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13. ABSTRACT ***(Maximum 200 Words)***

The Robert H. Lurie Comprehensive Cancer Center at Northwestern University is an NCI-funded comprehensive cancer center. One of the major accomplishments for the Cancer Center has been to establish a nationally recognized program in breast cancer in both laboratory research and clinical activity. Most notably, the Cancer Center successfully competed for a SPORE in Breast Cancer from the NCI in September 2000. In September 2000 the Cancer Center received a four year award from the US Army for comprehensive training of graduate students and postdoctoral fellows conducting breast cancer relevant research entitled, "The Molecular Biology of Breast Neoplasia" (DAMD17-00-1-0386). In Year 01 the Selection Committee reviewed applications and assisted in the selection of 4 graduate students and two postdoctoral fellows for appointment to the Training Program. Trainees and mentors actively participated in the weekly meetings of the Breast Cancer Journal Club, as well as Cancer Center seminars, symposium and Breast SPORE meetings. Year 02 appointments have just been completed.

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INTRODUCTION

The Robert H. Lurie Comprehensive Cancer Center has developed a premier breast cancer program at Northwestern University. In October, 1993, the Cancer Center recruited V. Craig Jordan, Ph.D., D.Sc. to direct the breast cancer laboratory research program and Monica Morrow to direct the clinical breast cancer research program and the Lynn Sage Comprehensive Breast Center at Northwestern. Dr. Jordan is an internationally recognized leader in breast cancer research. His most important contribution to the field has been in the research and development of the antiestrogen tamoxifen, an important drug used in the treatment and prevention of breast cancer. In September 1994, the Cancer Center successfully competed for a grant from the NCI to establish a breast cancer program (NCI P20 CA65764). The co-principal investigators of this grant were Drs. Jordan and Morrow. In August, 1996, the Cancer Center was selected as one of three institutions in the US to receive a four year breast cancer center grant from the US Army (DAMD17-96-2-6013, Principal Investigator: Monica Morrow, M.D). The title of the grant was "Increasing Access to Modern Multidisciplinary Breast Cancer Care". The award provided funds for eight research projects to address prevention and access to breast cancer care by minority women. The Cancer Center received a 4.2 million dollar award from the Avon Products Foundation Breast Cancer Research and Care Program in March 2000 for laboratory research and clinical treatment. This gift will also expand the breast cancer research programs to medically underserved minority women. In September 2000 the Cancer Center was selected by the National Cancer Institute to receive a SPORE in Breast Cancer (P50 CA89018). The Program Director of the SPORE is V. Craig Jordan, Ph.D. and the co-directors are Monica Morrow, M.D. and Ann Thor, M.D. The five-year award provides 13 million dollars in funding for six translational research projects, four core facilities, career development and developmental research opportunities in breast cancer.

BODY

In September 2000, the Cancer Center received a four-year award from the US Army for training of graduate students and postdoctoral fellows conducting breast cancer relevant research entitled, "Molecular Biology of Neoplasia". This program provides trainees with comprehensive training in breast cancer biology, utilizing the powerful tools of molecular biology, genetics and biochemistry to unravel the complex mechanisms of breast neoplasia. The program enables four graduate students and two postdoctoral fellows per year to be exposed to senior basic science faculty with research relevant to breast cancer research and to clinical investigators who provide a translational link. Training grant students participate and present at the weekly Breast Cancer Journal Club meetings (see attached schedule for 2000). Students present a selected breast cancer article from the basic and clinical literature and Dr. Jordan leads a discussion revolving around the topic. The journal club regularly attracts 15-25 graduate, postdoctoral and faculty participants in addition to the four predoctoral students and two postdoctoral fellows who are required to participate. Dr. Jordan provides further educational opportunities for the trainees in his role as Program Director for the SPORE in Breast Cancer. The SPORE investigators meet every two weeks and are required to present their progress in the diverse funded areas of breast cancer in the program. The breast cancer trainees sponsored by the US Army grant are exposed to the latest developments in breast cancer research nationwide.

Dr. Gerald Soff has been added to the list of preceptors on The Molecular Biology of Breast Neoplasia Training Grant. He replaces Dr. Noel Bouck who has retired from Northwestern University. Dr. Soff is a nationally recognized leader in the field of translational research in angiogenesis in breast and prostate cancer. He is a funded investigator on both the Breast and Prostate SPORE grants. Dr. Thor has recently left Northwestern University to assume a position as Chairman, Department of Pathology at Oklahoma University.

Each year the Cancer Center solicits applications from faculty in the Breast Cancer Program nominating students. The Training Grant Selection Committee is responsible for the admission of students and fellows. Committee members include: V. Craig Jordan, Ph.D., D.Sc., Program Director, Steven Rosen, M.D., Director, Cancer Center, Robin Leikin, Ph.D., Scientific Program Director; Kathleen Rundell, Ph.D., Professor, Microbiology-Immunology and Jonathan Jones, Ph.D., Professor, Cell and Molecular Biology. Students are selected based upon their academic credentials, the relevance of their research projects to breast cancer and their potential as future academicians in breast cancer research. The Training Grant Executive Committee provides final approval for the trainee selections.

The following predoctoral and postdoctoral students have been allocated funds in Year 01 of the Molecular Biology of Breast Neoplasia Training Grant:

Year 1:

Name	Preceptor	Department
Predocs:		
Lisa Salvador	Mary Hunzicker-Dunn	Cell and Molecular Biology
Hao Wang	Gerald Soff	Medicine
Michael Werner	Vince Cryns/Larry Jameson	Medicine
Yi Wu	Sharon Stack	Cell and Molecular Biology
Postdocs:		
Yongji Chung	Ann Thor	Pathology
Daisuka Tsuruta	Jonathan Jones	Cell and Molecular Biology

Yi Wu

Yi Wu is a student in the laboratory of Dr. Sharon Stack. He is studying the function of membrane type 1 matrix metalloproteinase (MT1-MMP) and its regulation in breast cancer cell invasion. Yi Wu has shown that the breast cancer cell line MDA-MB-231 expresses MT1-MMP and that the cells invade type I collagen matrix. Cellular invasion of type I collagen matrices requires collagenase activity, is blocked by tissue inhibitor of metalloproteinases-2, TIMP-2, and is unaffected by TIMP-1. This suggests that the cell surface proteolytic cascade is initiated by MT1-MMP. Yi Wu has also shown that a) MT1-MMP activation is inhibited by a peptide-based Furin inhibitor CMK, b) Brefeldin A, which prevent protein trafficking into Golgi, blocks MT1-MMP activation, suggesting MT1-MMP is activated in a post-ER compartment and c) an a1-PI mutant which carries the P4'-P2 peptide sequence of MT1-MMP activation site blocks MT1-MMP activation. The Stack laboratory is currently using biochemical approaches to identify the enzyme that may mediate MT1-MMP activation in vivo. They have demonstrated that MT1-MMP co-localizes with integrins including $\alpha 2\beta 1$, $\alpha 3\beta 1$ and partially $\alpha 5\beta 1$, but not $\alpha V\beta 3$ in MDA-MB-231 cells. The lab has identified a PKC phosphorylation site on the cytoplasmic domain of MT1-MMP that may potentially regulate MT1-MMP trafficking. Taken together, these studies provide insight into tumor cell functions including migration, invasion and matrix remodeling.

Lisa Salvador

Ms. Salvador is a graduate student in the laboratory of Dr. Mary Hunzicker-Dunn. Her research project is to examine the pathway by which cAMP/PKA signals in concert with estrogen to promote cellular proliferation and differentiation and to inhibit apoptosis. The specific molecules being studied are A kinase anchoring proteins (AKAPs). The AKAPs anchor PKAs to specific cellular sites, thereby promoting specific and efficient substrate phosphorylation in response to a specific stimulus. Ms. Salvador has determined that PKA activates

the mitogen activated protein kinase (MAPK) pathway. She is also characterizing an R1 AKAP that may be involved in breast cancer cell apoptosis.

Hao Wang

Hao Wang is pursuing his Ph.D. in the laboratory of Dr. Gerald Soff. He is studying the in vivo generation of angiostatin 4.5 (AS4.5) as an inhibitor of angiogenesis in breast cancer patients. Recent experiments have indicated that the "Angiostatic Cocktail" administered to cancer patients generated not only AS4.5, but also angiostatin related proteins and angiostatin-related complexes. Similar proteins and complexes are generated in human plasma given "Angiostatic cocktail" *in vitro*. The Soff data suggest that the complexes are plasmin-α2-antiplasmin complex and plasmin-α2-macroglobumin complex. Although these complexes do not show inhibiting effect in endothelial cell proliferation assay, their studies do suggest that α2-antiplasmin can protect AS4.5 from further degradation by plasmin and help to elevate AS4.5 concentration in the blood of cancer patients administered "Angiostatic cocktail." It is also suggested that α2-antiplasmin can influence the plasmin-angiostatin conversion through binding to plasmin. The purpose of Hao Wang's doctoral thesis is to determine the effect of α2-antiplasmin on plasmin-Angiostatin conversion and the structure and function of angiostatin-related proteins. These studies will improve our understanding of angiostatin and the effect of "Angiostatic cocktail" *in vivo*. This will be helpful to the application of "Angiostatic cocktail" in the treatment of cancer patients.

Michael Werner

Michael Werner is a graduate student studying caspases in the Cryns laboratory. Caspases are a conserved family of cysteine proteases that are universal effectors of programmed cell death. However, the molecular mechanisms by which caspases induce breast cancer cell death are poorly understood. The Cryns laboratory has developed a novel expression cloning strategy to identify cDNAs encoding caspase substrates. They identified integrin β4 as a new caspase-3 substrate that is cleaved in breast cancer cells undergoing chemotherapy-induced apoptosis. They have demonstrated that caspase cleavage of integrin β4 removes a large portion of its cytoplasmic tail. They hypothesize that caspase cleavage of integrin β4 promotes apoptosis by specifically disrupting integrin β4's ability to activate the PI3K/Akt cell survival pathway. Once the exact cleavage site is identified, they will generate breast cancer cells that stably express wild-type integrin β4, caspase-truncated integrin β4 (which lacks the tail portion removed by caspases), and caspase-cleavage resistant integrin β4 (which lacks the caspase cleavage site and cannot be cleaved by caspases). Mike will then compare the sensitivity of these breast cancer cells to chemotherapy-induced apoptosis and examine their ability to activate the PI3K/Akt cell survival pathway. Dr. Cryns predicts that the breast cancer cells expressing truncated integrin β4 will be most sensitive to apoptosis induction (because they will be unable to activate the PI3K/Akt cell survival pathway), while those expressing caspase cleavage-resistant integrin β4 will be the most resistant to chemotherapy-induced apoptosis (even more resistant than breast cancer cells expressing wild-type integrin β4). These experiments, then, will provide novel insights into the mechanisms of breast cancer apoptosis, and they may lead to new breast cancer therapies specifically targeting integrin β4.

Daisuka Tsuruta, Ph.D.

Dr. Tsuruta's research in the Jones laboratory has involved analyses of the regulation of breast cell behavior by extracellular matrix. Normal breast epithelial cells interact with the extracellular matrix, in part, via hemidesmosomes comprised of a complex of α6β4 integrin/laminin-5. This complex is the conduit for hemidesmosome-mediated cell signaling. During the past year, he has focused on hemidesmosome protein dynamics using green fluorescent protein (GFP)-tagged β4 integrin in live confluent and subconfluent epithelial

cells in order to learn more about their assembly. In confluent populations, rosette-like clusters of GFP-β4 remain stable over 1 hr. In subconfluent populations, clusters of GFP-β4 are not stable but assemble into and disassemble out of rosette-like arrays relatively rapidly in an energy-dependent fashion. Furthermore, Dr. Tsuruta monitored GFP-β4 in epithelial cells populating scrape wound sites. In these migratory cells, β4 integrin protein clusters progressively assemble and disassemble into primarily linear streaks located towards the periphery of the migrating cells. In summary this data provides the first evidence that hemidesmosomes are dynamic in both migratory cells and non-motile cells. Dr. Tsuruta speculates that this allows them to respond rapidly and reversibly to external stimuli. This has important implications for normal and aberrant breast epithelial tissue morphogenesis since hemidesmosomes may play the role of sensitive mechano-signal tranducers.

Yongji Chung, Ph.D.

