FITC. A confocal microscope was used to collect serial 1 µm sections through cells. Four sections from the series were chosen to illustrate cell surface staining: a) staining at the basal surface of the cells; b), c) staining of the cells in the middle of the series; d) staining at the dorsal surface of the cells.

Bar = 10 µm
Figure 5.6 HT1080 fibrosarcoma mRNA levels for MT1-MMP are not altered by culture on fibronectin or laminin-1

Northern blot of RNA from HT1080 cells cultured on fibronectin, laminin-1 or on plastic. Cells were cultured for 24 hours prior to extraction of total RNA. Samples (5 μg/lane) were separated on agarose gels, transferred to a nylon membrane and MT1-MMP mRNA detected by hybridisation with a digoxigenin-labelled riboprobe. The position of the 28S and 18S ribosomal RNA is shown. A GAPDH riboprobe was used as an internal standard. Cells were cultured on plastic (lane 1) on laminin-1 (lane 2); or cultured on fibronectin (lane 3).
Figure 5.7 The processing of MT1-MMP protein to a 45 kDa form by HT1080 fibrosarcoma cells is increased by culture on fibronectin
HT1080 cells were cultured under serum free conditions on substrates of fibronectin or laminin-1, or on culture plastic with or without PMA for 48 hours.
A. Western blot analysis of HT1080 cell lysates. Cells were lysed in a buffer containing Triton-X-100. Lysate proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose by electroblotting and probed with a polyclonal antibody to MT1-MMP. Cells cultured on fibronectin (lane 1), cells cultured on laminin-1 (lane 2); cells cultured on plastic in the presence or absence of PMA (lanes 3 and 4, respectively).
B. The conditioned media were analysed for gelatin degrading activity by zymography. Cells cultured on fibronectin (lane 1), cells cultured on laminin-1 (lane 2); cells cultured on plastic in the presence or absence of PMA (lanes 3 and 4, respectively).
Molecular mass markers are indicated on the right.
Figure 5.8  Cellular processing of MT1-MMP to the 45 kDa form is mediated by a matrix metalloproteinase

HT1080 cells were cultured on fibronectin under serum free conditions, with or without protease inhibitors for 48 hours. The conditioned medium was harvested for zymography and the cells lysed for Western blot analysis.

A. Western blot: HT1080 cell lysates were prepared and subjected to 10% SDS-PAGE. Proteins were transferred to nitrocellulose by electroblotting and probed with a polyclonal antibody to MT1-MMP. Cells cultured on fibronectin (lanes 1-4), alone (lane 1), or in the presence of metalloproteinase inhibitor CT1746 at 1 μM (lane 2), recombinant MMP inhibitors TIMP-2 and TIMP-1 at 150 nM (lanes 3 and 4, respectively). Cells cultured on laminin-1 (lane 5).

B. The conditioned medium was analysed by gelatin zymography. Cells cultured on fibronectin (lanes 1-4), alone (lane 1), or in the presence of metalloproteinase inhibitor CT1746 at 1 μM (lane 2), recombinant MMP inhibitors TIMP-2 and TIMP-1 at 150 nM (lanes 3 and 4 respectively). Cells cultured on laminin-1 (lane 5). Molecular mass markers are indicated on the right.
Figure 5.9  Cellular processing of MT1-MMP to the 45 kDa form is inhibited by TIMP-2, but not TIMP-1 or N-TIMP-1

HT1080 cells were cultured on fibronectin under serum free conditions, with or without added recombinant TIMPs (150 nM). The conditioned medium was harvested for zymography and the cells lysed for Western blot analysis.

A. The conditioned medium was analysed by gelatin zymography. Cells cultured on fibronectin (lanes 1-4), alone (lane 1), or in the presence of TIMP-2 (lane 2), TIMP-1 (lane 3) or N-TIMP-1 (lane 4). Cells cultured on laminin-1 (lane 5).

B. Western blot: HT1080 cell lysates were prepared and subjected to 10% SDS-PAGE. Proteins were transferred to nitrocellulose by electroblotting and probed with a polyclonal antibody to MT1-MMP. Cells cultured on fibronectin (lanes 1-4), alone (lane 1), or in the presence of TIMP-2 (lane 2), TIMP-1 (lane 3) or N-TIMP-1 (lane 4). Cells cultured on laminin-1 (lane 5), or treated with PMA (lane 6).

Molecular mass markers are indicated on the right.
Figure 5.10 The addition of anti-MT1-MMP antibodies to HT1080 cells cultured on fibronectin: effects on GLA activation and MT1-MMP processing to the 45 kDa form

HT1080 cells were cultured on fibronectin under serum free conditions, alone or in the presence of antibodies to MT1-MMP. The conditioned medium was harvested for zymography and the cells lysed for Western blot at 48 hours.

A. Gelatin zymography: cells cultured on fibronectin (lanes 1-4), in the presence of control antibody 2G12 (lane 1), anti-MT1-MMP antibody 4G6 (lane 2), non-immune sheep IgG (lane 3) or sheep anti-MT1-MMP antibody N175 (lane 4).

B. Western blot: HT1080 cell lysates were prepared and subjected to 10% SDS-PAGE. Proteins were transferred to nitrocellulose by electroblotting and probed with a polyclonal antibody to MT1-MMP. Lane order as for part A.

Molecular mass markers are indicated on the right.
Figure 5.11 The addition of a polyclonal anti-GLA antibody to HT1080 cells cultured on fibronectin: effects on GLA activation and MT1-MMP processing to the 45 kDa form

HT1080 cells were cultured on fibronectin under serum free conditions, in the presence of a sheep anti-GLA antibody (K399; lane 2), or in the presence of non-immune sheep IgG control (lane 1). The conditioned medium was harvested for zymography (A) and the cells lysed for Western blot at 48 hours. Lysate proteins were transferred to nitrocellulose by electroblotting and probed with a polyclonal antibody to anti-MT1-MMP (B). Molecular mass markers are indicated on the right.
Chapter 6

Purification of 45 kDa MT1-MMP
Chapter 6 Purification of 45 kDa MT1-MMP

6.1 Introduction

Precise details of the 45 kDa cleavage site in MT1-MMP would aid in determining the role of this molecule in cell surface proteolysis/MMP activation cascades. To obtain the 45 kDa MT1-MMP sequence, strategies are required for the purification of native MT1-MMP. Several difficulties may be encountered in the purification of integral membrane proteins. Large quantities of cells or tissue are required to compensate for tiny amounts of target protein present in the membrane. Often, high concentrations of detergents are needed throughout the purification procedure to maintain the solubility of the membrane protein. In this chapter, native MT1-MMP was extracted from detergent-solubilised HT1080 cells by immunoprecipitation, using a specific anti-MT1-MMP antibody. The yield from this technique was typically very low. Therefore, as an alternative, recombinant MT1-MMP was cleaved with MMPs in an attempt to generate the '45 kDa' truncation.

Knowledge of the N-terminal sequence of 45 kDa MT1-MMP is needed to engineer recombinant 45 kDa MT1-MMP. This could be used to predict whether MT1-MMP/TIMP-2 interactions are destroyed upon truncation to 45 kDa. Furthermore, the hypothesis that 45 kDa MT1-MMP is catalytically inactive could be tested with recombinant 45 kDa MT1-MMP. N-terminal sequence data would also aid in the design of neo-epitope antibodies. An antibody that recognises the newly exposed N-terminus of the 45 kDa form would enable tracking of 45 kDa MT1-MMP throughout the cell. As the generation of 45 kDa MT1-MMP is concomitant with GLA activation, a neo-epitope antibody may prove a useful marker of MT1-MMP/GLA activity in diseased tissue.
6.2 Results

6.2.1 Purification of native 45 kDa MT1-MMP for N-terminal sequencing

6.2.1a Cleavage of MT1-MMP in vitro to yield the 45 kDa form

A cell lysate or membrane preparation in which the majority of MT1-MMP is converted to 45 kDa would make an ideal starting material for purification purposes. As the cleavage of 60 kDa MT1-MMP to 45 kDa is MMP mediated, the ability of MMPs to process membrane-bound MT1-MMP to 45 kDa in vitro was tested.

Firstly, cell membranes were prepared from HT1080 cells stably transfected with wild type MT1-MMP (details of this cell line in Chapter 2). To monitor cleavage to 45 kDa, a membrane preparation enriched in 60 kDa MT1-MMP was generated by culturing the cells in the presence of the inhibitor CT1746 prior to harvest. The CT1746 was then washed from the membrane preparation (see methods, Chapter 2). A quenched fluorescent peptide assay was used to estimate the amount of free (active) MT1-MMP in the preparation (kindly performed by Dr G. Butler, University of East Anglia, Norwich, UK). Active MT1-MMP was estimated at 23 pmol/mg of membrane protein, which was considered a highly significant activity, as Butler et al. (1998) showed that a similar membrane preparation of 1.52 pmol active MT1-MMP per mg of protein was capable of activating recombinant proGLA.