Elevation of serum 17-β-estradiol (E_2) via a continuous release pellet between 8-16 weeks of age significantly shortens the latency period and increases the incidence of atypical hyperplasia and breast carcinogenesis in MMTV-wt-erbB-2 transgenic mice. Tumors from E_2 treated mice appeared more aggressive/invasive. Over 20 novel cell lines have now been generated from the mammary tumors of both E_2 treated and control animals. In vitro cell lines from E_2 associated tumors proliferate twice as fast, with an average doubling time of 1.2 days as compared to the 2.1 days of non-E_2 associated cell lines. Representative lines from the E_2 treated group also demonstrated a greater response to growth factors EFG, IGF-1, and βFGF but not estrogen. Subcutaneous implantation studies in the mammary fat pad of parental FVB mice demonstrated significantly shorter latency and a greater take of the E_2 associated, as compared to non-E_2 associated, cell lines. Preliminary analyses of differential gene expression by c-DNA array technology revealed greater than 8 fold differential expression of growth-related genes, such as MARK-1, MARK-3, cyclin A, and PCNA as well as several apoptosis related genes in the E_2 associated murine mammary breast cancer lines. Protein expression studies confirmed enhanced expression of several erbB-2 signaling pathway molecules.

KEY RESEARCH ACCOMPLISHMENTS

- Four predoctoral students and two postdoctoral fellows selected from a pool of candidates by Selection Committee
- Journal Club held on Tuesdays at 11 am throughout the academic year
- Students exposed to educational programs of the Cancer Center through seminars and symposium
- Students exposed to translational relevance of their research through Breast SPORE meetings
- Advisory committee reviewed progress of 6 trainees and selected 3 for renewal of their funding in Year 02

REPORTABLE OUTCOMES

The success of the Robert H. Lurie Comprehensive Cancer Center's Training Program is exemplified by the publications by the trainees as a direct result of their funding through the Molecular Biology of Breast Neoplasia Training Grant. Publications include:

Ellerbroek, S. M., **Wu, Y. I.**, Overall, C. M., and Stack, M. S. (2001). Functional interplay between type I collagen and cell surface matrix metalloproteinase activity, J Biol Chem *276*, 24833-42.

Ellerbroek, S. M., **Wu, Y. I.**, and Stack, M. S. (2001). Type I collagen stabilization of matrix metalloproteinase-2, Arch Biochem Biophys *390*, 51-6.

Ghosh, S., **Wu, Y. I.**, and Stack, M. S. (2000) Proteolysis in ovarian carcinoma. In *Cancer Treatment and Research: Ovarian Cancer.* Stack, M. S. and Fishman, D. A., Eds. Rosen, S. Series Editor. Kluwer Academic Publishers, Boston. (in press)

Tsuruta D, Kono T, Kutsuna H, Yashiro N and Ishii M. Granulomatous Slack Skin: An Ultrastructural Study. Journal of Cutaneous Pathology 2001, 28:44-48.

Gonzales M, Weksler B, **Tsuruta D**, Goldman RD, Ytoon KJ, Hopkinson SB, Flitney FW and Jones JCR. Structure and function of a vimentin-associated matrix adhesion in endothelial cells. Molecular Biology of the Cell 2001, 12:85-100.

Soff GA, **Wang H**, Schultz R, Kunz P, Cundiff D, French E, Hoppin EC, Rossbach HC. Therapeutic application of an angiostatic cocktail for patients with Refractory Cancer. *Nature Medicine* (Submitted), 2001.

CONCLUSIONS:

The Molecular Biology of Breast Neoplasia has enabled the Cancer Center to provide state of the art laboratory and didactic training to 4 predoctoral students and two postdoctoral fellows in Year 01. Dr. Jordan has established the Breast Cancer Journal Club to bring together the members of the Training Program on a weekly basis to discuss relevant journal articles and areas of research. Trainees on the Molecular Biology of Breast Neoplasia also participate in the Tumor Cell Biology and Carcinogenesis courses offered through the Integrated Graduate Program of Northwestern University Medical School as well as departmental seminars and journal clubs that have direct relevance to breast cancer. These include the Tumor Cell Biology Seminar Series, Cell and Molecular Biology Seminars and the Molecular Endocrinology Seminars. The Cancer Center's SPORE in Breast Cancer Program provides further educational opportunities. Plans are underway to implement an integrated course in breast cancer biology and treatment beginning in the Winter Quarter 2002. The monthly lectures will present an integrated overview of clinical breast cancer for laboratory scientists. The goal is to enhance the understanding of clinical breast cancer so that the relevance of the research conducted by trainees can be stimulated.

Table 6

Robert H. Lurie Comprehensive Cancer Center
Breast Cancer Program Journal Club

Vanderwicken Library
Olson Pavilion 8260
Tuesdays, 11 a.m.

October 10	Craig Jordan, Ph.D.
October 17	Csaba Gajdos
October 24	Ana Levenson, Ph.D.
October 31	Sue Clare, M.D.
November 7	Pei-Yu Chien
November 14	Ruth O'Regan, M.D.
November 21	Barry Gehm, Ph.D.
November 28	Seema Khan, M.D.
December 5	**No Journal Club (San Antonio Symposium)**
December 12	Yi Wu
December 19	Mike Werner
December 26	**No Journal Club (X-mas)**
January 2	**No Journal Club (New Year)**
January 9	Sunil Badve, M.D.
January 16	Sharon Stack, Ph.D.
January 23	Lisa Salvador
January 30	Peter Gann, M.D.
February 6	Robert Chatterton, Ph.D.
February 13	Yongji Chung
February 20	Rita Dardes
February 27	Yasmin Ahmed, Ph.D.
March 6	Sam Pappas, M.D.
March 13	Mari Hunzicker-Dunn, Ph.D.
March 20	Jonathan Jones, Ph.D.
March 27	**No Journal Club (AACR)**
April 3	Daisuke Tsuruta, Ph.D.
April 10	Woo-Chan Park
April 17	Jessica Manela
April 24	Debra Tonetti, Ph.D.

May	1	Hong Liu, Ph.D.
May	8	Robyn Pike
May	15	Sue Gapstur, Ph.D.
May	29	Monica Jakacka
June	5	Gerald Soff, Ph.D.
June	12	Vince Cryns
June	19	Hao Wang
June	26	Presentation by students

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Functional Interplay between Type I Collagen and Cell Surface Matrix Metalloproteinase Activity*

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Type I collagen stimulation of pro-matrix metalloproteinase (pro-MMP)-2 activation by ovarian cancer cells involves β_1 integrin receptor clustering; however, the specific cellular and biochemical events that accompany MMP processing are not well characterized. Collagenolysis is not required for stimulation of pro-MMP-2 activation, and denatured collagen does not elicit an MMP-2 activation response. Similarly, DOV13 cells bind to intact collagen utilizing both $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins but interact poorly with collagenase-treated or thermally denatured collagen. Antibody-induced clustering of $\alpha_3\beta_1$ strongly promotes activation of pro-MMP-2, whereas $\alpha_2\beta_1$ integrin clustering has only marginal effects. Membrane-type 1 (MT1)-MMP is present on the DOV13 cell surface as both an active 55-kDa TIMP-2-binding species and a stable catalytically inactive 43-kDa form. Integrin clustering stimulates cell surface expression of MT1-MMP and co-localization of the proteinase to aggregated integrin complexes. Furthermore, cell surface proteolysis of the 55-kDa MT1-MMP species occurs in the absence of active MMP-2, suggesting MT1-MMP autolysis. Cellular invasion of type I collagen matrices requires collagenase activity, is blocked by tissue inhibitor of metalloproteinases-2 (TIMP-2) and collagenase-resistant collagen, is unaffected by TIMP-1, and is accompanied by pro-MMP-2 activation. Together, these data indicate that integrin stimulation of MT1-MMP activity is a rate-limiting step for type I collagen invasion and provide a mechanism by which this activity can be down-regulated following collagen clearance.

The MMP family is composed of at least 25 zinc-dependent extracellular endopeptidases whose activities are regulated predominantly by expression as inactive precursors, or zymogens (1–3). Although precise physiological activators of MMPs[1] are unknown, a variety of serine proteinases and other MMPs in the extracellular milieu execute the initial propeptide cleavage events *in vitro* (2). An exception to serine protease activation is pro-MMP-2 (72-kDa gelatinase A), which lacks the necessary basic amino acid cleavage sites in its pro-domain (4). A primary mechanism of pro-MMP-2 activation involves zymogen association with the cell surface via formation of a ternary complex containing tissue inhibitor of metalloproteinase (TIMP)-2 and membrane type 1-MMP (MT1-MMP, MMP-14) (3–6). Following trimeric complex formation, it is hypothesized that a neighboring MT1-MMP molecule that is not associated with TIMP-2 cleaves pro-MMP-2 at the Asn^{37}–Leu^{38} peptide bond within the pro-domain (7). Intermediately processed MMP-2 (Leu^{38}–MMP-2) undergoes further concentration-dependent autolytic cleavage(s) to generate mature enzymes that can be released into the soluble phase or remain surface-associated (8). Although biological mechanisms of active MMP-2 release from the cell surface are not well characterized and dissociation kinetics provide little insight, cellular binding affinities may shift following pro-MMP-2 cleavage.

Culturing a variety of cell types within a three-dimensional gel of type I collagen stimulates cellular activation of pro-MMP-2 (9–12). Although MT1-MMP is implicated in MMP-2 processing, regulation of cellular events that promote MMP processing are poorly understood. As cellular interaction with type I collagen is mediated largely through integrin receptors, it has been postulated that collagen stimulation occurs either directly or indirectly through integrin signaling (12–16). In support, we have previously demonstrated that culturing DOV13 ovarian cancer cells in a three-dimensional collagen gel elicits a strong pro-MMP-2 activation response that can be mimicked by clustering of β_1 integrin receptors (12). Furthermore, pro-MMP-2 activation coincides with the processing of MT1-MMP into truncated 55- and 43-kDa forms on the cell surface. In this study, we utilize a variety of approaches to elucidate the biochemical requirements of type I collagen stimulation of MMP zymogen activation, characterize processed forms of MT1-MMP that are generated in this response, and examine the proteinase requirements for cellular invasion of type I collagen gels. Our findings illustrate a general mechanism by which cells may regulate cell surface-associated MMP activity via interactions with pericellular collagen matrix.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin, gelatin, cell culture reagents, human placental type I collagen, aminophenylmercuric acetate, anti-(rabbit IgG)-peroxidase conjugates, purified mouse immunoglobulins, 2.97-μm diameter latex beads, concanavalin A (ConA), and *ortho*-phenanthroline were all purchased from Sigma. Anti-human β_1 integrin

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[1] The abbreviations used are: MMP, matrix metalloproteinase; MT1-MMP, membrane type-1 matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase; ConA, concanavalin A; rCBD123, recombinant collagen binding domain; rCD, recombinant carboxyl hemopexin domain; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; BSA, bovine serum albumin; mAb, monoclonal antibody; CR, collagenase-resistant; MES, 4-morpholineethanesulfonic acid; MMPI, MMP inhibitor; CHO, Chinese hamster ovary.

mAb clones 21C8 and P5D2, anti-human α_2 mAb clones P1E6 and AK7, anti-human α_3 integrin mAb clones P1B5 and ASC-6, anti-human $\alpha_3\beta_1$ heterodimer integrin mAb clone M-KD102, anti-human (carboxyl domain) TIMP-2 mAb clone 67-4H11, and anti-human MT1-MMP polyclonal antibody (AB815, hinge domain) were all obtained from Chemicon (Temecula, CA). Hydrobond-P:polyvinylidene difluoride membrane was obtained from Amersham Pharmacia Biotech. Super-Signal-enhanced chemiluminescence reagents were purchased from Pierce. The general hydroxamic acid MMP inhibitor INH-3850-PI (MMPI) was purchased from Peptides International (Louisville, KY). Purified TIMP-2 and TIMP-1 and anti-MT1-MMP (raised against amino acids 160–173/catalytic domain) polyclonal antibody MTK3 were generous gifts of Dr. Hideaki Nagase (Kennedy Institute of Rheumatology, Imperial College School of Medicine, UK). Recombinant MMP-2 type II fibronectin domain repeats (rCBD123) and hemopexin carboxyl domain (Gly^{417}–Cys^{631}) were generated as described previously (17, 18). Collagenase-resistant (CR) murine type I collagen (α_1(I) chain mutations, $Gln^{774} \rightarrow Pro^{774}$, $Ala^{777} \rightarrow Pro^{777}$, and $Ile^{776} \rightarrow Met^{776}$) and wild type murine type I collagen were the generous gifts of Dr. Stephen Krane (Harvard University) (19).