Based on the value of 23 pmol/mg protein, active MMPs were added at a 1:1 molar ratio with native MT1-MMP, with the exception of recombinant MT1-MMP, which was added at a 5:1 molar ratio. Incubation at 37°C for 4 hours resulted in the generation of the 45 kDa form of MT1-MMP, with active preparations of GLA, MT1-MMP, SL-1 and CL-3 all capable of cleaving native MT1-MMP to a form apparently identical to 45 kDa MT1-MMP (Fig. 6.1). Incubation of the membrane preparation alone also resulted in the generation of a 45 kDa band, but to a lesser extent than when active MMPs were added, demonstrating intrinsic MT1-MMP processing activity in the membrane preparation (Fig. 6.1).
The proteolytic processing of 60 kDa MT1-MMP was monitored over 8 hours at 37°C, to select a time point at which the majority of MT1-MMP is cleaved to 45 kDa. As expected from the results in Figure 6.1, a three hour incubation with GLA resulted in a considerable increase in the level of the 45 kDa band (Fig. 6.2). However, incubations of five hours or longer resulted in a loss of both 60 and 45 kDa MT1-MMP, presumably due to non specific proteolysis.

It was apparent that cleavage of native MT1-MMP in the test tube would not lead to a preparation of MT1-MMP that was predominantly 45 kDa. This method showed no advantage over stimulating HT1080 cells in culture to produce 45 kDa MT1-MMP prior to harvest. PMA treatment was chosen as the best option for stimulating 45 kDa MT1-MMP production; culturing the cells on fibronectin was considered impractical due to the costs involved in coating the 175 cm² culture flasks.

6.2.1b Immunoprecipitation of MT1-MMP and Protein-G Sepharose purification

The polyclonal antibody to MT1-MMP (N175) was used to immunoprecipitate MT1-MMP from HT1080 cell lysates. Lysates prepared from PMA treated HT1080 cells were pre-cleared with pre-immune sheep IgG, treated with N175, and the resulting immunoprecipitates collected by Protein-G Sepharose chromatography. Western blot analysis of the immunoprecipitates showed that 63, 60 and 45 kDa MT1-MMP were precipitated by N175 (Fig. 6.3). Unfortunately, diffuse bands of IgG at approximately 55 kDa overshadowed the 63 and 60 kDa forms of MT1-MMP, a consequence of using the same antibody for both immunoprecipitation and Western analysis. In this case, the anti-sheep IgG secondary antibody used in the Western blot detected the N175 IgG added during immunoprecipitation (Fig. 6.3). This problem could have been avoided if a second anti-MT1-MMP antibody (raised in different species) was available. However, the mouse anti-MT1-MMP peptide antibody 113 failed to immunoprecipitate MT1-MMP from cell lysates (S.J. Atkinson, personal communication), and was not suitable for the Western blot detection of immunoprecipitated MT1-MMP. 113 has a weak affinity for the 45 kDa form of MT1-MMP, the reason for which is unclear.
MT1-MMP was immunoprecipitated from lysates with N175 and transferred to PVDF membrane. The membrane was stained with Ponceau S, and the 45 kDa band of MT1-MMP positively identified by Western blot analysis (data not shown). This band was excised from the PVDF membrane, subjected to N-terminal sequencing (performed by Dr Bryan Smith, Celltech Ltd, Slough, UK), and found to be N-terminally blocked. Further purifications of MT1-MMP using this technique also proved to be N-terminally blocked.

Reverse-phase HPLC chromatography was investigated as an alternative method for the purification of MT1-MMP from immunoprecipitated cell lysates. Immunoprecipitates were adsorbed to a column of Protein-G Sepharose, and bound proteins were eluted with guanidinium-HCl. A second elution of Laemmli reducing sample buffer was used to remove any proteins remaining on the column. Western blot analysis of the column fractions showed that guanidinium-HCl was successful in eluting the bound protein (Fig. 6.4), but distorted the gel, making it difficult to distinguish MT1-MMP and IgG bands. The proteins eluted with guanidinium-HCl were applied to a C4 column, and subjected to reverse-phase HPLC (performed by W. English, School of Biological Sciences, University of East Anglia, Norwich, UK). A gradient of 0-100% A-B (A=H2O/ 0.1% trifluoroacetic acid, B= 60% isopropanol/40% acetonitrile/0.1% trifluoroacetic acid) eluted a single peak (data not shown). The peak was analysed by W. English, using SDS-PAGE (reducing conditions), followed by silver stain. Two bands were visible at approximately 55 kDa and 29 kDa, the molecular mass of the heavy and light chains of IgG respectively (Harlow and Lane, 1988). No 45 kDa MT1-MMP was apparent.

In this case, it is likely that the level of MT1-MMP applied to the C4 column was too low. Fig. 6.4, lanes 2 and 3 show that most of the MT1-MMP present in the immunoprecipitation mixture did not bind Protein-G Sepharose. It would appear that the problem did not lie with N175/Protein-G Sepharose binding, as there were no IgG bands of 55 kDa apparent in the unbound material (Fig. 6.4, lane 2). Rather, the data suggests that much of the MT1-MMP was not immunoprecipitated by N175. This antibody was raised to partially refolded Δ511–582 TM-MT1-MMP (d'Ortho et al., 1998). By dot-blot analysis
N175 recognised reduced, SDS-denatured Δ502-559 TM-MT1-MMP very well, but reacted poorly with untreated ('native') Δ502-559 TM-MT1-MMP (data not shown). In conclusion, immunoprecipitation of MT1-MMP from HT1080 cell lysates with N175 did not yield sufficient material to support further purification by HPLC.

Chromatographic procedures used to purify other MMPs were considered, in order to bypass the immunoprecipitation step prior to HPLC purification. Red Sepharose CL-6B has been used to purify recombinant human stromelysin (Koklitis et al., 1991), but does not perform well in concentrated detergent conditions. The concentration of Triton X-100 in the lysis buffer was reduced from 1% to 0.1% (v/v), but unfortunately MT1-MMP did not extract into the aqueous phase (data not shown).

6.2.2 MMP cleavage of recombinant MT1-MMP

An alternative approach was needed to generate 45 kDa MT1-MMP for sequencing. As native MT1-MMP is susceptible to cleavage by MMPs to yield a 45 kDa form, recombinant MT1-MMP should likewise be susceptible. A soluble recombinant transmembrane deletion mutant of MT1-MMP (Δ502-559 TM-MT1-MMP) was incubated with active MMPs for four hours at 37°C. Cleavage products were analysed by SDS-PAGE and silver stain. Incubation of pro-ΔTM-MT1-MMP alone resulted in a drop in molecular mass of from 60 kDa to 52 kDa (Fig. 6.5A, lanes 1 and 2), probably corresponding to the loss of the pro-peptide (Will et al., 1996). Further processing products at 34 and 32 kDa were evident (Fig. 6.5A, lane 2). Allowing for the lack of a transmembrane domain (approximately 5 kDa) in ΔTM-MT1-MMP, the predicted molecular mass of the '45 kDa' form of MT1-MMP was approximately 40 kDa. However, no band of 40 kDa was apparent when ΔTM-MT1-MMP was incubated alone (Fig. 6.5A, lane 2), or when ΔTM-MT1-MMP was incubated with equimolar quantities of pre-activated ΔTM-MT1-MMP (Fig. 6.5A, lane 4). This indicated that self cleavage of ΔTM-MT1-MMP to 40 kDa did not occur. Incubation of ΔTM-MT1-MMP with equimolar concentrations of active SL-1 or active CL-3 (Fig. 6.5A, lanes 5 and 6 respectively) also did not result in bands of 40 kDa. Very faint bands between 35 and 45 kDa were apparent
in GLA treated MT1-MMP (Fig. 6.5A, lane 3), however, these bands corresponded to GLA break down products, and were also present in the active GLA control (Fig. 6.5A, lane 7). Interestingly, incubation with active GLA and CL-3 resulted in the processing of 60 and 52 kDa bands of ΔTM-MT1-MMP to the 34/32 kDa doublet (Fig. 6.5A, lane 6). Active CL-3 incubated alone autocatalytically degraded (lane 10), as has been observed previously in our laboratory (Knäuper et al., 1997).

It is possible that low levels of 40 kDa fragment were generated by MMP cleavage, but not detected by silver stain. Cleavage products were analysed by Western blot with N175 antibody, which detects nanogram quantities of MT1-MMP. Incubation of pro-ΔTM-MT1-MMP alone (Fig. 6.5B, lane 2), or with active ΔTM-MT1-MMP (Fig. 6.5B, lane 4) did not generate bands of 40 kDa. The autocatalytic breakdown of active ΔTM-MT1-MMP (incubated alone for four hours) also did not apparently proceed via a 40 kDa step (Fig. 6.5B, lane 2). Active GLA, SL-1 and CL-3 did not cleave soluble ΔTM-MT1-MMP to 40 kDa (Fig. 6.5B, lanes 3, 5 and 6, respectively). The chemiluminescent detection step for the Western blot was left to develop overnight, but no bands of 40 kDa appeared (data not shown). ΔTM-MT1-MMP was incubated with active GLA for longer periods, after 18 hours the ΔTM-MT1-MMP was processed entirely to the 34/32 kDa doublet, but no 40 kDa was detected by silver stain analysis (data not shown).

Native MT1-MMP may be complexed with TIMP-2, which could alter the susceptibility of MT1-MMP to MMP cleavage. Recombinant active ΔTM-MT1-MMP/TIMP-2 complexes were formed, and then incubated with active GLA for five hours at 37°C. Silver stain analysis of the cleavage products (Fig. 6.6) show that the addition of TIMP-2 did not alter the susceptibility of ΔTM-MT1-MMP to GLA action, and no 40 kDa bands were detected. This data does not rule out a rapid transition from the 52 kDa recombinant MT1-MMP to 34 kDa via an unstable 40 kDa intermediate. It is possible that the 40 kDa form of MT1-MMP is rapidly broken down to 34 kDa.
6.3 Discussion

Several predictions can be made about the nature of the 45 kDa form of MT1-MMP. It is associated with the membrane fraction of cells, implying that the transmembrane domain is intact. Consistent with this observation, Lohi et al. (1996) described a 43 kDa form of MT1-MMP that was detected using a peptide antibody raised to the C-terminal intracellular domain of MT1-MMP. Simple calculations therefore predict that the proteolytic truncation occurs in the catalytic domain.

Pin-pointing the exact cleavage site in MT1-MMP is difficult due to discrepancies between the predicted molecular mass (calculated from the amino acid sequence) and the molecular mass estimated by Western blot. In HT1080 cell lysates, active MT1-MMP has an apparent molecular mass of 60 kDa; calculations from the amino acid sequence predict that the catalytic, hinge and C-terminal domains (including the transmembrane region and cytoplasmic tail) account for 54 kDa. MT1-MMP has two potential glycosylation sites: one in the catalytic domain at position Asn 130, and the other in the hinge region at position Asn 311 (Fig. 6.7). Each glycosylation has the potential to add 3-5 kDa to the molecular mass of MT1-MMP. One could assume, therefore, that at least one of the sites is glycosylated. However, results from our laboratory would indicate otherwise, for treatment of native MT1-MMP with N-glycanase (a de-glycosylating enzyme), did not lead to a reduction in molecular mass (Dr G. Butler, S. Atkinson, personal communication).

A fragment of MT1-MMP comprising residues 187-582 would have a mass of 45 kDa, assuming that MT1-MMP is not glycosylated. Several authors describe a truncated form of MT1-MMP at 43 kDa (Lohi et al., 1996; Cowell et al., 1998), which would correspond to residues 207-582. The estimation of molecular mass from SDS-PAGE is subject to a reasonable degree of error. Therefore, taking an average of 44 kDa, and assuming a +/- 2 kDa deviation, the cleavage site in MT1-MMP may occur anywhere between residues 178 and 215 (see Fig. 6.7). Further predictions can be made on the basis that proteolysis of MT1-MMP to the 45 kDa form is MMP dependent. Nagase et al. (1991) published a listing of seventy ECM, MMP and serpin sequences cleaved by MMPs (but not MT-
MMPs). These sequences were compared by eye with residues 178-215 of MT1-MMP, but there was no obvious sequence homologous to the cleavage site sequences published in the report by Nagase and co-authors. However, a set of fluorogenic peptides has been used to examine the P1' specificity of MT1-MMP itself, and the rate of hydrolysis was greatest for peptide Dnp-Arg-Pro-Leu-Ala-Tyr-Trp-Arg-Ser-NH₂₂, with the cleavage occurring between Ala and Tyr, at P₁ and P₁' respectively (Imper and van Wart, 1998). An Ala-Tyr pair occurs at position 202-203 in the sequence of MT1-MMP (Fig. 6.7), furthermore, residue 204 is a phenylalanine, and bulky hydrophobic residues are favoured by MMPs in the P₂' position (Imper and van Wart, 1998). There is limited information available regarding the P₃' specificity of MMPs, so it is difficult to predict how the proline at residue 205 would affect cleavage specificity; however the P₄' position on the substrate probably does not interact with the enzyme (Imper and van Wart, 1998). Therefore, based on the information available, a cleavage between Ala 202 and Tyr 203 residues is the best prediction.

Any cleavage between residues 178-215 of the catalytic domain of MT1-MMP would lead to a loss of the insert unique to MT-MMPs (Fig. 6.6), but would leave the zinc box intact. Furthermore, cleavage between residues 178-215 could interfere with the coordination of the second zinc ion and the two calcium ions. These ions are essential for maintaining the stability of the catalytic site of the homologous structures stromelysin and neutrophil collagenase (Bode et al., 1994; van Doren et al., 1995). Most of the residues that coordinate 'structural' zinc and calcium ions in neutrophil collagenase are conserved in the MMPs, and are located between residues 180 and 200 of MT1-MMP. Therefore, it is hypothesised that if MT1-MMP is cleaved to 45 kDa somewhere between residues 178 and 215, the severe disruption to the catalytic site would not only render it catalytically inactive, but could also interfere with the binding of TIMP inhibitors.

To test these hypotheses, quantities of purified 45 kDa MT1-MMP suitable for N-terminal sequencing were required. Methods for generating 45 kDa MT1-MMP enriched cell lysates/membranes were investigated. The first approach taken was to incubate
membranes enriched in the 60 kDa form of MT1-MMP with active MMPs in vitro. Interestingly, bands apparently identical to the 45 kDa form were generated by all of the MMPs tested, including a gelatinase, stromelysin, collagenase and MT1-MMP itself. Clearly the cleavage site is very susceptible to MMP action. After 4 hours incubation with the enzymes, only a proportion of the MT1-MMP had been cleaved to 45 kDa, and so longer incubations were attempted. Unfortunately, extending the incubation did not increase the quantities of 45 kDa MT1-MMP generated. In fact, a 3 hour incubation yielded, at best, a membrane preparation that was approximately 1:1 of 60/45 kDa, according to the Western blot. Therefore, the next approach was to promote cleavage to 45 kDa in cell culture.

PMA treatment proved the simplest method for promoting conversion to 45 kDa MT1-MMP in HT1080 cells. The polyclonal anti-MT1-MMP antibody N175, which performed well in Western blots, was used to immunoprecipitate 45 kDa MT1-MMP from PMA treated cell lysates. 45 kDa MT1-MMP was transferred to PVDF membrane; Ponceau S staining of the membrane revealed that sufficient 45 kDa MT1-MMP was obtained for N-terminal sequencing. However, repeated attempts to sequence the excised band were frustrated by N-terminal blocking of the protein. Assuming that the 45 kDa form of MT1-MMP is not naturally blocked, a blockage must have occurred during the lysis, immunoprecipitation or electroblotting procedures. Care was taken to use high quality reagents, and to remove reactive species from the SDS-polyacrylamide gel, but to no avail.

Immunoprecipitation procedures were repeated, but reverse-phase HPLC was substituted for SDS-PAGE as a means of separating the MT1-MMP. Material eluted from the Protein-G Sepharose column did not contain sufficient MT1-MMP to obtain a peak from the HPLC column. Failure to purify enough MT1-MMP by immunoprecipitation reflected the inability of N175 to recognise epitopes on native MT1-MMP, as this antibody was raised to denatured material. Other antibodies were sought that have a strong affinity for the 45 kDa form of MT1-MMP. At the time of writing, there were no suitable antibodies available.
Alternative strategies were needed to partially purify MT1-MMP, ready for a final step of reverse-phase HPLC purification. The high concentrations of detergent used in the cell lysis ruled out chromatographic procedures traditionally used for MMP purification. Other extraction methods were tried, but those that successfully extracted MT1-MMP all contained detergents such as Lubrol, NP40 and sodium deoxycholate. MMP/hydroxamate-inhibitor interactions have been used to purify MMPs (Moore et al., 1986). Although severe disruption of the catalytic domain is predicted on truncation of MT1-MMP to 45 kDa, binding to hydroxamate inhibitors may be retained. Future experiments could test the binding of membrane-bound MT1-MMP forms to Pro-Leu-Gly-Leu linked to Sepharose. This is known to be an affinity ligand for the soluble form of MT1-MMP.

Surprisingly, attempts to generate a '45 kDa' form of MT1-MMP from cleavage of recombinant material failed. The recombinant ΔTM-MT1-MMP employed for these studies was susceptible to MMP action; incubation with GLA and CL-3 generated a doublet of 34/32 kDa. Incubation of active ΔTM-MT1-MMP alone also generated the doublet, but the proteolytic degradation did not appear to proceed via a form equivalent to the 45 kDa MT1-MMP. The MT1-MMP/TIMP-2 'receptor' hypothesis discussed in Chapter 5 predicts that the majority of MT1-MMP is bound to TIMP-2 at the cell surface, with adjacent, proteolytically active TIMP-2 'free' MT1-MMP molecules. It is an open question as to which MT1-MMP molecule is cleaved to 45 kDa, however, it is possible that TIMP-2 acts to stabilise MT1-MMP in a conformation that exposes the 45 kDa cleavage site. In an attempt to cleave recombinant ΔTM-MT1-MMP to a '45 kDa' equivalent, the recombinant active MT1-MMP was complexed with TIMP-2 prior to incubation with active GLA, but no 45 kDa equivalent was generated.

Alternatively, transmembrane anchorage of MT1-MMP may induce a conformation that exposes the 45 kDa cleavage site. One option that was not explored was the linkage of ΔTM-MT1-MMP to a solid phase, such as heparin-agarose. Data from our laboratory has indicated that both ΔTM-MT1-MMP and GLA have heparin binding sites in the
hemopexin domain (Butler et al., 1998). The addition of soluble heparin potentiates the rate of proGLA activation by ΔTM-MT1-MMP, presumably by concentrating and colocalising the two components (Butler et al., 1998). Future experiments could investigate whether the binding of ΔTM-MT1-MMP to heparin or heparin-agarose is enough to induce a conformational change, such that the molecule is cleaved to 45 kDa, either autolytically, or by heparin bound active GLA.