Cell Culture—The ovarian carcinoma cell line DOV13 was provided by Dr. Robert Bast, Jr. (M.D. Anderson Cancer Center, Houston, TX). Cell culture was maintained under standard conditions in 75-cm^2 cell culture flasks (20).

Quantification of Cell Adhesion—96-well cluster plate chambers were coated with 50 μl of 10 μg/ml type I collagen, ¾,¼ collagen fragments (prepared as described below), or type I gelatin (1.6 μg/cm^2) in sterile phosphate-buffered saline (PBS) for 4 h at 25 or 37 °C, blocked with 3% BSA in minimal essential medium for 1 h at 37 °C, washed with PBS, and air-dried. Cells (1×10^5 cells/ml in serum-free medium) were incubated for 20 min at 37 °C with specific integrin function-blocking antibodies or nonspecific control antibodies, plated at a density of 2×10^4 cells/well, and allowed to adhere for 75 min. Unbound cells were removed by washing with PBS, and adherent cells were fixed in ethanol (10 min), stained with 0.5% crystal violet (20 min), washed extensively with water, and solubilized with 100 μl 1% SDS. Relative adhesion was quantified by monitoring the absorbance of released dye at 540 nm ($n = 5$).

Isolation of Collagen Fragments and Preparation of Collagen-containing Surfaces—Human placental collagen (7 mg) was cleaved into ¾ and ¼ fragments by incubating with 2 μg of MMP-1 for 16 h at 25 °C in Tris-buffered saline (TBS) (pH 7.4) containing 60 nM $CaCl_2$. Cleaved collagen was isolated using a modified ammonium sulfate precipitation protocol (21). Ammonium sulfate (12%) was added to collagen suspension on ice at 4 °C under constant stirring for 1 h and then centrifuged (15,000 × *g*, 1 h, 4 °C). The 12% pellet containing intact collagen was washed with ice-cold TBS containing 12% ammonium sulfate, re-centrifuged (15,000 × *g*, 1 h, 4 °C), solubilized with 0.2 M acetic acid, and dialyzed against PBS. To isolate the ¾ and ¼ fragments, ammonium sulfate was added to the 12% supernatant to a final concentration of 25%, incubated for 1 h, and centrifuged (15,000 × *g*, 1 h, 4 °C). The 25% pellet containing ¾ and ¼ collagen fragments was washed with ice-cold TBS containing 25% ammonium sulfate, re-centrifuged (15,000 × *g*, 1 h, 4 °C), solubilized, and dialyzed as described above. Type I gelatin was produced by thermal denaturation of type I collagen for 20 min at 60 °C. Protein concentrations were determined using the Bio-Rad D_C kit and bovine albumin as a standard.

Assays using thin deposits of type I collagen were performed by dialyzing acid-solubilized collagen against PBS, diluting to 10 μg/ml in 100 mM sodium carbonate (pH 9.6), and coating 24-well cluster plate chambers with 200 μl (10 μg/ml, 1.1 μg/cm^2) of collagen. Chambers were incubated for 1 h at 37 °C, washed with sterile PBS, and air-dried. For three-dimensional collagen gel experiments, dialyzed type I collagen was diluted to 1.5 mg/ml with cold minimum essential medium containing 20 mM Hepes (pH 7.4). Diluted collagen (200 μl, 158 μg/cm^2) was added to 24-well plates and allowed to gel at 37 °C for 30 min before the addition of cells (2.5×10^5) to wells. Cells were incubated for 48–72 h in serum-free medium at 37 °C before collection of conditioned media.

Integrin Clustering—Anti-integrin subunit-specific antibodies or control IgG were passively adsorbed onto 2.97-μm diameter latex beads as described previously using the following modifications (12, 24). Latex beads were incubated at a final suspension of 0.1% in 100 mM MES buffer pH 6.1), with 25 μg/ml appropriate antibody in 1-ml volumes overnight at 4 °C under agitation, and then blocked with 10 mg/ml BSA for 2 h at room temperature. Blocked beads were pelleted (3,000 × *g*, 3 min, 25 °C), washed twice with 1 ml of serum-free media, and resuspended to 1% by volume. Protein concentration assays using a BCA detection kit (Sigma) indicated 60–70% adsorption of immunoglobulins. Cells were plated at a density of 2.5×10^5 cells/well in 24-well cluster plates (Becton Dickinson) overnight in serum-containing medium, incubated for 2 h in serum-free medium prior to the addition of fresh medium containing soluble antibodies (10 μg/ml), concanavalin A (20 μg/ml), (25) or antibody-adsorbed latex beads (3–4 μg/ml; 0.02% beads by final volume) for 18–20 h. All final volumes were 500 μl/well.

Gelatin Zymography—Gelatinase activities in conditioned media were determined using SDS-polyacrylamide gel electrophoresis zymography. Conditioned media (20 μl) from an equivalent number of cells were electrophoresed without reduction on SDS-polyacrylamide gel electrophoresis gels prepared with 9% acrylamide containing 0.1% gelatin (23). SDS was removed through a 1-h incubation in 2.5% Triton X-100, and gels were incubated in 20 mM glycine, 10 mM $CaCl_2$, 1 μM $ZnCl_2$ (pH 8.3), at 37 °C for 24 h prior to staining for gelatin with Coomassie Blue. Enzyme activity was visualized as zones of gelatin clearance within the gels.

MMP-2 Competition Experiments—Cells were grown to confluency in 24-well chamber plates and incubated for 2 h in serum-free medium. Fresh medium containing various concentrations of rCBD123 (17) or recombinant hemopexin carboxyl domain (18) were added to cells in a 500-μl volume. After 2 h, ConA was added to a final concentration of 20 μg/ml, and cells were incubated for an additional 18 h. Conditioned media and cell lysates were collected and processed as described above.

MT1-MMP Immunoblots—Cells were incubated under various conditions, collected with lysis buffer, and protein concentration of lysates was analyzed using the Bio-Rad D_C detection kit and bovine albumin standards. Cell lysates (5–15 μg) were electrophoresed on 9% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membrane, and blocked with 3% BSA in 50 mM Trizma (Tris base) (pH 7.5), 300 mM NaCl, 0.2% Tween 20 (TBST). Membranes were incubated for 1 h at room temperature with a 1:4000 dilution of anti-human MT1-MMP polyclonal antibody in 3% BSA/TBST. Immunoreactive bands were visualized with a peroxidase-conjugated anti-(rabbit-IgG) (1:5000 in 3% BSA/TBST) and enhanced chemiluminescence.

Isolation of Biotin-labeled Cell Surface Proteins—Cells were grown to confluency in 6-well cluster dishes, washed with PBS, and incubated for 2 h in serum-free medium. Fresh serum-free medium (1 ml) containing a 0.06% antibody-coated bead suspension or 20 μg/ml concanavalin A was added, and cells were incubated for 20 h. Conditioned medium was removed; cells were washed with 2 × 2 ml of PBS, and surface proteins were labeled with a non-cell-permeable sulfo-NHS biotin analog (500 μl at 500 μg/ml PBS, Pierce) under gentle shaking at 4 °C for 30 min. After washing, cells were incubated with 1 ml of 100 mM glycine/PBS for an additional 20 min under gentle shaking at 4°C. Washed cells were lysed with 500 μl of lysis buffer (50 mM sodium phosphate buffer (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% Triton X-100, 1 μg/ml aprotinin, 1 μM pepstatin, 10 μM leupeptin, and 10 μM E64), collected with a cell scraper, and clarified by centrifugation (10,000 × *g*, 10 min, 4 °C). Protein concentrations were calculated as described. To precipitate biotin-labeled cell surface proteins, lysate (1 ml, 750 μg/ml) was added to either ImmunoPure immobilized monomeric avidin or multimeric streptavidin gel (40 μl) (Pierce) and incubated overnight at 4 °C on a rotator. Gels were washed 5× with lysis buffer. In some experiments, 10 mM free D-Biotin/PBS (Pierce) was added to aliquots of monomeric avidin gel-immobilized protein to compete off bound protein at 4 °C overnight. Eluates (30 μml) were analyzed by gelatin substrate zymography or immunoblotting. Control conditions utilized non-biotinylated cell lysates.

Isolation of Plasma Membranes—DOV13 cells were grown to confluency in 15-cm culture dishes, washed with PBS, switched to serum-free medium for 2 h, and incubated in 5 ml of fresh serum-free medium containing 20 μg/ml ConA for 20 h. Cells were washed twice with 5 ml of PBS, collected with a cell scraper, and pelleted by centrifugation (1500 × *g*, 10 min, 4 °C). Cells were resuspended in ice-cold 5 mM Tris/HCl (pH 7.8) and incubated for 10 min prior to homogenization by 30 passages through a 26¼-gauge needle (8). Crude membrane preparations were isolated by centrifuging whole cell lysates (10,000 × *g*, 10 min, 4 °C), retaining the supernatant, and centrifuging the supernatant (100,000 × *g*, 1 h, 4 °C). The recovered pellet was washed in 20 mM Tris/HCl (pH 7.8), 10 mM $CaCl_2$, 0.05% Brij 35 and recentrifuged (100,000 × *g*, 45 min, 4 °C). The plasma membrane-containing pellet was resuspended in washing buffer, and protein concentration was assessed as described above.

Cross-linking and Immunoprecipitation—To isolate TIMP-2-binding MT1-MMP species, cell surface proteins were cross-linked, and TIMP-2 complexes were isolated through immunoprecipitation. Confluent cells were incubated for 16 h in 5 ml of serum-free medium containing 20 μg/ml ConA and 125 ng/ml TIMP-2. After washing (2 × 5 ml of PBS),

cell surface proteins were cross-linked with 2 mM 3,3'-dithiobis(sulfosuccinimidylpropionate) (Pierce) in PBS at 4 °C for 25 min under gentle shaking. Cells were washed (2 × 10 ml of PBS), and lysates were collected in cold lysis buffer. Clarified cell lysates (700 μg/ml) were incubated with 5 μg of anti-TIMP-2 (carboxyl-terminal) mAb clone 67-4H11 or murine IgG (κ) for 2.5 h at 4 °C on a rotator prior to the addition of a 50% slurry of protein A-agarose (20 μl) for 90 min. Immunoprecipitates were centrifuged (10,000 × *g*, 3 min, 4 °C), washed with lysis buffer (5 × 1 ml), and protein solubilized with 50 μl of 5× Laemmli buffer and processed for MT1-MMP immunoblotting as described above. To evaluate MT1-MMP activity, immunoprecipitations using anti-MT1-MMP hinge antibodies were performed using lysates from cells that were not exposed to cross-linking agent. Lysates were evaluated by gelatin zymography as described above.

Stable Transfection of MT1-MMP—The eukaryotic plasmid vector pCR3.1-Uni (Invitrogen, Carlsbad, CA) containing the MT1-MMP gene under a cytomegalovirus promoter was the generous gift of Dr. Duaniqng Pei (University of Minnesota). A 6 histidine repeat was incorporated 3' of the carboxyl terminus using a polymerase chain reaction based approach with a 5' primer of ATgggCAgCgATgAAgTC and 3' primer of CgTCTAgATCAgTgATgATggTggTgATggACCTTgTCCAgCAgggA. The amplified polymerase chain reaction product was cloned back into the vector using a unique *Fse*1 restriction site and the *Xba*I restriction site in the 3' polylinker region of the vector. Plasmid DNA was sequenced and then isolated for transfection experiments using a Qiafilter maxi-kit (Qiagen, Valencia, CA). The vector control used in experiments is the pCR3.1-Uni vector driving the chloramphenicol transferase gene (pCR3.1/CAT, Invitrogen). pCR3.1/MT1-His_6 and control pCR3.1/CAT were transfected with LipofectAMINE (Pierce) as a delivery vehicle and isolated transformants screened for neomycin resistance selection (450 μg/ml G418). Soluble recombinant MT1-MMP lacking the linker from the carboxyl-terminal hemopexin domain through the cytoplasmic tail was expressed in CHO cells using the pW1HG vector. Soluble MT1-MMP purified from CHO serum-free conditioned medium was examined by electrophoresis on SDS-polyacrylamide gels and silver staining to visualize proteins.

Immunocytochemistry—Cells were cultured on glass coverslips overnight prior to addition of a 1-ml volume of serum-free medium containing 0.005% β_1 integrin mAb-coated beads. Cells were fixed with 3.7% formaldehyde without permeabilization and incubated with anti-MT1-MMP polyclonal antibody (MTK3) or normal rabbit serum in PBS. Immunoreactivity was visualized with a fluorescein-conjugated anti-rabbit secondary antibody previously purified against cross-reactivity to mouse immunoglobulins (Chemicon). Phase contrast and indirect fluorescent images were collected using a Zeiss fluorescence microscope (model II) and Adobe PhotoShop Software (version 4.0).