In conclusion, future attempts to generate sufficient MT1-MMP for N-terminal sequencing will require either i) an antibody that has a high affinity for native 45 kDa MT1-MMP for immunoprecipitation purposes, or ii) the development of other chromatographic procedures to partially purify 45 kDa MT1-MMP from cell lysates/membranes. A cell line overexpressing MT1-MMP would be an advantage, due to increased levels of the 45 kDa form in overexpressing cells.
6.4 Addendum

Subsequent to the completion of this chapter, a report was published describing the purification and N-terminal sequencing of a recombinant soluble MT1-MMP truncated to 43 kDa. The authors (Lehti et al., 1998) also reported difficulties with obtaining the N-terminal sequence of the wild type truncated (43 kDa) form of MT1-MMP. Therefore, a novel approach was used to obtain sufficient enzyme for sequencing. A recombinant ΔTM-MT1-MMP vector was engineered with six additional histidine residues at the C-terminal end and transfected into HT1080 cells. Stably transfected clones were selected and the conditioned medium immunoblotted to show that the main forms of MT1-MMP migrated on SDS-PAGE at slightly less than wild-type 60 and 43 kDa forms of MT1-MMP. The soluble '43' kDa form was then purified from the conditioned medium using a metal-affinity resin, transferred to PVDF membrane and analysed by N-terminal sequencing. This form of MT1-MMP had an N-terminal amino acid of Ile 256, with a calculated molecular mass of 37.8 kDa, although it migrated at approximately 43 kDa on SDS-PAGE. As we had predicted, this truncated form of MT1-MMP lacked the insert region in the catalytic domain that is unique to MT1-MMP, but also, surprisingly, the zinc-binding region (Fig. 6.7). Of great concern, the predicted Ala 255-Ile 256 cleavage occurs in the region of the 'methionine-turn', a conserved region in the MMPs. This tight turn at Met 257 is buried adjacent to the highly hydrophobic active site helix (Fernandez-Catalan et al., 1998) and one would predict that it is not accessible for MMP cleavage. In view of our experience that soluble forms of MT1-MMP are processed differently to the membrane associated enzyme, it is not clear whether this soluble cleavage event is comparable to the membrane-bound product. Efforts to sequence wild-type 45 kDa MT1-MMP are ongoing in our laboratory, for comparison with the sequence reported by Lehti and coworkers.
Figure 6.1 Incubation of membrane bound native MT1-MMP in vitro with MMPs

Cell membranes enriched in the 60 kDa form of MT1-MMP were prepared from cultures of MT1-MMP transfected HT1080 cells grown in the presence of CT1746 inhibitor. Membrane preparations were incubated alone, or in the presence of active MMPs for 4 hours at 37°C. The cleavage reaction was stopped by the addition of Laemmli sample buffer, and the protein mixture separated by 10% SDS-PAGE, electroblotted to nitrocellulose and probed with the polyclonal antibody to MT1-MMP. Cell membranes incubated alone (lane 2), or in the presence of active GLA (lane 3), active ΔTM MT1-MMP (lane 4), active SL-1 (lane 5), active CL-3 (lane 6). Cell membranes, unincubated (lane 1). Recombinant active ΔTM MT1-MMP, incubated alone (lane 7). Molecular mass markers are indicated on the right.
Figure 6.2 Cleavage of native MT1-MMP with MMPs: a time course study

Cell membranes enriched in the 60 kDa form of MT1-MMP were prepared from cultures of MT1-MMP transfected HT1080 cells grown in the presence of CT1746 inhibitor. Membrane preparations were incubated alone, or in the presence of active GLA at 37°C for the times indicated. The cleavage reaction was stopped by the addition of Laemmli sample buffer, and the protein mixtures separated by 10% SDS-PAGE, electroblotted to nitrocellulose and probed with the polyclonal antibody to MT1-MMP. Molecular mass markers are indicated on the right.
Figure 6.3 Immunoprecipitation of MT1-MMP from HT1080 cell lysates

Cell lysate was prepared from HT1080 cells treated with PMA. MT1-MMP was immunoprecipitated from the lysate using a polyclonal antibody to MT1-MMP (N175). Immunoprecipitates were reduced and separated by 10% SDS-PAGE, transferred to nitrocellulose by electroblotting and probed N175. The blue arrow indicates the immunoprecipitated MT1-MMP and the black arrows indicate the N175 IgG present in the immunoprecipitation mixture, which was detected by the secondary antibody. Molecular mass markers are indicated on the right.
Figure 6.4 Purification of MT1-MMP immunoprecipitates for reverse-phase HPLC
Lysates from HT1080 cells treated with PMA were immunoprecipitated with a sheep anti-MT1-MMP antibody. Immunoprecipitates were passed down a column of Protein G Sepharose and eluted with 25 mM Tris/HCl pH 7.5 containing 8 M guanidinium-HCl (eluate 1), followed by Laemmli reducing buffer (eluate 2). Column fractions (10 µl) were separated by 10% SDS-PAGE, proteins transferred to nitrocellulose by electroblotting and probed with the sheep anti-MT1-MMP antibody. PMA treated HT1080 lysate (lane 1); proteins not bound by Protein G Sepharose (lane 2); column washes (lanes 3-7); eluate 1 (lanes 8-11); column washes (lanes 12-14) and eluate 2 (lane 15). Molecular mass markers are indicated on the left.
Figure 6.5 Cleavage of recombinant ΔTM MT1-MMP with active MMPs

Recombinant ΔTM MT1-MMP was incubated alone, or in the presence of recombinant MMPs for 4 hours at 37°C. The cleavage reaction was stopped by the addition of Laemmli sample buffer, and the protein mixture separated by 10% SDS-PAGE.

A. Silver stain analysis: recombinant ΔTM MT1-MMP incubated alone (lane 2) or in the presence of active GLA (lane 3), active ΔTM MT1-MMP (lane 4), active SL-1 (lane 5), active CL-3 (lane 6). Control MMPs, unincubated: pro-ATM MT1-MMP (lane 1), active GLA (lane 7), active SL-1 (lane 8), active CL-3 (lane 9). Molecular mass markers are indicated on the right.

B. Western blot analysis: proteins separated by SDS-PAGE were electroblotted onto nitrocellulose and probed with a polyclonal antibody to MT1-MMP. Recombinant ΔTM MT1-MMP incubated alone (lane 2) or in the presence of active GLA (lane 3), active ΔTM MT1-MMP (lane 4), active SL-1 (lane 5), active CL-3 (lane 6). Control MMPs, unincubated: pro-MT1-MMP (lane 1), active GLA (lane 7), active SL-1 (lane 8), active CL-3 (lane 9). Molecular mass markers are indicated on the right.
Figure 6.6 Cleavage of recombinant ΔTM MT1-MMP/TIMP-2 complex with GLA

Recombinant active ΔTM MT1-MMP was complexed with recombinant TIMP-2 for 1 hour at 25°C. Active ΔTM MT1-MMP/TIMP-2 complexes were then incubated alone or in the presence of recombinant active GLA for 5 hours at 37°C. The cleavage reaction was stopped by the addition of Laemmli sample buffer, the protein mixture separated by 10% SDS-PAGE and analysed by silver stain. Active ΔTM MT1-MMP/TIMP-2 complexes incubated alone (lane 5), or in the presence of active GLA (lane 6). Active ΔTM MT1-MMP/TIMP-2 complexes unincubated (lane 4). Control lanes: unincubated pro-ΔTM MT1-MMP (lane 1); pro-ΔTM MT1-MMP incubated alone (lane 2), pro-ΔTM MT1-MMP incubated with active GLA (lane 3); unincubated TIMP-2 alone (lane 7) and unincubated active GLA (lane 8). Molecular mass markers are indicated on the right.
Figure 6.7 Schematic representation and amino acid sequence of MT1-MMP

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A. Schematic representation of MT1-MMP domains.

B. Amino acid sequence, modified from Sato et al, 1994. The colour code used for the sequence corresponds to the colours used in A., which represent the various functional domains of MT1-MMP. The furin cleavage site is marked with an arrow and the potential glycosylation sites are marked with stars. The arrowhead indicates the Ala 255-Ile 256 cleavage site at the N-terminus of the 43 kDa form of MT1-MMP reported by Lehti et al., 1998.
Chapter 7

General Discussion
Chapter 7 General Discussion

Cell-matrix interactions affect a diverse range of cellular functions including cell differentiation, migration, proliferation and survival. Information provided by the ECM via cell surface receptors can control processes of embryonic growth and differentiation, tissue remodelling and repair. It follows that localised proteolytic degradation of the ECM, and the release of matrix fragments and matrix bound growth factors and cytokines will have a profound effect on cell behaviour. This thesis examines the role of MMPs in the cellular response to the pericellular environment, with particular reference to GLA and its activators. Data from this thesis has led to two main publications and this discussion has been divided accordingly.

A. The recognition sites of the integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ within collagen IV are protected against GLA attack in the native protein

The aim of this study was to assess the susceptibility of three different solubilised forms of type IV collagen to GLA cleavage, and to investigate the concomitant effects on cell binding to the collagen. The study was based on the observation that GLA cleaves collagen IV in close proximity to the CB3[IV] region of collagen IV, that contains the recognition sites for $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins. It was hypothesised that cleavage in this region might impair integrin/collagen IV interactions, thus affecting cell attachment. The approach of the study was to compare cell adhesion to intact collagen IV preparations with cell adhesion to collagen IV fragments generated by prior incubation with GLA. Collagen IV preparations solubilised by different methods were compared.

This study yielded unexpected results, which are of significance to the design of future experimental models incorporating solubilised collagen IV. Two preparations of collagen IV monomers, extracted from EHS tumours using ostensibly identical conditions, proved to have markedly different susceptibilities to GLA action. Preparation B was not cleaved by GLA even when the temperature was raised to 37°C. A closer examination of the preparations by non-reducing SDS-PAGE, revealed that the intramolecular disulphide bonds were reduced to a greater extent in preparation A than in preparation B. Cell
adhesion studies showed that the integrin binding sites in preparation A were largely destroyed by GLA action, whereas the integrin binding sites in preparation B remained intact.

Clearly, with regards to our original hypothesis, the interpretation of the data depended upon the preparation of collagen IV chosen for the study. This was of great concern, as the preparations of collagen IV monomers available commercially are isolated from EHS tumours under the same conditions as those used for preparations A and B. Several commercial preparations of collagen IV monomers were investigated using non-reducing SDS-PAGE, and cleaved with GLA. The results confirmed that the commercial preparations are subject to the same degree of variation in disulphide bonding as preparations A and B.

Collagen IV preparations solubilised by alternative extraction methods were then investigated. Dimeric collagen IV extracted from human placenta by bacterial collagenase proved resistant to GLA attack, reflecting the protection offered by the intact disulphide bonds in the NC1 region. Cell attachment to the collagen IV dimers was unaffected by GLA treatment. In contrast, tetrameric collagen IV isolated from the EHS tumour by pepsin treatment was very susceptible to cleavage by GLA, and this was attributed to the loss of the C-terminal NC1 domains upon pepsin extraction. Despite the extensive degradation by GLA apparent on SDS-PAGE analyses, the cell attachment to collagen IV was not affected.

It would make an interesting study to compare the collagen IV preparations as barriers in a cell invasion study, using a cell line that expresses high levels of active GLA (such as HT1080 cells). The original hypothesis for tumour cell migration put forward by Liotta and Stetler-Stevenson (1991) predicted that proteolytic dissolution of the matrix is required to allow movement of the cell through tissue barriers. Based on this hypothesis, one would predict that collagen IV preparations with intact intramolecular disulphide bonds would prove to be a greater barrier to cell invasion than preparations in which the disulphide
bonds were reduced, as the latter would be more susceptible to protease action. Such studies might highlight the need for careful choice of soluble collagen IV preparations when planning investigations in which GLA degradation is an issue.

Studies reported in this thesis shed light on some of the discrepancies in the literature regarding the susceptibility of collagen IV to gelatinase action. For instance, it can be surmised that the study by Mackay et al. (1990) in which collagen IV monomers were found to be resistant to GLA attack, reflects a high degree of intramolecular disulphide bonds in the CB3[IV] region of the monomers used. These authors make the point that GLA is a poor 'type IV collagenase' in comparison with other members of the MMP family such as stromelysin. Studies carried out in our laboratory agree with this statement, as both SL-1 and CL-3 proved to more efficient at degrading preparation B monomers than GLA or GLB.

Studies have shown that the presence of active GLA correlates with the invasive phenotype of breast and non-small lung carcinoma (Brown et al., 1993a; 1993b). This has been attributed to the type IV collagenolytic properties of GLA, which may facilitate invasion through host membranes. Support for this hypothesis has come from studies showing that TIMP-2 inhibits tumour cell invasion through amnion basement membrane, smooth muscle cell generated basement membrane, and Matrigel (Albini et al., 1991; DeClerck et al., 1991; DeClerck et al., 1992). It remains to be seen whether collagen IV incorporated into the macromolecular network is susceptible to GLA action. As the tumour cell invasion studies did not distinguish between GLA action and action of other MMPs, future studies should address whether the combined action of several MMPs leads to invasion of basement membranes.
B. The activation of GLA by HT1080 cells is promoted by culture on a fibronectin substrate and is concomitant with an increase in processing of MT1-MMP to a 45 kDa form

The aim of this study was to search for ECM molecules that influence the cellular activation of GLA. The study focussed on fibronectin, which is a multifunctional ECM protein that controls diverse cellular functions such as adhesion, migration, differentiation and cell fate. Fibronectin in solution is a highly flexible molecule, but adsorption to plastic or deposition into the matrix exposes activities of fibronectin that are latent in the soluble molecule (discussed in Chapter 1). Studies have shown that adsorption of plasma fibronectin onto plastic exposes cell adhesive activities (Ugarova et al., 1995). The approach taken in the current study was to examine GLA activation by HT1080 cells cultured on adsorbed fibronectin. Analysis of gelatinase expression by zymography revealed that the processing of GLA to the active form was promoted by fibronectin. A substrate of laminin-1 proved to be a suitable reference for these studies, as the GLA secreted by HT1080 cells cultured on laminin-1 was mostly in the pro-form. It has been published previously that PMA induces GLA activation by HT1080 cells (Lim et al., 1996; Lohi et al., 1996) and a comparison of the GLA activation induced by fibronectin and that induced by PMA treatment indicated that fibronectin had a potent effect on the GLA activation pathway. The fibronectin effect was a novel finding, as previously the induction of GLA activation had been attributed to collagen matrices only (Azzam and Thompson, 1992; Seltzer et al., 1994; Gilles et al., 1997; Tomasek et al., 1997; Haas et al., 1998).

The next approach was to use peptide fragments of fibronectin to determine whether the up-regulation of GLA activation was linked to interactions with a particular domain of fibronectin. Several fragments from the central cell binding domain encompassing the RGD integrin binding site of fibronectin induced GLA activation when adsorbed to culture plastic. This implied that integrin receptors might be signalling GLA activation. Cell adhesion studies to full length fibronectin confirmed that HT1080 cells adhere to fibronectin via α5β1 integrin only. Accordingly, antibodies to the α5 and β1 integrin subunits were used as substrates for HT1080 cell adhesion. The activation of GLA was
induced by both mAb 16 (anti-α5) and mAb 13 (anti-β1), both of which are known to cause integrin receptor clustering (Akiyama et al., 1989). For comparison, HT1080 cells were cultured on an antibody to the α6 integrin subunit, as HT1080 cells attach to laminin-1 via α6β1 integrin. The GLA secreted by cells cultured on this antibody was mostly in the pro-form, which correlated well with the level of GLA activation effected by a substrate laminin-1. The data implied that signals via the α5β1 integrin promote GLA activation by HT1080 cells. Further insight into the integrin signalling mechanism involved was gained by a comparison of two different anti-α5 integrin antibodies as culture substrates. GLA activation was promoted when the cells were cultured on mAb 16, which clusters integrins and mimics the action of the fibronectin ligand, whereas mAb 11, which clusters integrins but does not mimic ligand occupancy (Akiyama et al., 1989) failed to effect GLA activation.

Current models for integrin mediated signalling postulate that integrin cytoplasmic tails are associated with large aggregates of signal transduction molecules, immobilised on the membrane-associated cytoskeleton. Clustering of integrins caused by a multivalent ligand such as an ECM molecule, or an anti-integrin antibody has been shown to cause the transmembrane accumulation of a wide variety of cytoskeletal proteins and signalling molecules (reviewed by Yamada and Geiger, 1997). Anti-integrin antibodies have proved to be an invaluable tool in dissecting out these associations. Miyamoto et al. (1995b) used latex beads coated with mAb 11 in a fibroblast culture model to show that integrin clustering triggers the accumulation of twenty signal transduction molecules (including RhoA, Rac1, Ras, Raf1, ERK1 and 2, JNK1, PI3-kinase and PLC-γ), along with focal adhesion kinase (FAK) and the cytoskeletal protein tensin. Significantly, beads coated with mAb 16 or mAb 13 induced the further accumulation of the cytoskeletal proteins vinculin, talin, and α-actinin, as well as F-actin, filamin and paxillin. This process appeared to require tyrosine kinase activity in addition to cytoskeletal organisation, as in the presence of tyrosine kinase inhibitors, mAb 11 induced the transmembrane accumulation of tensin and FAK only, and mAb 16 and mAb 13 induced the accumulation of tensin, vinculin, talin, α-actinin and FAK only (see Fig. 7.1). As discussed in Chapter 4,
the addition of a monovalent ligand (such as an RGD peptide) to cells stimulated with mAb 11 restored the full complement of cytoskeletal proteins and signal transduction molecules attributed to treatment with mAb 16. Future studies could be aimed at investigating the importance of tyrosine kinase activity in the signalling of GLA activation in the HT1080 model. The tyrosine kinase inhibitors genistein and herbimycin A would be useful tools for preliminary studies (Miyamoto et al., 1995b), and might give some insight into the types of cytoskeletal proteins and signalling models required for GLA activation to proceed.

An exciting development over the last few years has been the discovery that integrin receptors colocalise and act in synergy with growth factor receptors. Many of the signalling pathways downstream of integrin clustering are in common with signalling pathways activated by growth factor receptor ligation (for review, see Dedhar and Hannigan, 1996). Using a similar approach to that described above, Miyamoto et al. (1996) used beads coated with fibronectin, mAb 13 or mAb K20 (similar in action to mAb 11) to show that the ERK class of MAP kinases is activated by integrin aggregation alone, or by various growth factors. However, in the presence of integrin receptor occupancy the growth factors EGF, PDGF or bFGF caused a marked and additive increase in ERK activity. Each of the receptors for EGF, PDGF or bFGF were induced to accumulate at sites of integrin-ligand aggregation, even in the absence of the growth factor ligands. The combination of integrin occupancy and growth factor ligand also led to a synergistic increase in growth factor receptor tyrosine phosphorylation (Miyamoto et al., 1996). Others have also reported colocalisation and synergism between growth factors and integrin receptors (Plopper et al., 1995; Schwartz et al., 1995; Schneller et al., 1997). Fibroblast adhesion to fibronectin in the presence of PDGF stimulates phosphatidyl inositol hydrolysis (McNamee et al., 1993). Specifically, McNamee and coworkers reported that integrin ligation increases the activity of PI5-kinase via the regulatory molecule Rho, generating the substrate 4,5-PIP2, while PDGF concomitantly activates PLC-γ to hydrolyse the substrate and generate second messengers. These studies highlight the need to consider the presence of endogenous growth factors in the current experimental
model. However, it must be stressed that the cultures were serum-free and it has been reported that after extensive serum starvation integrins cannot synergise with growth factors for MAP kinase activation (Miyamoto et al., 1996).