Cellular Migration and Invasion—Type I collagen was dissolved in 0.5 M acetic acid at a concentration of 2 mg/ml. For invasion experiments, the collagen stock was neutralized with 100 mM Na_2CO_3 (pH 9.6) to a final concentration of 0.4 mg/ml. Transwell inserts (0.8 μm, Becton Dickinson, Bedford, MA) were coated on the underside with 500 μl of collagen diluted to a concentration of 100 μg/ml at 37 °C for 1 h. Collagen gels were prepared in the inner well by adding 50 μl of collagen (20 μg) at room temperature and allowing gels to air-dry overnight. Collagen-coated inserts were then washed with minimum essential medium three times to remove salts and used immediately. In some experiments, transwell inserts were coated with collagenase-resistant (19), rather than wild type, collagen. Cells were trypsinized, washed with serum-free medium, and 1 × 10^5 cells were added to the inner invasion chamber in a volume of 200 μl. The outer wells contained 400 μl of culture medium (serum-free, except in experiments using murine collagen). To evaluate the MMP dependence of invasion, after a 2-h incubation, the MMP inhibitors TIMP-1 (10 nM), TIMP-2 (10 nM), or MMPI (10 μM) were added to the inner and outer chambers as indicated. Control wells for MMPI contained the solvent Me_2SO. Cells were allowed to invade for 24–48 h as indicated; non-invading cells were removed from inner wells using a cotton swab, and invading cells adherent to the bottom of membrane were fixed and stained using a Diff-Quick staining kit (DADE AG, Miami, FL). Invading cells were enumerated by dividing membranes into 4 quadrants and counting the number of cells in 3 distinct areas for each quadrant under a 10× objective using an ocular micrometer. Assays were performed in triplicate. To evaluate the integrin dependence of invasion, after a 2-h incubation anti-integrin antibodies (15 μg/ml) or control IgG (15 μg/ml) were added to the inner and outer chambers, as indicated. To evaluate the collagen structural requirements of invasion, wells were coated with thermally denatured collagen (gelatin, 50 μg/well in a 50-μl volume) and allowed to invade for 24 h in the presence of TIMP-1 (10 nM), TIMP-2 (10 nM), or aprotinin (20 μg/ml), as indicated. Invasion of native and denatured collagen was also evaluated in the presence of the MMP-2 carboxyl hemopexin-like domain (rCD, 100 nM) (18) or the fibronectin type II-like domain (rCBD, 100 nM) (17) under the conditions described above.

To assess cell motility, migration through transwell membranes coated with a thin layer of collagen (100 μg/ml, 37 °C, 1 h) on both the upper and lower surfaces was evaluated as described above using an incubation time of 5.0 h. Haptotactic motility was assessed as described previously by plating cells on coverslips coated with colloidal gold overlaid with type I collagen (100 μg/ml) (22). Cells were allowed to migrate for 18 h, and phagokinetic tracks were monitored by visual examination using a Zeiss microscope with dark-field illumination. Semi-quantitative analysis of phagokinetic tracks was performed by measuring track area using computer-assisted image analysis and NIH Image.

TABLE I

Rapid attachment of DOV13 cells requires $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins

DOV13 cells (2 × 10^4/well) were preincubated with the indicated concentration of antibodies for 20 min under standard cell culture conditions prior to addition to collagen-coated wells. Cell attachment was measured as described under "Experimental Procedures." DOV13 cells utilize both $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins to bind native type I collagen. Values were corrected for control binding to BSA-coated wells.

Matrix + condition	Absorbance at 540 nm
Type I collagen	226 ± 41
+ 15 μg/ml mouse IgG	196 ± 51
+ 15 μg/ml anti-β_1 integrin (P5D2)	48 ± 4[a]
+ 5 μg/ml anti-α_2 integrin (P1E6)	167 ± 40
+ 15 μg/ml anti-α_2 integrin	126 ± 24[b]
+ 5 μg/ml anti-α_3 integrin (P1B5)	149 ± 10[b]
+ 15 μg/ml anti-α_3 integrin	117 ± 23[b]
+ 7.5 μg/ml anti-α_2 integrin/μg/ml anti-α_3 integrin	83 ± 12[a]

[a] $p < 0.05$.
[b] $p < 0.005$ relative to IgG controls.

RESULTS

Collagen Structure Regulates Cell Adhesion—To evaluate the relative contribution of $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins to collagen binding, attachment of DOV13 cells to type I collagen in the presence of integrin function-blocking antibodies was evaluated. DOV13 cells adhere rapidly to type I collagen-coated microtiter wells in an integrin-dependent fashion (Table I). Addition of anti-β_1 integrin function-blocking monoclonal antibodies (15 μg/ml, clone P5D2) significantly reduced binding to collagen relative to a nonspecific IgG control (75%), whereas equivalent amounts of either α_2 (P1E6) or α_3 (P1B5) integrin-blocking antibodies inhibited adhesion by 35–40% (Table I). Combination of α integrin blocking antibodies (7.5 μg/ml each) together reduced adhesion nearly 60%, supporting the conclusion that both $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrin heterodimers contribute to DOV13 cell attachment to type I collagen.

To assess the collagen structural requirements for integrin-mediated binding, adhesion to both collagenase-cleaved and thermally denatured collagen was analyzed. Native type-I collagen was incubated with collagenase-1 (MMP-1) and the ¾ and ¼ digestion products isolated through ammonium sulfate precipitation (21). Intact collagen heterofibrils, also isolated through this approach, were gelatinized by thermal denaturation. Intact collagen, ¾ and ¼ collagen fragments, or gelatin were coated onto microtiter wells at either 25 or 37 °C, and adhesion was evaluated as described above. Both intact collagen and ¾ and ¼ fragments support adhesion of DOV13 cells when matrices are coated at 25 °C; however, adhesion to ¾ and ¼ fragments was significantly reduced when matrices were coated at physiologic temperature (Table II). Although cells will slowly adhere to thermally denatured collagen (3–4 h), little adhesion to this matrix was observed under any coating condition over the course of the assay (75 min) (Table II). These results indicate that ¾ and ¼ collagen fragments coated at

TABLE II
Intact collagen is required for DOV13 attachment

Type I collagen, ¾ and ¼ collagen fragments, and type I gelatin were coated at the indicated temperature (1.6 $\mu g/cm^2$), blocked with BSA, and cell attachment (2×10^4/well) measured as described under "Experimental Procedures." Values are reported as the percent absorbance at 540 nm in comparison to intact type I collagen, coated at the specified temperature.

10 μg/ml matrix	% Bound relative to collagen
	%
Coating at 25 °C	
Type I collagen	100
Type I gelatin	28.1 ± 10.7[a]
¾ and ¼ type I collagen	94.3 ± 11.7
Coating at 37 °C	
Type I collagen	100
Type I gelatin	9.7 ± 9.2[a]
¾ and ¼ type I collagen	9 ± 6.3[a]

[a] $p < 0.005$ relative to intact type I collagen coated at the specified temperature.

25 °C retain a triple helical conformation that is required for recognition by $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins, whereas coating at 37 °C results in destabilization of fragment helices (21). Together, these data suggest that collagenase activity at the cell/matrix interface can reduce the efficiency of integrin-mediated adhesion on the surface of DOV13 cells, thereby potentially affecting matrix-induced signaling events that are involved in cellular invasion.

Integrin Clustering Promotes Cell Surface MMP-2 Processing—To evaluate the matrix structural requirements for collagen induction of pro-MMP-2 activation, cells were cultured in the presence of native or thermally denatured collagen. Processing of pro-MMP-2 was not observed by cells cultured on type I gelatin (Fig. 1, *lanes 4* and *5*). However, culturing cells with collagenase-resistant collagen (19) stimulated pro-MMP-2 processing as efficiently as wild type collagen (Fig. 1, *lanes 6* and *7*), indicating that collagen processing is not necessary to promote a cellular gelatinase activation response. Similar to the wild type protein, gelation of collagenase-resistant collagen abrogated the ability to elicit pro-MMP-2 activation (Fig. 1, *lane 5*). In conjunction with cell adhesion studies, these data indicate that integrin interaction with intact triple-helical collagen is necessary to stimulate pro-MMP-2 activation and that destabilization of the collagen matrix following collagenase activity will reduce the ability of pericellular collagen to elicit an MMP response.

We have demonstrated previously that β_1 integrin clustering stimulates a pro-MMP-2 processing event that correlates with enhanced gelatinolytic activity in conditioned media, indicating that integrin signaling is sufficient to elicit pro-MMP-2 activation (12). As adhesion of DOV13 cells to type I collagen is supported by both $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins (Table I), integrin clustering was induced through the α-subunit to evaluate the effects of clustering each specific integrin heterodimer on pro-MMP-2 activation. Consistent with adhesion blocking assays, α_2, α_3, and β_1 integrin antibody-coated beads attached with similar efficacy (data not shown); however, analysis of conditioned media by zymography demonstrates that clustering α_3 or β_1 integrins elicits a stronger pro-MMP-2 activation response than α_2 integrins (Fig. 2*A*, *lower panel*). The observed difference in α integrin subunit specificity was independent of the antibody clone used to promote clustering, and utilization of antibodies recognizing an $\alpha_3\beta_1$ heterodimer-specific epitope also resulted in a pro-MMP-2 activation response (Fig. 2*A*, *lower panel, lane 8*). Soluble integrin antibodies did not influence pro-MMP-2 expression or activation (Fig. 2*A*, *upper panel*), supporting the hypothesis that multivalent integrin aggregation is necessary for proteinase induction.

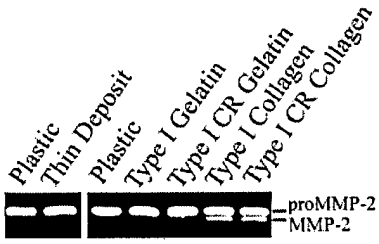

FIG. 1. **Collagen stimulation of pro-MMP-2 activation.** DOV13 cells (2.5×10^5 cells/well) were incubated on plastic or a thin deposit of type I collagen (1.1 $\mu g/cm^2$), in the presence of thermally denatured wild type or collagenase-resistant (*CR*) collagen (Gelatin or CR Gelatin, respectively, 158 $\mu g/cm^2$), or within wild type or CR type I collagen gels (Collagen or CR Collagen, respectively, 158 $\mu g/cm^2$), as indicated, in serum-free medium for 48 h. Conditioned media were analyzed by gelatin zymography. The relative migration positions of pro- and active MMP-2 are indicated.

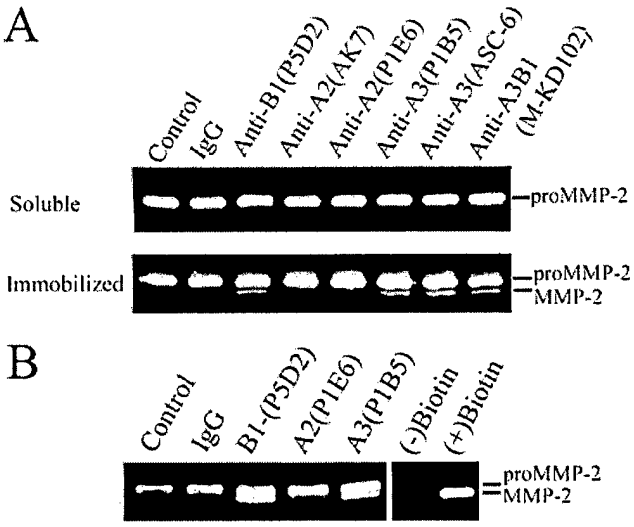

FIG. 2. **$\alpha_3\beta_1$ integrin clustering promotes MMP-2 processing and surface association.** *A*, DOV13 cells were treated with the indicated soluble (10 μg/ml) (*upper panel*) or bead-immobilized (4 μg/ml) (*lower panel*) antibodies for 20 h, and conditioned media were analyzed by gelatin zymography. Antibody clone numbers are given in *parentheses*. The relative migration positions of pro- and active MMP-2 are indicated. *B*, cells were untreated (*Control*) or treated with the indicated bead-immobilized antibodies (12 μg/ml) for 20 h prior to biotinylation of surface proteins using a non-cell-permeable biotin and cell lysis. Labeled protein (750 μg) was captured with monomeric avidin gels, eluted with D-biotin, and eluates analyzed by gelatin zymography. The lack of activity eluted from non-biotinylated ConA-treated (20 μg/ml) cell lysates (*−Biotin*) demonstrates the specificity of the system for cell surface proteins, as shown in the biotinylated, ConA-treated (20 μg/ml) control (*+Biotin*).

As the transmembrane proteinase MT1-MMP is predicted to catalyze cell surface integrin-mediated pro-MMP-2 activation, MMP-2 association with the plasma membrane was evaluated by surface labeling with cell-impermeable s-NHS-biotin and isolating biotinylated proteins with monomeric avidin-agarose gels. Proteins were eluted from avidin-agarose gels using 10 mM free D-biotin and analyzed by zymography for MMP activity. Clustering of both α_3 and β_1 integrins promoted a significant association of MMP-2 to the cell surface (Fig. 2*B*, *lanes 3* and *5*) relative to cells subjected to α_2 integrin clustering (Fig. 2*B*, *lane 4*) or controls (Fig. 2*B*, *lanes 1* and *2*), confirming that $\alpha_3\beta_1$ integrins elicit a more robust pro-MMP-2 processing response.

Release of MMP-2 from the Cell Surface—Secreted pro-MMP-2 binds to the cell surface through the carboxyl-terminal hemopexin domain, and an intermediately processed form is

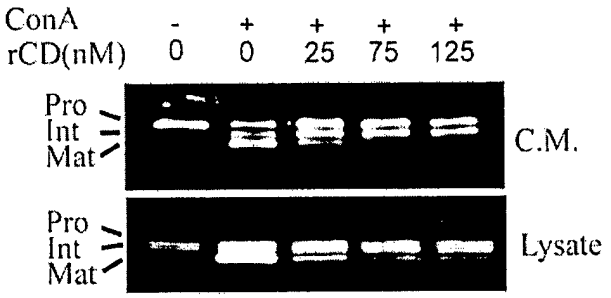

FIG. 3. **Competition of MMP-2 from the cell surface.** Cells were cultured for 20 h in the presence or absence of ConA (20 μg/ml) and recombinant MMP-2 carboxyl hemopexin domain (*rCD*, 0–125 nM), as indicated. Conditioned media (*C.M.*) and cell lysates (5 μg of total protein) were analyzed by gelatin zymography for processed forms of MMP-2. *Pro*, pro-MMP-2 (66 kDa); *Int*, intermediately processed MMP-2 (64 kDa); *Mat*, mature MMP-2 (62 kDa).

generated through MT1-MMP-mediated cleavage of the MMP-2 pro-domain at the Asn^{37}–Leu^{38} peptide bond (7). The MMP-2 intermediate is then predicted to undergo maturation to an active enzyme through concentration-dependent autolysis at the cell surface (8). Although formation of the trimeric MT1-MMP·TIMP-2·MMP-2 activation complex at the cell surface is well established (reviewed in Ref. 3), the mechanism by which active MMP-2 is released from the plasma membrane is presently unclear. Saturation binding studies are not technically feasible due to the instability of the intermediate and mature forms of the activated enzyme in concentrated solution. Thus, the ability of the MMP-2 hemopexin domain to dissociate surface-bound MMP-2 was evaluated. In the presence of increasing concentrations of recombinant hemopexin domain, active MMP-2 is not associated with the cell surface (Fig. 3*A*). In control experiments, competition of MMP-2 from the cell surface was not observed with rCBD123 at the same molar concentrations (data not shown).

Integrin Clustering Promotes Cell Surface MT1-MMP Processing—The appearance of active MMP-2 on the cell surface implicates the involvement of MT1-MMP in integrin stimulation of pro-MMP-2 processing. To evaluate the effect of α integrin clustering on events further upstream in the zymogen activation pathway, cells were treated with integrin antibody-coated beads, and cell lysates were immunoblotted with an antibody reactive against the hinge domain of MT1-MMP. Consistent with previous observations (12), stimulation of DOV13 cells with ConA promotes accumulation of 55- and 43-kDa forms of MT1-MMP in cell lysates (Fig. 4*A*, *lane 6*). Clustering of β_1 integrins, and to a lesser extent α_3 integrins, promoted processing of MT1-MMP to a 43-kDa form (Fig. 4*A*, *lanes 3* and *5*, respectively), whereas little or no change in MT1-MMP expression was observed following clustering of α_2 integrins (*lane 4*) or under control conditions with nonspecific IgG (*lane 2*). Furthermore, β_1 integrin clustering results in a small, but reproducible, accumulation of 55-kDa MT1-MMP in cell lysates (*lane 3*).

To address whether integrin clustering stimulates MT1-MMP expression on the cell surface, biotin-labeled cell surface proteins were isolated and immunoblotted for MT1-MMP species. ConA treatment promoted a strong accumulation of 55- and 43-kDa MT1-MMP species on the cell surface (Fig. 4*B*, *lane 1*). Higher molecular weight species that were detected in immunoblots of cell lysates (*brackets*, Fig. 4*A*) were absent in cell surface preparations (*brackets*, Fig. 4*B*), indicating that these proteins are likely intracellular. Consistent with cell lysate results, clustering of β_1 integrins promoted cell surface expression of 55- and 43-kDa forms of MT1-MMP (Fig. 4*B*, *lane 5*), whereas clustering of either α integrin subunit was not sufficient to promote detectable changes in the cell surface expression profile of MT1-MMP (Fig. 4*B*, *lanes 6* and *7*). Together, these data suggest that clustering of β_1 integrins promotes pro-MMP-2 cell surface binding and activation through increased expression of cell surface-localized MT1-MMP. Furthermore, cell surface gelatinolytic profiles indicate that $\alpha_3\beta_1$ integrins potentiate a stronger MMP response than $\alpha_2\beta_1$ integrins (Fig. 2*B*). The ability to detect enhanced levels of cellular, but not cell surface, MT1-MMP following α_3 integrin clustering likely reflects the technical limitations of the assay, as β_1 integrin clustering induces a more robust overall response. To evaluate further the pool of MT1-MMP species that reside on the cell surface, cells were stimulated with ConA, surface

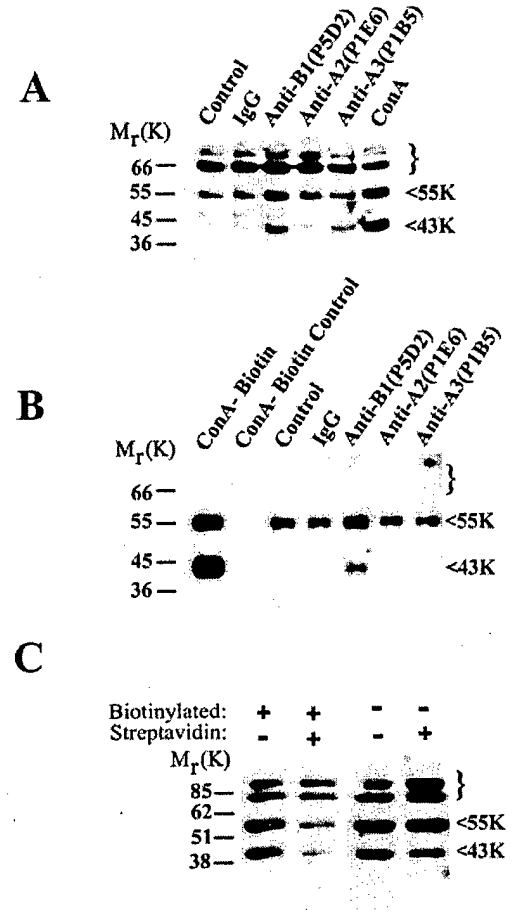

FIG. 4. **Integrin clustering promotes MT1-MMP cell surface expression and processing.** *A*, integrin-induced MT1-MMP processing. Cells were treated for 20 h with bead-immobilized antibodies (4 μg/ml) as indicated, and lysates (10 μg for all samples except ConA, 7 μg) were electrophoresed on 8% polyacrylamide gels and Western-blotted using an antibody to the MT1-MMP hinge region. Blots were developed using enhanced chemiluminescence. The migration position of molecular weight standards is indicated in the left margin, and the migration positions of the 55- and 43-kDa species of MT1-MMP are indicated in the *right margin*. *B*, cells were treated with ConA (20 μg/ml) or the indicated bead-immobilized antibodies (12 μg/ml) for 20 h prior to biotinylation of surface proteins using a non-cell-permeable biotin and cell lysis. Labeled protein (750 μg) was captured with monomeric avidin gels, eluted with D-biotin, and eluates analyzed by electrophoresis and Western blotting for MT1-MMP. The lack of protein eluted from non-biotinylated ConA-treated cell lysates (lane designated *ConA-Biotin Control*) demonstrates the specificity of the system. *C*, to evaluate what fraction of the total pool of 55- and 43-kDa MT1-MMP species resides on the cell surface, cells were stimulated with ConA (20 μg/ml) for 20 h, surface-biotinylated, lysed, and the lysate (750 μg, + Biotin) depleted of biotin-labeled proteins through precipitation using multimeric streptavidin-conjugated agarose gels (+ *Streptavidin*). Streptavidin-depleted or control lysates (7.5 μg) were analyzed by electrophoresis and Western blotting for MT1-MMP.

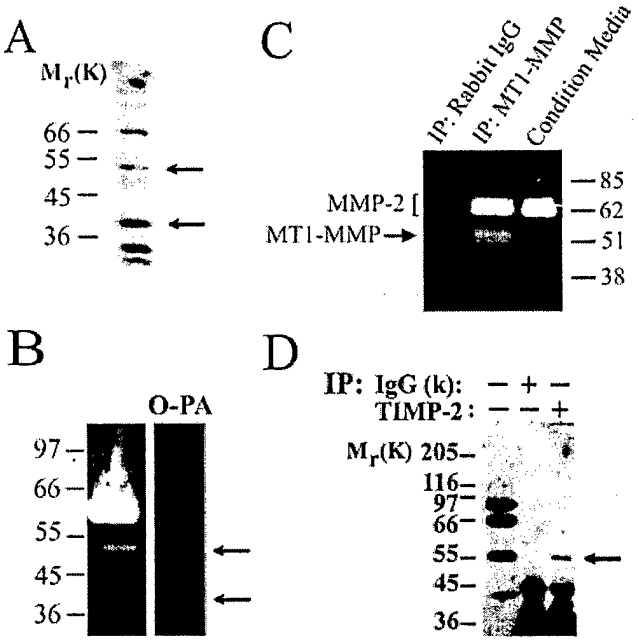

FIG. 5. **Identification of active MT1-MMP species.** DOV13 cells were cultured in the presence of ConA (20 μg/ml) for 20 h, lysed, and homogenized. Plasma membranes were isolated by centrifugation, and aliquots (20 μg) were analyzed for MT1-MMP protein (*A*) and activity (*B*) by immunoblotting and gelatin zymography, respectively. *A*, Western blot of plasma membranes showing proteins cross-reactive with MT1-MMP antibody. The *arrows* indicate the migration positions of the 55- and 43-kDa MT1-MMP species. *B*, zymogram depicting MT1-MMP gelatinolytic activity associated with the 55-kDa species (*upper arrow*). (Note that the prevalent gelatinase activity in this sample is due to plasma membrane-bound MMP-2.) All gelatinase activity is inhibited by the zinc chelating agent *ortho*-phenanthroline (*O-PA*). *C*, immunoprecipitation (*IP*) of DOV13 cell lysates. Lysates (non-denatured) were immunoprecipitated with either IgG control or anti-MT1-MMP antibodies as indicated and analyzed by gelatin zymography. Although the immunoprecipitating antibody recognizes both the 43- and 55-kDa forms of MT1-MMP by immunoblotting (*A*), gelatinase activity corresponding to only the 55-kDa species is observed (*arrow*). MMP-2 co-precipitated as a component of the ternary complex, as evidenced by the co-migration with an MMP-2 standard (*bracket*). Lane designated *conditioned media*, DOV13 conditioned medium to designate migration position of MMP-2 (not subjected to immunoprecipitation). *D*, cross-linking and immunoprecipitation. Cells were treated with ConA (20 μg/ml) and an excess of free TIMP-2 (125 ng/ml) for 18 h. After washing, cell surface proteins were cross-linked with a reducible cross-linking agent (2 mM 3,3′-dithiobis(sulfosuccinimidylpropionate), 25 min, 4 °C). Clarified cell lysates (700 μg) were incubated with 5 μg of anti-TIMP-2 (carboxyl-terminal) mAb clone 67-4H11 or murine IgG (κ) as indicated and precipitated using protein A-agarose. Immunoprecipitates were solubilized with Laemmli sample buffer, electrophoresed under reducing conditions on 8% polyacrylamide gels, and immunoblotted with the MT1-MMP hinge polyclonal antibody. The *arrow* designates the migration position of 55-kDa MT1-MMP, and the *left margin* indicates the migration position of molecular weight standards.