Foda et al. (1996) reported that PMA induction of GLA activation by human umbilical vein endothelial cells is mediated by protein kinase C (PKC) and is accompanied by an increased synthesis of MT1-MMP. Others have shown that PKC mediates the IL-1α induced increase of CL-1 and SL-1 expression in cervical fibroblasts (Takahashi et al., 1993) and the collagen stimulated induction of CL-1 expression in a keratinocyte cell model (Sudbeck et al., 1994). Although it has yet to be demonstrated whether PKC is associated with the accumulation of signalling molecules induced by integrin clustering, the activation of PKC has been shown to precede α5β1 integrin mediated cell spreading on fibronectin (Vuori and Ruoslahti, 1993). Given these associations, future experiments could determine whether PKC mediates fibronectin induced GLA activation, by incorporating PKC inhibitors such as chelythrine chloride (De Nichilo and Yamada, 1996) into the HT1080 culture model.

In a recent report, Kheradmand et al. (1998) examined the role of the Rho family of GTP-binding proteins in signalling changes to the actin cytoskeleton that up-regulate MMP expression. They demonstrated two divergent signalling pathways involved in the α5β1 mediated up-regulation of CL-1 synthesis by rabbit synovial fibroblasts. Surface-bound 120 kDa fragment of fibronectin, or surface-bound anti-α5 integrin subunit antibody promoted cell spreading and increased CL-1 expression by the fibroblasts. In contrast, addition of soluble anti-α5 antibody to cells cultured on fibronectin caused cell rounding and disruption of the cytoskeleton, but also induced CL-1 expression. As members of the Rho family (Rho, Rac and Cdc42) control the organisation and dynamics of the actin cytoskeleton, an investigation of the contribution of Rho family members to CL-1 expression was made. The transient transfection of dominant interfering mutants of Rho, Rac and Cdc42 into synovial fibroblasts revealed that Rac mutants (but not Rho or Cdc42 mutants) inhibited the induction of CL-1 by soluble anti-α5 integrin antibodies.
Furthermore, the transient expression of constitutively activated Rac 1 reduced actin stress fibres, increased the formation of actin-rich lamellipodia and stimulated CL-1 expression by the cells. Transient expression of Rac 1 also induced the rapid nuclear translocation and constitutive activation of the transcription factor NF-κB. This in turn promoted IL-1 gene transcription. Finally, study including the IL-1 receptor antagonist confirmed that IL-1 is an autocrine mediator involved in the induction of CL-1 expression by soluble anti-α5 antibodies in these cells. This report clearly and comprehensively showed that soluble anti-α5 integrin antibodies induce Rac activity in fibroblasts, which disrupts the cytoskeleton, and activates NF-κB mediated IL-1 synthesis, leading to increase in CL-1 expression.

Other studies have also shown that MMP expression and activation is linked to changes in the cytoskeleton. Cytoskeletal disrupting agents such as the cytochalasins have been shown to increase GLA activation by fibroblasts cultured in collagen lattices (Tomasek et al., 1997) and increase CL-1 synthesis by synovial fibroblasts (Aggeler et al., 1984). Tomasek and coworkers showed that activation of GLA is associated with the mechanical relaxation of collagen lattices. In contrast, in a similar study, work with two adhesion blocking anti-β1 integrin antibodies led to the observation that lattice contraction and GLA activation were separate events (Seltzer et al., 1994). Both events were controlled by the β1 integrin subunit, but specifically stimulated by different antibodies. The authors speculated that one antibody might cause more torsion of the β1 integrin than the other, leading to two sets of intracellular signals. Alternatively, there may be branch points in the signal transduction pathway that lead to GLA activation (Seltzer et al., 1994).

It is apparent that surface bound anti-α5 integrin antibody may signal changes to MMP expression via separate mechanisms to soluble anti-α5 integrin antibody. The study by Kheradmand and coworkers (1998) did not identify the signalling pathway involved in the up-regulation of CL-1 induced by surface-bound anti-α5 integrin antibody. However, immobilised antibody did not induce Rac1 or NF-κB activity, suggesting a second distinct pathway promoting CL-1 synthesis in synovial fibroblasts. Studies for this thesis showed
that GLA activation by HT1080 cells was induced by fibronectin, peptide fragments of fibronectin, or by anti-α5β1 integrin antibodies adsorbed to culture plastic. All of these ligands promoted cell spreading, and the activation of GLA appeared to proceed independently of discernible changes in cell shape. Immunolocalisation of actin microfilaments with rhodamine-phalloidin would confirm whether actin stress fibre formation was associated with GLA activation in this model. However, future studies could address whether agents that disrupt the cytoskeleton, such as cytochalasin D also stimulate GLA activation. The effect of soluble anti-α5 integrin antibodies added to cells cultured on fibronectin should be tested. A positive link between cytoskeletal re-organisation and GLA activation could be followed up by transfection of the dominant-interfering mutants of the Rho family members into HT1080 cells, as detailed by Kheradmand and co-workers (1998). The requirement for an IL-1 autocrine loop in the α5β1 integrin-mediated up-regulation of GLA activation could easily be tested by the addition of IL-1 neutralising antibodies or the IL-1 receptor antagonist to HT1080 cells cultured on fibronectin. Both approaches have been used to show that fibronectin fragments or anti-α5 integrin antibodies stimulate cellular expression of MMPs via an IL-1 autocrine loop (Homandberg et al., 1997; Kheradmand et al., 1998).

Several members of our laboratory cultured other cell types on fibronectin and did not observe up-regulation of GLA activation. These cell types included A375M human melanoma cells, human foreskin fibroblasts, human endothelial cells and the human chondrosarcoma cell line SW1353. This may reflect the differing integrin expression patterns between HT1080 cells and the other cell types tested. Other studies have shown that A375M cells, foreskin fibroblasts and endothelial cells express αvβ3 integrin (Massia and Hubbell, 1991; Seftor et al., 1993; Scatena et al., 1998), which also recognises the RGD sequence in fibronectin. In contrast, our HT1080 cell line did not appear to express αvβ3 integrin, and adhered to fibronectin solely via α5β1. One could hypothesise that the other cell types tested used predominantly αvβ3 integrin to adhere to fibronectin, and that signals via α5β1 integrin were not activated. A study by Seftor et al. (1993) lends support to this hypothesis. These authors compared the moderately invasive A375M cells, which
express higher levels of αvβ3 integrin than α5β1 integrin, with highly invasive C8161 melanoma cells, which express higher levels of α5β1 than αvβ3 integrin. The C8161 cells responded to anti-α5β1 integrin antibodies by increased levels of proGLA synthesis and increased invasion through a Matrigel barrier. In contrast, the invasion of A375M cells through Matrigel was not increased in the presence of anti-α5β1 integrin antibodies. A375M cells were subjected to fluorescence activated cell sorting to select a population of cells deficient in αvβ3 integrin. These cells proved to be 30-50% more invasive than the parent A375M line. It is worth noting that, in general, αvβ3 integrin is associated with highly invasive melanomas (Marshall et al., 1991). Nevertheless, in the study by Seftor and coworkers, it would have been interesting to compare the proGLA secretion by parent and cloned A375M cells cultured on a fibronectin substrate, but this was not addressed. However, the current study could be extended by transfection of HT1080 cells with αvβ3 integrin, as one would predict that transfected cells would activate less GLA on fibronectin than the parent HT1080 cells.

The apparently unique response of HT1080 cells to fibronectin may also reflect their tumourigenicity. Although these cells synthesise fibronectin, they do not lay down a fibronectin matrix (Mardon et al., 1993). Overexpression of plasma-type fibronectin in HT1080 cells was shown to promote the deposition of a fibronectin matrix, but reduced cell motility and cell proliferation (Akamatsu et al., 1996). The levels of α5β1 integrin on the surface of cells overexpressing fibronectin were apparently unchanged. Fukai et al. (1995) showed that proteolytic cleavage of fibronectin by GLA releases the C-terminal fibrin binding domain of fibronectin that can simulate cell migration. It is possible that the HT1080 cells in the current study secreted increased levels of active GLA in response to a fibronectin matrix in order to maintain cell motility. This hypothesis could be tested by time lapse video microscopy of migrating cells. To assess the importance of GLA activity, HT1080 cell motility could be monitored in the presence and absence of blocking antibodies to GLA (such as K399). Similarly, HT1080 cells stably transfected with MT1-MMP (used in studies for Chapter 6) may migrate on fibronectin more efficiently than the parent line, as these cells activate greater levels of GLA than the parent line (Dr J.
Gavrilovic, personal communication). It would also make a very interesting study to include soluble fibronectin fragments in HT1080 cultures, particularly as the study by Fukai and coworkers identified a fragment that stimulates cell migration. There are well established methods for the purification of fibronectin fragments, and many fragments are now available commercially.

Studies such as those described above would aid our understanding of the significance of increased GLA activation in response to fibronectin. Other studies have shown that HT1080 cells and various breast cancer cell lines activate GLA in response to collagen I (Azzam and Thompson, 1992; Gilles et al., 1997). The synthesis and activation of MMPs in response to matrix may be crucial for the invading tumour cell to pass through host interstitial matrices which are rich in fibrillar collagens and fibronectin. As discussed earlier, proteolysis of these matrices may lead to the release of fibronectin fragments that stimulate migration (Fukai et al., 1995; Fabunmi et al., 1996); the exposure of cryptic RGD sites in collagen I (Mackay et al., 1990; Montgomery et al., 1994); or the remodelling of the matrix such that directional migration is maintained (Pilcher et al., 1997). Many parallels can be drawn between normal physiological remodelling processes and tumour cell invasion. However, the migration of untransformed cells during physiological remodelling is tightly controlled by various factors including the surrounding matrix. For instance, the migration of keratinocytes in a wound healing model is dependent upon intact collagen I, which induces CL-1 activity (Pilcher et al., 1997). Laminin-1, which is deposited in the newly formed basement membrane of the epidermis, represses the collagen-mediated induction of keratinocyte CL-1 (Sudbeck et al., 1997). The authors speculate that the accumulation of laminin-1 may effect the signal to halt keratinocyte migration as re-epithelialisation progresses (Sudbeck et al., 1997). Similarly, tenascin-C inhibits oligodendrocyte precursor migration, by a process which is independent of its anti-adhesive properties (Kiernan et al., 1996). As tenascin-C is concentrated at the retina-optic nerve junction, it may serve as a barrier to inhibit the migration of oligodendrocyte precursor cells into the retina (ffrench-Constant et al., 1988). In the current study, laminin-1 did not effect GLA activation by HT1080 cells, and preliminary studies indicated that
tenascin-C bound to fibronectin substrates down-regulated fibronectin-induced GLA activation (data not shown). Accordingly, the effect of laminin-1 and tenascin-C on HT1080 cell migration could be tested by time lapse videomicroscopy of cells on mixed substrates.

Having established that fibronectin induces GLA activation in HT1080 cells, the next step was to determine at what point in the GLA activation cascade fibronectin exerts its effect. As discussed in Chapter 1, proGLA activation is initiated by interaction of the C-terminal domain of proGLA with cell membranes (Murphy et al., 1992b; Strongin et al., 1993; Ward et al., 1994). Several models for GLA binding to the cell surface were postulated by researchers during the course of this study. Itoh et al. (1998) published recently that TIMP-2 binds to the cell surface independently of MT1-MMP and acts as a receptor for GLA (discussed in detail later in the chapter), although it is not clear how this binding contributes to GLA activation. GLA binding to cell-surface associated collagen via the fibronectin type II domains has also been observed (Steffensen et al., 1998), however the authors considered it unlikely that these interactions contribute to GLA activation. Direct binding between αvβ3 integrin and the C-terminal domain of GLA has been reported using isolated integrin and GLA in solid-phase assays. Furthermore, full length GLA interferes with the binding of a functionally-blocking anti-αvβ3 integrin antibody to the αvβ3 integrin ligand binding site (Brooks et al., 1996; Brooks et al., 1998). It has been suggested by these authors that this interaction localises proteolytically active GLA to discrete regions on the cell surface, facilitating angiogenesis and potentiating invasive behaviour. A parallel can be drawn with the well defined interactions between αvβ3 integrin and urokinase plasminogen activator receptor (reviewed by Chapman, 1997). It has yet to be determined whether αvβ3/GLA binding contributes to the activation of GLA, or whether MT1-MMP colocalises with αvβ3 integrin, although one possibility suggested is that GLA binds to αvβ3 integrin after activation has occurred (Brooks et al., 1998). As discussed in Chapter 5, it was considered unlikely that this mechanism was important in the current study, for immunolocalisation studies of HT1080 cells cultured on fibronectin did not detect αvβ3 integrin (A. Messent and J. Gavrilovic, personal communication). It is
of concern that studies in our laboratory by A. Messent were unable to show direct interaction between isolated ανβ3 integrin and either recombinant chicken GLA (used in the studies by Brooks et al., 1996, 1998) or human recombinant GLA (Messent, 1997). Much work is needed to determine whether the ανβ3 integrin/GLA interactions observed by Brooks et al. are a general mechanism, whether GLA colocalises with ανβ3 in all ανβ3-expressing cells, and whether ανβ3 integrin/ligand interactions are destabilised by the presence of bound GLA. It would also be interesting to determine whether GLA interacts with other integrins, although it has been reported by Brooks et al. (1996) that GLA does not bind immobilised α5β1 integrin receptor.

It is clear that the association of TIMP-2 with the cell surface is a key event in the activation of GLA. This study favoured the hypothesis that MT1-MMP and TIMP-2 complex together to form a receptor for GLA (Strongin et al., 1995; Butler et al., 1998; Kinoshita et al., 1998). Efforts were made to measure the TIMP-2 secreted by HT1080 cells, however the levels were at the detection limit of several of the techniques tried. Nevertheless, there was no apparent variation in the levels of TIMP-2 secreted by cells cultured on fibronectin or laminin-1 substrates. The levels of TIMP-2 associated with the cells at the end of the culture period were also similar for the two substrates. The data does not rule out rapid changes in the concentration of TIMP-2 at the cell surface over the culture period, and it is possible that the fibronectin substrate (in comparison to laminin-1) promoted the rapid recycling of TIMP-2 'receptors' for GLA. Pulse chase analyses would be required to detect such changes.

Fibronectin substrates appeared to exert an effect on the post-transcriptional regulation of MT1-MMP. A new antibody to MT1-MMP (N175) was characterised for use in these studies and examination of HT1080 cell lysates with N175 revealed that in addition to the 63 and 60 kDa forms of MT1-MMP reported by others (Strongin et al., 1995; Cao et al., 1996), a band at 45 kDa was detected. The level of this form of MT1-MMP was up-regulated when cells were cultured on fibronectin, or in the presence of PMA, and appeared concomitantly with processing of GLA to the active form. The 60 kDa form corresponded
most likely with active MT1-MMP isolated from HT1080 cells and N-terminally sequenced by Strongin et al. (1995). However, only one other group had reported a similar truncated form of MT1-MMP (43 kDa), detected by Western blot analyses of HT1080 cell lysates using an antibody to the cytoplasmic portion of MT1-MMP (Lohi et al., 1996). Assuming therefore, that the truncated form of MT1-MMP must still be membrane bound, calculations based on the apparent molecular weight predicted that the cleavage occurred in the catalytic domain of MT1-MMP. It was hypothesised that this form was catalytically inactive.

The ability to process MT1-MMP to 45 kDa was not restricted to HT1080 cells. Work in our laboratory showed that human foreskin fibroblasts, human microvascular endothelial cells, a human chondrosarcoma cell line and several human melanoma cell lines also processed MT1-MMP to 45 kDa, but unlike the HT1080 cells, processing was not constitutive, and required concanavalin A or PMA as a cell stimulus. Zymography of the conditioned media from all cell types showed that processing to 45 kDa was accompanied by GLA activation, with the notable exception of PMA treated microvascular endothelial cells, which processed MT1-MMP to 45 kDa in the absence of processing of pro-GLA (C. Lindsay, personal communication). MT1-MMP truncation to 45 kDa appeared to be a common mechanism in stimulated cells, and we hypothesised that it might represent an important regulatory step in MT1-MMP mediated MMP activation cascades.

To purify sufficient 45 kDa MT1-MMP for N-terminal sequencing, several approaches were tried. Immunoprecipitation of MT1-MMP from cell lysates using N175 antibody did not provide sufficient material for further purification by reverse-phase HPLC chromatography, and transferring the immunoprecipitates to PVDF for microsequencing was confounded by N-terminal blocking of the protein. Attempts were made to generate 45 kDa MT1-MMP in vitro, based on the observation that MT1-MMP is cleaved to 45 kDa by MMPs (discussed below). Cleavage of recombinant soluble MT1-MMP with MMPs did not appear to proceed via a '45 kDa' form and this was of concern. This indicated either that soluble ΔTM-MT1-MMP was folded in such a way that the cleavage site was
not accessible to protease action, or that the transmembrane domain and/or other membrane-bound molecules were required to facilitate MT1-MMP cleavage. After work was completed for this thesis, Lehti et al. (1998) published an N-terminal sequence of a truncated form of mutant MT1-MMP. These authors constructed a soluble mutant MT1-MMP construct, tagged C-terminally with histidines and expressed the construct in HT1080 cells. This approach was innovative, as it relied on the 'machinery' present within the HT1080 cell to process MT1-MMP to 45 kDa, but had the added advantage of a histidine tag on the recombinant molecule that could be utilised during subsequent purification. However, as the cleavage site was located to the hydrophobic 'methionine-turn' of MT1-MMP, which is expected to be buried within the molecule, it remains an important question as to whether wild-type MT1-MMP is cleaved at this sequence.

Further studies were undertaken to identify the enzyme(s) involved in processing MT1-MMP to 45 kDa. The addition of MMP inhibitors to HT1080 cells cultured on fibronectin identified the protease activity as an MMP. Work with isolated cell membranes from HT1080 cells showed that MMPs from all subclasses were capable of cleaving MT1-MMP to 45 kDa in vitro, implying that the cleavage site is particularly MMP sensitive. For future studies it would be interesting to use protease inhibitors specific for other classes of protease to determine whether other proteases (such as plasmin) also cleave MT1-MMP to 45 kDa.