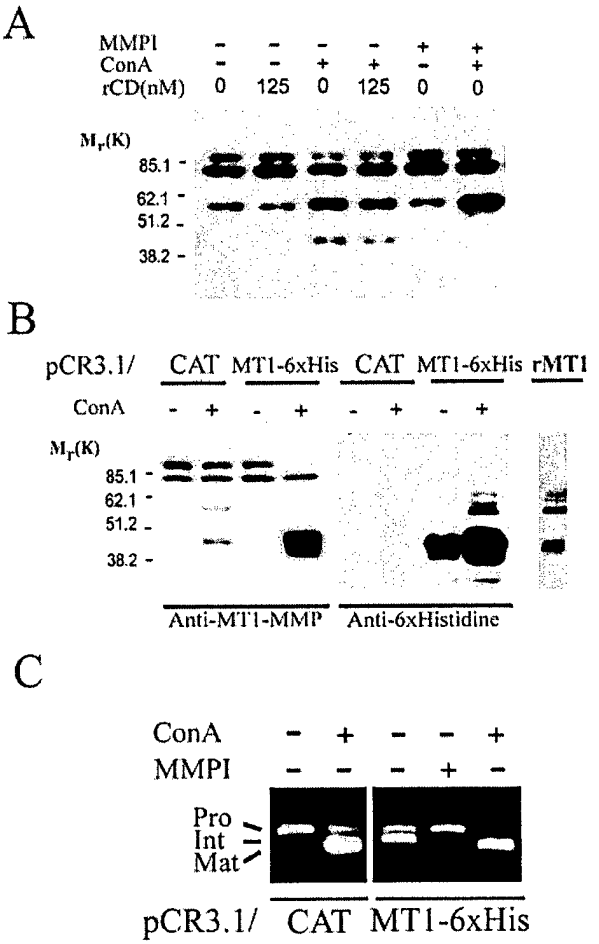

FIG. 6. **MT1-MMP processing in the absence of cell surface MMP-2.** *A*, cells were cultured in the presence or absence of MMPI (10 μM), ConA (20 μg/ml), or rCD (125 nM) as indicated. Lysates (7.5 μg) were electrophoresed on 8% polyacrylamide gels and immunoblotted for MT1-MMP. *B*, stable DOV13 transfectants expressing polyhistidine-tagged MT1-MMP (*MT1-6xHis*) or control vector (*CAT*) were isolated, cultured in the presence or absence of ConA (20 μg/ml) as indicated, and 7.5 μg of cell lysates analyzed by electrophoresis and immunoblotting for MT1-MMP (*lanes 1–4*) or polyhistidine (*lanes 4–8*). *Lane 9* contains soluble recombinant MT1-MMP (*rMT1*) lacking the transmembrane and cytoplasmic domain purified from CHO cell-conditioned medium and visualized by silver staining. *C*, DOV13 stable transfectants expressing polyhistidine-tagged MT1-MMP (*MT1-6xHis*) or vector controls (*CAT*) were isolated, cultured in the presence or absence of ConA (20 μg/ml) or MMPI (10 μM) as indicated, and conditioned media analyzed for pro-MMP-2 activation by gelatin zymography. The relative migration positions of the pro-, intermediate (*Int*), and activated mature (*Mat*) forms of MT1-MMP are indicated in the *left margin*.

biotin-labeled, and lysates were depleted of biotinylated cell surface proteins using multimeric streptavidin-conjugated agarose. A selective depletion of only the 55- and 43-kDa forms of MT1-MMP was observed in streptavidin-treated samples compared with non-treated controls (Fig. 4*C*, *lane 2*), supporting the conclusion that these species, but not the higher molecular weight immunoreactive material present in Fig. 4*A*, are stably expressed on the cell surface.

Characterization of Cell Surface MT1-MMP—As MT1-MMP has been reported to have weak gelatinase activity (30, 31), plasma membrane preparations of ConA-treated DOV13 cells were analyzed by gelatin zymography to determine whether either of the surface-associated MT1-MMP species are proteolytically active. In addition to surface-bound MMP-2, an ortho-phenanthroline-sensitive gelatinolytic band that co-migrated with the non-reduced 55-kDa form of MT1-MMP was observed (Fig. 5*B*), whereas no gelatinase activity was attributable to the 43-kDa species. Control immunoblots demonstrate that both the 55- and 43-kDa species were prevalent in the experimental sample (Fig. 5*A*). To confirm that the observed 55-kDa gelatinolytic activity is a property of MT1-MMP, cell lysates were immunoprecipitated with an anti-MT1-MMP-specific antibody and the immunoprecipitates were analyzed by gelatin zymography. A 55-kDa gelatinolytic activity was recovered together with MMP-2, suggesting that both proteinases co-precipitate as components of the ternary complex (Fig. 5*C*). The relative ratio of MT1-MMP to MMP-2 gelatinase activity is enhanced by the immunoprecipitation approach (Fig. 5, *C versus B*). Similar to results obtained in whole plasma membrane preparations, there was no observable gelatinolytic activity attributable to

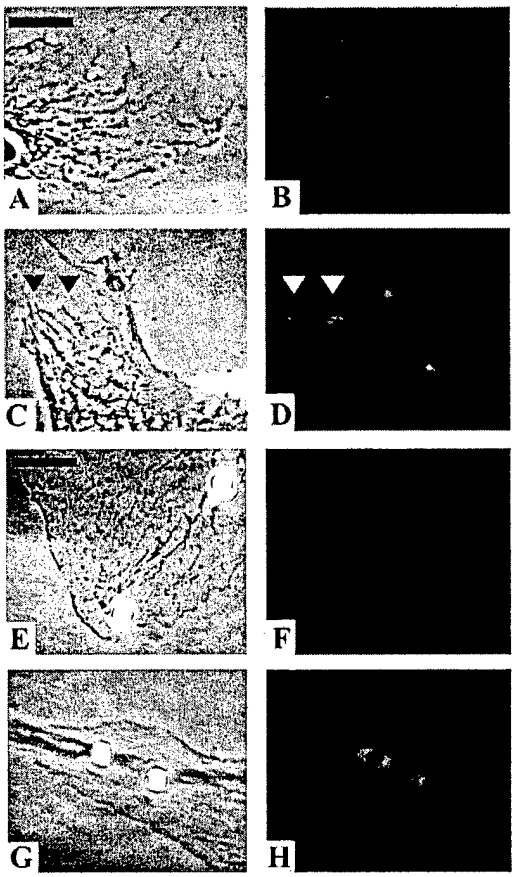

FIG. 7. **MT1-MMP localizes to cellular processes and redistributes to clustered β_1 integrins.** Cells were cultured on glass coverslips in the absence (*A–D*) or presence (*E–H*) of anti-β_1 integrin (clone 21C8)-coated beads for 8 h and then processed for immunofluorescence as described under "Experimental Procedures." Cells were incubated with normal rabbit serum (*B* and *F*) or anti-MT1-MMP antibody (*D* and *H*). Immunoreactivity was visualized with fluorescein-conjugated anti-rabbit secondary antibody previously purified against cross-reactivity to mouse immunoglobulins. Phase contrast images *A, C, E,* and *G* correspond with *B, D, F,* and *H,* respectively, and identify cellular processes and 2.97 μm diameter latex beads. *Arrowheads* denote MT1-MMP immunoreactivity in cell surface projections. *Magnification bar* is 5 μm (*A–D*) or 10 μm (*E–H*), and images were collected with a 63× objective using a Zeiss fluorescence microscope.

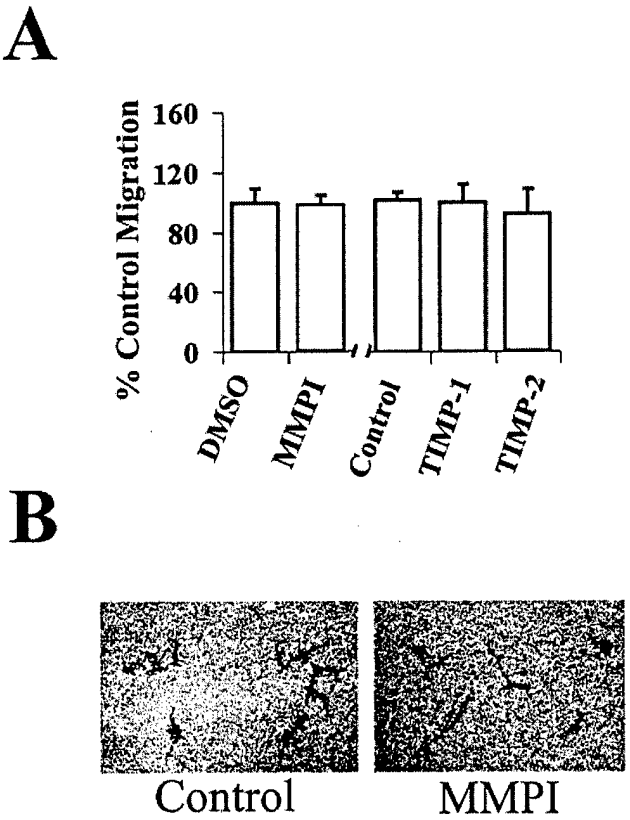

FIG. 8. **Migration of DOV13 cells is MMP-independent.** *A,* cells (2.5×10^5) were seeded onto Transwell filters (8 μm pore) coated with a thin layer of collagen (as described under "Experimental Procedures") in the presence or absence of MMPI (10 μM), Me_2SO (*DMSO*, vehicle for MMPI), TIMP-1 (10 nM), or TIMP-2 (10 nM) as indicated and incubated for 5 h to permit migration. Non-migrating cells were removed from the upper chamber; filters were stained, and migrating cells adherent to the underside of the filter were enumerated using an ocular micrometer. Data are expressed as % of control migration (Me_2SO for MMPI and PBS for TIMP-1 and -2). There is no significant difference in migration of inhibitor-treated samples relative to respective controls ($p > 0.05$). *B,* haptotactic motility of DOV13 cells. Cells (1000) were plated onto coverslips coated with colloidal gold overlaid with type I collagen (100 μg/ml) in the presence or absence of MMPI (10 μM) as indicated, allowed to migrate for 24 h, and phagokinetic tracks visualized using dark field illumination.

the 43-kDa form of MT1-MMP. As these results indicated that the 55-kDa form of MT1-MMP is an active species on the cell surface, the ability to bind TIMP-2 was assessed. Exogenous TIMP-2 was added directly to ConA-treated cells, followed by cross-linking with a reducible, cell-impermeable cross-linker. Cell lysates were then immunoprecipitated with an antibody specific to the carboxyl-terminal domain of TIMP-2, reduced, and analyzed by electrophoresis and immunoblotting for MT1-MMP. The 55-kDa species of MT1-MMP was specifically precipitated through TIMP-2 (Fig. 5*D, lane 3*), providing additional evidence that it is an active, TIMP-2-binding protein.

MT1-MMP Processing in the Absence of Cell Surface MMP-2—As reported previously (12), active 55-kDa MT1-MMP is converted to an inactive 43-kDa form through MMP-dependent proteolysis in DOV13 cells (Fig. 6*A*, compare *lanes 3* and *6*) (12). In a regulated cellular system generating an endogenous MMP-2/MT1-MMP activation response, it is unclear whether this cleavage is mediated by activated MMP-2 or through concentration-dependent autolysis of MT1-MMP. As active MMP-2 is effectively removed from the cell system with 125 nM hemopexin domain (Fig. 3), cellular MT1-MMP processing in the absence of MMP-2 was assessed by immunoblotting. Conversion of 55-kDa MT1-MMP to the 43-kDa form was not affected by the loss of active MMP-2 from the cell surface (Fig. 6*A, lanes 3* and *4*), indicating that MMP-2 is not required for proteolytic processing of endogenous 55-kDa MT1-MMP in DOV13 cells.