Notably, processing of MT1-MMP to 45 kDa by HT1080 cells was not inhibited by TIMP-1, which implied MT1-MMP autolysis to 45 kDa. Unfortunately, antibodies that block MT1-MMP activity were not available to test this hypothesis. The transient transfection of a catalytically inactive mutant of MT1-MMP into MT1-MMP null cells would provide an alternative method for testing the hypothesis, as one would predict that mutant MT1-MMP would not be processed to 45 kDa. However identifying a suitable cell line for transient transfection that does not express MT1-MMP could prove difficult, as the cell lines currently used in our laboratory for this style of study (Chinese hamster ovary cells, COS cells) express low levels of MT1-MMP. This study identified a new blocking antibody to
GLA (K399) and work with this antibody indicated that processing of MT1-MMP to 45 kDa by HT1080 cells is independent of GLA activity. As mentioned earlier, observations by C. Lindsay in our laboratory supported this concept, as PMA treated microvascular cells processed MT1-MMP to 45 kDa in the absence of detectable GLA activation. Hence it is apparent that whilst GLA activation and MT1-MMP processing to 45 kDa are often concomitant, they are not interdependent events.

Other questions remain with respect to the biology of 45 kDa MT1-MMP. What is its 'life cycle', does it have a biological role, or is it simply an inactive byproduct of MT1-MMP activity that is resorbed back into the cell? With regards to the truncation of MT1-MMP to 45 kDa, evidence suggests that it is associated with the routing of MT1-MMP to the cell surface. Cell surface biotinylation studies by Lehti et al. (1998) showed that the 63, 60 and 43 kDa forms of MT1-MMP are all present at the surface of HT1080 cells. In our laboratory, circumstantial evidence that processing to 45 kDa is associated with the cell surface was provided by a comparison between concanavalin A and PMA treated human foreskin fibroblasts. Immunolocalisation studies showed that concanavalin A treatment of the fibroblasts stimulated MT1-MMP localisation to the cell surface, and concurrent Western blot analysis of the cell lysates indicated that MT1-MMP was processed to 45 kDa (R. Hembry, H. Stanton, G. Murphy, unpublished data). In contrast, however, PMA treatment of the foreskin fibroblasts caused the intracellular accumulation of MT1-MMP in the perinuclear region, with no cell surface staining apparent (d'Ortho et al., 1998). Strikingly, no 45 kDa MT1-MMP was detected by Western blot analysis of the cell lysates, and GLA activation was not stimulated. Although PMA treatment appeared to increase the expression of MT1-MMP in the fibroblasts, it did not signal the routing of MT1-MMP to the cell surface, or the processing of MT1-MMP to 45 kDa. Clearly, the response to PMA is cell type specific, as evidenced by the differences between the fibroblast model, the endothelial cell model studied by C. Lindsay and the HT1080 cell model used in this study.

Notably, the HT1080 cells were the only cell type studied that showed constitutive staining for MT1-MMP on the cell surface. Staining was apparent over the entire cell surface,
which differs markedly from the staining patterns reported by Nakahara et al. (1997) who observed MT1-MMP localised to the invadopodia of human melanoma cells. Clustering of MT1-MMP molecules at the leading edge of migrating cells may be essential for the localised degradation of matrix required for directional migration. It is likely that a sequence in the transmembrane domain/cytoplasmic domain of MT1-MMP targets it to invadopodia, as a chimeric MT1-MMP containing the transmembrane and cytoplasmic domains of the interleukin-2 receptor alpha chain was not localised to invadopodia, and localised ECM degradation attributable to MT1-MMP was not observed (Nakahara et al., 1997). Furthermore, concanavalin A prevented the invadopodial localisation of MT1-MMP and matrix degradation. In the light of these studies, it would be interesting to investigate the matrix-degrading abilities of HT1080 cells, which do not appear to localise MT1-MMP molecules at the leading edge. A model system such as that used by d'Ortho et al. (1998) may be suitable for this investigation.

Pulse chase analyses by Lehti et al. (1998) have shown that 45 kDa form of MT1-MMP is relatively short-lived. Processing of MT1-MMP to 45 kDa by PMA treated HT1080 cells occurred within 1.5 hours of stimulation, and levels of 45 kDa MT1-MMP decreased to barely detectable levels after 16 hours of stimulation. For the current study, attempts to label newly synthesised MT1-MMP with $^{35}$S-methionine failed, compounded by problems with using the antibody N175 for immunoprecipitation. However, a preliminary time-course study analysed by Western blotting showed that PMA treatment of HT1080 cells stimulated MT1-MMP processing to 45 kDa during the first 2 hours of stimulation, and that the levels of 45 kDa in the cell lysates then remained constant (data not shown). It is possible that continued PMA stimulation caused a constant turnover of 45 kDa MT1-MMP. Certainly the levels of 45 kDa MT1-MMP detected in the lysates of PMA treated HT1080 cells were significant after 48 hours of culture (as shown in Chapter 5).

With regards to the biological role of MT1-MMP, it is tempting to speculate that truncation of MT1-MMP to 45 kDa accompanies the release of active GLA (although it can also proceed independently of GLA activation). It is possible that TIMP-2 'free' MT1-MMP on
the cell surface is involved with processing the MT1-MMP/TIMP-2 receptor complex to release GLA, creating 45 kDa MT1-MMP in the process. However, as the 45 kDa cleavage site in MT1-MMP is apparently in a highly hydrophobic region of the molecule (Lehti et al., 1998), it is difficult to predict the molecular dynamics of the process. It is also possible that MT1-MMP is cleaved to 45 kDa following release of active GLA/TIMP-2, as a means of regulating the number of 'receptor' complexes available for GLA binding to the cell surface.

One question that must be addressed is what is the fate of GLA upon activation at the cell surface? Is it immediately inhibited by TIMP-2, or is TIMP-2 degraded upon release from the MT1-MMP receptor complex? The recent report by Itoh et al. (1998) presents an intriguing hypothesis. Using concanavalin A treated cells, these authors demonstrated that TIMP-2 is bound to cell membranes via hydroxamate inhibitor sensitive and insensitive mechanisms, implying that TIMP-2 binds to the cell membrane independently of MT1-MMP. In addition, TIMP-2 bound to cell membranes in a hydroxamate insensitive way specifically inhibited active GLA, but not active SL-1 or GLB. The data implied that upon release of active GLA/TIMP-2 from the cell surface 'receptor', the N-terminal domain of TIMP-2 then inhibited the catalytic domain of GLA. It follows that all GLA released in this manner would be rendered inactive by bound-TIMP-2, which begs the question of how catalytically active, TIMP-2-free GLA is produced. However, using zymography to analyse the conditioned medium from concanavalin A treated cultures, the authors noted the presence of the 45 kDa truncated form of GLA lacking a C-terminal domain, that was shown to be catalytically active. GLA can be processed to 45 kDa autolytically, or by other MMPs, and the authors speculated that this form of GLA was responsible for GLA catalytic activity, not the 65 kDa 'active' form of GLA seen by zymography. In the current study, using fibronectin or PMA treated HT1080 cells, there was no 45 kDa GLA detected in the conditioned media. In our laboratory, this form of GLA has only been detected in membrane preparations from comparable intact cell studies, but not in the conditioned medium from concanavalin A treated cultured fibroblasts, although it may be at levels too low to detect.
In summary, Chapter 4-6 of this thesis show that a substrate of fibronectin promotes GLA activation by HT1080 cells and that signals via α5β1 integrin are likely to be involved. This data adds to a growing body of evidence that integrin receptors play a role in transducing signals to regulate MMP synthesis and activity. Both α5β1 integrin receptor clustering and α5β1 integrin receptor occupancy appeared to be required to signal GLA activation, and defining the intracellular signalling events involved will form the basis of a future project. Data also suggested that fibronectin influences MT1-MMP activity in HT1080 cells, stimulating the truncation of MT1-MMP to a 45 kDa form. Further work is required to obtain the N-terminal sequence of wild-type 45 kDa MT1-MMP, to compare with the sequence of a similarly truncated mutant form of MT1-MMP (Lehti et al., 1998). However, it is predicted that the 45 kDa form is catalytically inactive, and as the processing of MT1-MMP to 45 kDa is common to many cell types, it may represent an important end-point in MT1-MMP activation cascades. Studies presented in this thesis implicated MT1-MMP self-processing to 45 kDa, however further work is required to confirm this hypothesis. As the truncation of MT1-MMP occurs concomitantly with the activation of GLA, future studies should focus on how truncation of MT1-MMP to 45 kDa relates to the broader question of GLA activation at the cell surface. The mechanism of release of active GLA from the cell also needs to be defined. Certainly, the observation made in this thesis that MT1-MMP is processed to a 45 kDa form is a new issue in MT1-MMP biology and one that deserves further exploration.
Figure 7.1 Integrin-mediated transmembrane signalling and organisation of the cytoskeleton
(adapted from Yamada and Miyamoto, 1995)

A hierarchy of accumulation of molecules has been established, based on whether an integrin is clustered, occupied with a ligand, or both.

(a) If an integrin binds a monovalent ligand it becomes redistributed into a pre-existing site of integrin and cytoskeletal protein accumulation, such as a focal adhesion

(b) If integrin aggregation occurs, certain types of cytoskeletal proteins accumulate, depending on the state of occupancy. If tyrosine phosphorylation can proceed, a large accumulation of signal transduction molecules occurs, accompanied by signalling via MAP kinases of the ERK and JNK families.

(c) A combination of occupancy, aggregation and tyrosine phosphorylation results in the maximal effect, including accumulation of F-actin and cytoskeletal proteins in a massive adhesive, cytoskeletal and signalling complex.
References