To determine whether MT1-MMP degradation proceeds through autolysis or through activity of an unidentified metalloprotease, DOV13 cells that stably overexpress full-length histidine-tagged MT1-MMP were generated. Overexpression of MT1-MMP resulted in accumulation of 40–45-kDa species of recombinant enzyme as detected by Western blotting with anti-MT1-MMP (Fig. 6*B, lane 4*) and confirmed using an antibody against the polyhistidine label (Fig. 6*B, lanes 7* and *8*). Similar results were obtained following expression of soluble recombinant MT1-MMP lacking the transmembrane and cytoplasmic domain, in which autolytic processing to 40–45-kDa species was also observed (Fig. 6*B, lane 9, labeled rMT1*), supporting the conclusion that the 55-kDa MT1-MMP-processing activity is directly attributable to the level of cell surface MT1-MMP activity (32). Together, these data indicate that active 55-kDa MT1-MMP undergoes concentration-dependent autolysis to an inactive 43-kDa form on the surface of DOV13 cells. Control experiments demonstrated MMP-dependent processing of pro-

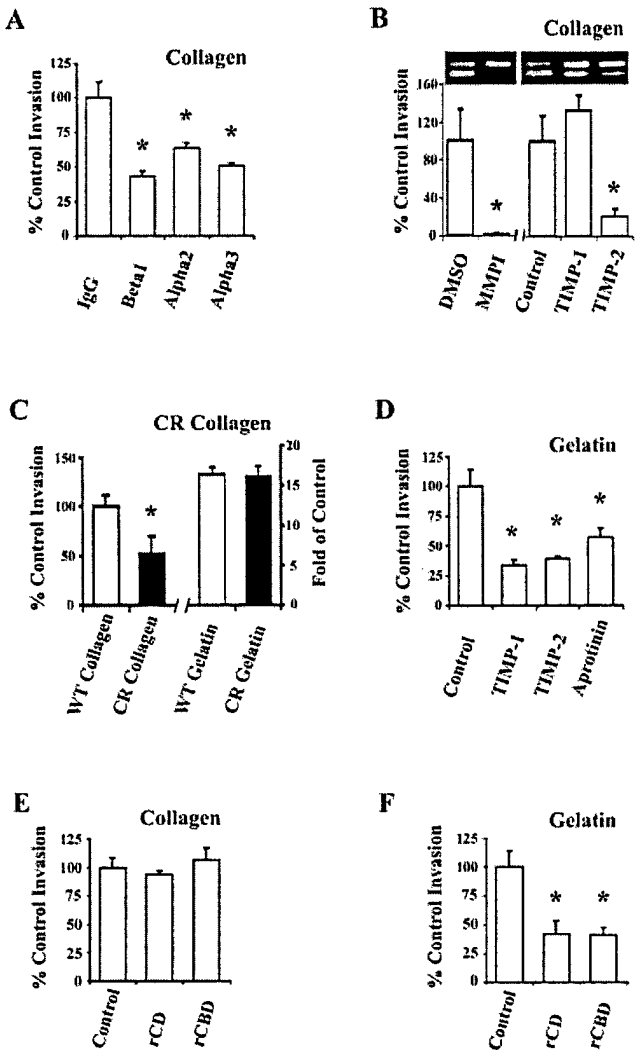

FIG. 9. **Characterization of the proteinase requirements for invasion.** *A–C* and *E*, invasion of collagen. Cells (1.0×10^5) were added to collagen-coated Transwell chambers (20 μg) in serum-free medium for 2 h prior to addition of reagents indicated below and allowed to invade for 48 h under the indicated conditions. Following invasion, cells were removed from the top chamber with a cotton swab; membranes were stained, and invading cells were enumerated using an ocular micrometer. *C, D,* and *F,* invasion of gelatin. Cells (1.0×10^5) were added to gelatin-coated Transwell chambers (50 μg) in serum-free medium for 2 h prior to addition of reagents indicated and allowed to invade for 24 h under the indicated conditions. Invading cells were quantified as described. *A,* integrin subunit-specific antibodies block invasion of collagen. Integrin subunit-specific antibodies or control IgG (15 μg/ml) were added as indicated. Results are expressed as % of control invasion (IgG), normalized to 100%. (*, $p < 0.05$ relative to control.) *B,* effect of MMP inhibitors on collagen invasion. Invasion was quantified in the presence or absence of MMPI (10 μM), TIMP-1 (10 nM), or TIMP-2 (10 nM), as indicated. Results are expressed as % of control invasion. MMPI data are normalized to Me_2SO controls (designated 100%), and TIMP-1 and -2 data are normalized to PBS controls (designated 100%). (*, $p < 0.05$ relative to control.) *Inset,* analysis of conditioned media from invasion chambers by gelatin zymography. *C,* invasion of wild type and CR collagen gels. Cells were allowed to invade gels composed of wild type or CR collagen or wild type and CR gelatin, as indicated. Invasion of CR collagen is expressed as % of control invasion (with wild type collagen designated 100%). Invasion of gelatin is designated as fold increase relative to wild type collagen control. (*, $p < 0.05$ relative to control.) *D,* effect of proteinase inhibitors on invasion of gelatin. Invasion was evaluated in the presence or absence of TIMP-1 (10 nM), TIMP-2 (10 nM), or aprotinin (20 μg/well), as indicated. Invasion of gelatin is expressed as % of control invasion (designated 100%). (*, $p < 0.05$ relative to control.) *E,* effect of MMP-2 domains on collagen invasion. Invasion was quantified in the presence or absence or rCD (100 nM) or rCBD (100 nM), as indicated. Results are expressed as % of control invasion (designated 100%). *F,* effect of MMP-2 domains on gelatin invasion. Invasion was quantified in the presence or absence or rCD (100 nM) or rCBD (100 nM), as indicated. Results are expressed as % of control invasion (designated 100%). (*, $p < 0.05$ relative to control.)

MMP-2, confirming surface expression of the recombinant enzyme in DOV13 cells (Fig. 6*C*).

Localization of Cell Surface MT1-MMP—In previous studies, MT1-MMP has been localized to membrane protrusions, termed invadopodia, which are rich in matrix proteinases and integrins, including $\alpha_3\beta_1$ integrins (27–29). Immunocytochemical analysis of MT1-MMP surface staining in non-permeabilized DOV13 cells demonstrates MT1-MMP localization to distinct cell surface projections (Fig. 7*D*), characteristic of integrin-rich sites of cell-matrix contact. Following β_1 integrin clustering using antibody-coated beads, MT1-MMP immunoreactivity is substantially redistributed to the periphery of the aggregated integrins (Fig. 7*H*). These data indicate that MT1-MMP can be actively recruited to membrane sites containing clustered β_1 integrins on the surface of DOV13 cells.

MMP Dependence of Migration and Collagen Gel Invasion—Three-dimensional collagen culture and clustering of collagen binding integrins up-regulate surface MMP activity in DOV13 cells. To assess the functional consequences of matrix-enhanced proteolytic potential, the ability of cells to migrate and to invade a three-dimensional collagen matrix was evaluated. MMP activity was not required for general cell motility, as cells migrated through uncoated transwell filters with equal efficiency in the presence or absence of a broad spectrum MMP inhibitor (MMPI), TIMP-1, or TIMP-2 (Fig. 8*A*). Haptotactic motility over collagen-coated colloidal gold surfaces is also unaffected by a broad spectrum MMP inhibitor (Fig. 8*B*). Semiquantitative analysis of phagokinetic tracks from 30 cells in the absence and presence of MMPI using computer-assisted image analysis gave relative migration areas of 1.4 ± 0.1 and 1.5 ± 0.3, respectively, indicating that MMP activity does not contribute to collagen-driven migration of DOV13 cells.

DOV13 cells efficiently penetrate three-dimensional collagen gels via $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrin receptors, and obstructing collagen-induced integrin clustering using integrin function blocking antibodies inhibits invasion (Fig. 9*A*). Invasion correlates with collagen-induced pro-MMP-2 processing (Fig. 9*B, inset*), and in contrast to cellular migration, invasion of collagen is MMP-dependent, as both a broad spectrum MMPI and exogenous TIMP-2 abrogate invasion and reduce or eliminate pro-MMP-2 processing (Fig. 9*B, lanes 2* and *5*). TIMP-1, a poor inhibitor of MT1-MMP (26), failed to reduce either collagen invasion or MT1-MMP-mediated pro-MMP-2 processing (Fig. 9*B*), suggesting that MT1-MMP collagenolytic activity may potentiate invasion. The serine proteinase inhibitor aprotinin had no effect on invasion of intact collagen gels (not shown). Furthermore, although collagenase-resistant collagen is sufficient to stimulate pro-MMP-2 activation (Fig. 1), cellular penetration of this matrix is significantly inhibited relative to wild type collagen (Fig. 9*C*), providing further support for the hypothesis that collagenolysis is required for invasive activity. Disrupting the triple helical structure of collagen by thermal denaturation removes the requirement for collagenase activity, as no difference in invasion of cells through gelatin derived from either wild type or collagenase-resistant collagen is observed (Fig. 9*C*). This is supported by data using wild type gelatin, in which invasion is effectively blocked by both TIMP-1 and -2 as well as by the serine proteinase inhibitor aprotinin (Fig. 9*D*), indicating that additional (non-MMP) gelatinolytic proteinases potentiate invasion following destabilization of collagen triple helical structure. Collagen invasion is unaltered in

the presence of either the rCD or rCBD of MMP-2 (Fig. 9*E*). However, both the rCD, which inhibits MMP-2 cell surface activation (Fig. 3) (6, 8), and rCBD, which prevents MMP-2 binding to native and denatured collagen (17), effectively inhibit the MMP-2-dependent component of gelatin invasion (Fig. 9*F*). Together these data suggest that the stimulation of MT1-MMP collagenolytic activity (31) through collagen-binding integrins is a rate-limiting step for invasion of native type I collagen-rich matrices.

DISCUSSION

MT1-MMP is a cell surface activator of pro-MMP-2 and has been implicated in collagen invasion and turnover (33–36). In this study, DOV13 ovarian cancer cells activate MT1-MMP as a consequence of culture in type I collagen gels and display MMP-dependent invasion of type I collagen, indicating that MMP activity is required for removal of collagen matrix constraints during invasion. However, migration over two-dimensional collagen is not impeded by MMP inhibitors. Although TIMP-1 does not interact with MT1-MMP, TIMP-2 specifically binds the proteinase, functioning in both inhibition and stabilization of the enzyme on the cell surface (26, 32). The ability of exogenous TIMP-2, in contrast to TIMP-1, to inhibit DOV13 collagen gel invasion implicates a cell surface proteolytic cascade initiated by MT1-MMP. This is further supported by data demonstrating that inhibition of MMP-2 cellular activation or collagen binding using rCD and rCBD (17, 18), respectively, has no effect on collagen invasion. As the cellular events that govern the collagen-induced MMP-2/MT1-MMP response are unclear and technically difficult to assess in three-dimensional collagen gel systems, a variety of biochemical approaches were employed in this study to dissect the interplay between collagen-cell interactions and regulation of cell surface MMP activity.

DOV13 cells bind type I collagen via $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins. Recognition of collagen by these integrins depends on retention of the triple helical conformation, as thermal gelation of collagen abrogates cellular adhesion. Collagenase-cleaved type I collagen produces ¾ and ¼ fragments that display a lower T_m than intact fibrils (37). Adhesion data in the current study demonstrate that the triple helical conformation of the collagen fragments is stabilized at low coating temperatures but is lost under physiological coating conditions (21). Together, these data suggest that pericellular type I collagenolysis will reduce $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrin-mediated cell-matrix contacts. By using a similar approach, the appearance of cryptic $\alpha_V\beta_3$ integrin-binding sites (RGD) in collagenase-generated collagen fragments was reported (21). However, DOV13 cells adhere weakly to type I gelatin or ¾ and ¼ fragments, and aggregation of $\alpha_V\beta_3$ integrins on the surface of DOV13 cells does not elicit a cellular MMP processing response (12, 20). Nevertheless, the exposure of cryptic $\alpha_V\beta_3$- or $\alpha_V\beta_5$-binding sites in collagenase-cleaved collagen may further influence MMP expression in a cell type-specific manner. In support of this observation, we have previously demonstrated that vitronectin-induced aggregation of melanoma cell $\alpha_V\beta_3$ integrins up-regulates MMP-2 expression (38). Relative to collagen gel penetration, DOV13 cells rapidly invade a gelatin matrix. Although pro-MMP-2 activation is not up-regulated over basal levels under these conditions, additional proteinases of other mechanistic classes can provide gelatinase activity (1, 39, 40). Together these data indicate that collagenase activity provided by MT1-MMP is critical to invasion of an intact collagen matrix. Subsequent clearance of resultant fragments can then proceed by activation of cell surface MMP-2 along with contributions from other cell surface proteinases including seprase (39) and the components of the plasminogen activator/plasmin system (40), which have been implicated in DOV13 cellular invasion of Matrigel (22).

Stimulation of pro-MMP-2 activation does not require collagenolysis, as collagenase-resistant collagen is as efficacious as wild type type I collagen at inducing pro-MMP-2 processing. However, thermal denaturation of either collagen abolishes the ability to enhance MMP activation, suggesting that $\alpha_2\beta_1$ and/or $\alpha_3\beta_1$ integrin binding to intact triple-helical collagen mediates the MMP activation response in DOV13 cells. By using subunit-specific antibodies to dissect integrin requirements for MMP processing, our data demonstrate that clustering of α_3 integrins promotes a stronger cellular MMP processing response than α_2 integrin aggregation. A potential role for α integrin-specific regulation has been demonstrated previously for type I collagen-induced cellular responses, including those involving MMP-1 expression (41, 42). Administration of function blocking antibodies against either α integrin subunit reveals a role for both receptors during invasion. Although this study implicates the $\alpha_3\beta_1$ heterodimer in mediating the MT1-MMP response, it is likely that both $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins provide a mechanical advantage to the migration component of invasion. Furthermore, it is unclear at this level of investigation whether $\alpha_2\beta_1$ integrins are required for effective dispersal of $\alpha_3\beta_1$ into focal adhesions (43, 44). Interestingly, $\alpha_3\beta_1$ integrins have recently been hypothesized to play a major organizational role in the formation of invadopodia in response to cellular engagement of type I collagen (45). As MT1-MMP also localizes to invadopodia (27–29), it is possible that $\alpha_3\beta_1$ clustering in DOV13 cells selectively initiates cellular events that mimic formation of invadopodial projections, which in turn regulate MT1-MMP activity. Moreover, our data demonstrate localization of MT1-MMP immunoreactivity to the periphery of clustered β_1 integrins, indicating that MT1-MMP redistribution occurs during integrin clustering events. This observation, together with previous reports of MT1-MMP localization to integrin-rich cellular protrusions, suggests a cellular regulatory mechanism for MT1-MMP aggregation, thereby promoting effective pro-MMP-2 processing and efficient matrix degradation. As MT1-MMP can function as a collagenase (31) and MT1-MMP null mice exhibit severe deficiencies in collagen remodeling (33), localization of the enzyme to cellular collagen receptors could clearly influence physiologic events such as collagen gel contraction, adhesion, and invasion. In addition, pro-MMP-2 bound to intact peri-cellular collagen may readily infiltrate the MT1-MMP activation pathway, resulting in a switch from a collagenase to a gelatinase environment as pro-MMP-2 activation and collagen triple helix denaturation ensues (17).

It has recently been demonstrated that exogenous MT1-MMP overexpressed in a MMP-2 null background undergoes autolysis to a 43-kDa form, the rate of which is regulated by TIMP-2 (32). Similarly, endogenously expressed MT1-MMP in DOV13 cells exists in two major forms of 55 and 43 kDa (12). The current data indicate that the 55-kDa form of MT1-MMP is the active TIMP-2-binding species, whereas the 43-kDa form is an inactive autolysis product. This result is consistent with the amino-terminal sequences of similar MT1-MMP species obtained from overexpression systems, which demonstrate a loss of essential amino acids in the zinc-binding consensus sequence (30, 32). Although catalytically inactive, the 43-kDa form of MT1-MMP is nevertheless retained on the cell surface. As this 43-kDa species contains the carboxyl-terminal domains necessary for invadopodial localization and enzyme aggregation, it is interesting to speculate that MT1-MMP-mediated proteolysis may be down-regulated through the dilution of active enzyme with truncated proteinase.

In summary, our data support the hypothesis that as DOV13

cells interact with type I collagen, integrin receptors cluster on the cell surface, resulting in up-regulation of MT1-MMP and pro-MMP-2 processing, recruitment of MT1-MMP to sites of cell-matrix contact, MMP-2 surface association, and MT1-MMP-dependent collagen gel invasion. As a consequence of MT1-MMP collagenolysis, the resulting collagen cleavage products thermally denature, providing a substrate for a number of proteinases. In addition, MMP-2 is released from the cell surface to further advance matrix clearance through directed gelatinase activity on denatured collagen fragments. As $\alpha_2\beta_1$ or $\alpha_3\beta_1$ integrin occupancy is reduced, collagen matrix stimulation of proteolysis is attenuated. Furthermore, MT1-MMP activity can be down-regulated by autolytic processing to a stable, inactive 43-kDa form that may functionally dilute productive enzyme-substrate interactions. Together, these data support an hypothesis wherein matrix status influences cell surface matrix-degrading potential to facilitate cellular functions including migration, invasion, and matrix remodeling.

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Type I Collagen Stabilization of Matrix Metalloproteinase-2

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The activity of matrix metalloproteinase-2 (MMP-2) is regulated stringently on the posttranslational level. MMP-2 efficiently undergoes autolysis into inactive polypeptides *in vitro*, prompting the hypothesis that MMP-2 autolysis may function as an alternative mechanism for posttranslational control of MMP-2 *in vivo*. Moreover, MMP-2 binds to intact type I collagen fibrils; however, the functional consequences of this interaction have not been fully elucidated. To test the hypothesis that MMP-2 binding to type I collagen functions as a positive regulator of MMP-2 proteolytic potential, the effect of type I collagen on MMP-2 activity, inhibition by tissue inhibitor of metalloproteinase-2 (TIMP-2), and enzyme stability was examined. Here, we report that purified MMP-2 binds but does not cleave intact type I collagen. The presence of type I collagen affects neither enzymatic activity against a quenched fluorescent peptide substrate nor the kinetics of inhibition by TIMP-2. However, MMP-2 is stabilized from autolysis in the presence of type I collagen, but not by elastin, fibrinogen, or laminin. These data provide biochemical evidence that MMP-2 exosite interactions with type I collagen may function in the posttranslational control of MMP-2 activity by reducing the rate of autolytic inactivation. © 2001 Academic Press

Key Words: **type I collagen; matrix metalloproteinase-2; gelatinase A; proteinase.**

Matrix metalloproteinase-2 (MMP-2,[2] gelatinase A) is a member of a family of zinc-dependent metalloendopeptidases that functions in the degradation of collagen types IV, V, VII, X, and XIV, gelatins, elastin, fibronectin, and aggrecan (1–3). The proteinase is composed of five structural domains including an amino-terminal propeptide, a zinc-binding catalytic domain, fibronectin type II (FNII) repeats, a hinge region, and a carboxyl-terminal hemopexin-like domain (1–3). Both the fibronectin type II repeats and the hemopexin-like domain have been implicated in substrate recognition and targeting (4, 5). Unike other MMP family members, MMP-2 production is largely refractory to stimulation by most biologic agents and is constitutively expressed by numerous cell types. Thus, the activity of MMP-2 is stringently regulated predominantly on the posttranslational level and many studies have focused on zymogen activation and enzyme-inhibitor binding as primary mechanisms for control of MMP-2-mediated proteolysis (reviewed in 1–3, 6). Activation of secreted proMMP-2 occurs at the cell surface via formation of a ternary complex between proMMP-2, tissue inhibitor of metalloproteinase-2 (TIMP-2) and a transmembrane MMP designated membrane type 1-MMP (MT1-MMP) (7–10). Pericellular TIMP-2 plays a dual role, as it is critical to promote ternary complex formation for zymogen activation, but can also function to inhibit either MMP-2 or MT1-MMP catalytic activity via formation of a 1:1 noncovalent inactive enzyme–inhibitor complex (7–10).

Active MMP-2 undergoes concentration-dependent autolysis at Pro^{394}–Ile^{395}, resulting in cleavage of the hemopexin-like domain from the catalytic domain (11). This observation suggests that the active state of MMP-2 is unstable in the absence of substrate and indicates that autolytic inactivation may function as an alternative mechanism for posttranslational control of MMP-2 activity (11). This hypothesis is supported by the observation that a hemopexin-like domain fragment of MMP-2 can be recovered from tumor tissues (12). Moreover, intermolecular autolytic cleavage of MMP-2 also occurs on the cell surface (13), indicating that a functional consequence of zymogen activation

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[2] Abbreviations used: MMP-2, matrix metalloproteinase-2; FNII, fibronectin type II; TIMP-2, tissue inhibitor of metalloproteinase-2; MT1-MMP, membrane type 1-MMP; APMA, amino-phenylmercuric acid; MMPI, MMP inhibitor; BSA, bovine serum albumin; DMSO, dimethylsulfoxide.

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may be the generation and sequestration of sufficient MMP-2 to support autolytic processing.

In addition to TIMP-2-mediated regulation of zymogen activation and substrate cleavage, exosite interactions between MMP-2 and pericellular macromolecules may also influence enzyme activity. Previous studies have demonstrated that the FNII-like modules inserted within the MMP-2 catalytic domain promote protein–protein interaction between MMP-2 and type I collagen fibrils, gelatins, and insoluble elastin (14–17). While binding to gelatins may be predicted to promote enzyme activity, the significance of type I collagen binding is unclear, as only avian MMP-2 has been shown to function as an interstitial collagenase (18), while MMP-2 from other species is inactive against type I collagen (19–22). However, processing of MMP-2 by neutrophil elastase into inactive products is dramatically slowed in the presence of type I collagen or gelatin, suggesting that exosite interactions between the FNII-like domains of MMP-2 and pericellular matrix proteins such as type I collagen may protect the enzyme from proteolytic degradation (23). This hypothesis is supported by studies showing that addition of soluble type I collagen or gelatin to purified MMP-2 decreased the rate of autolysis (10). Furthermore, cellular MT1-MMP-mediated proMMP-2 activation is enhanced in the presence of exogenous recombinant FNII-like domain, suggesting that disruption of the proMMP-2/pericellular collagen interaction may promote filtration of proMMP-2 into the MT1-MMP activation pathway (5). Together, these data suggest that MMP-2 binding to type I collagen functions as a positive regulator of MMP-2 proteolytic potential. To advance this hypothesis, the effect of type I collagen on MMP-2 activity, inhibition by TIMP-2, and enzyme stability was examined. These data indicate that collagen binding does not alter net MMP-2 enzymatic activity, but functions to significantly reduce the rate of autolysis.

MATERIALS AND METHODS

Materials. Purified human MMP-1, MMP-2 (TIMP-2-free), and TIMP-2 were generous gifts of Dr. Hideaki Nagase (Kennedy Institute of Rheumatology, Imperial College School of Medicine, United Kingdom). Bovine serum albumin and type I gelatin, amino-phenylmercuric acetate (APMA), dimethyl sulfoxide, and human type I collagen, fibrinogen, elastin, laminin-1, and pepsin were purchased from Sigma (St. Louis, MO). The quenched fluorescent peptide substrate, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2, and peptide standard, Mca-Pro-Leu-OH were acquired from BACHEM BioSciences, Inc. (King of Prussia, PA). The serine protease inhibitor aprotinin was purchased from Alexis Biochemicals (San Diego, CA). The broad spectrum hydroxamic acid-based MMP inhibitor designated MMP INH-3850-PI (MMPI) was purchased from Peptides International (Louisville, KY).

Collagenase assays. Cleavage of native or denatured type I collagen was evaluated by incubating type I collagen or gelatin (10 ug) with the indicated concentrations of MMPs in Tris–glucose buffer (50 mM Tris, 200 mM glucose, 200 mM NaCl, pH 7.4) for 18 h (25°C), followed by electrophoretic resolution on 8–15% SDS–polyacrylamide gels (24). Collagen degradation was visualized by staining gels with Coomassie blue. Prior to incubation with collagen or gelatin, MMPs were diluted to a concentration of 1 μM and incubated for 30 min (37°C) with 2 mM APMA to promote enzyme auto-activation. Type I gelatin was prepared by thermal denaturation of human type I collagen (60°C, 20 min).

Collagen binding. Microtiter plates (high binding; Greiner, Lake Mary, FL) were coated overnight by passive adsorption at 4°C in assay buffer (100 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij-35) containing 100 μg of the indicated matrix protein. Nonspecific binding sites on coated wells were blocked by incubation with a 200-μl volume of 3% BSA in assay buffer for 2 h (37°C). To evaluate the structural integrity of immobilized collagen or gelatin, wells were incubated with MMP-2 (~1 nM, 35°C, 18 h) or pepsin (1 unit in 0.1% acetic acid, pH 2.6, 37°C, 15 min) prior to solubilization in reducing Laemmli sample dilution buffer (24) and electrophoresis on 8% SDS–polyacrylamide gels. Protein degradation was visualized by staining gels with Coomassie blue. To evaluate the activity of collagen-bound MMP-2, purified MMP-2 was activated with APMA as described above, diluted to 50 nM in assay buffer, and incubated overnight (4°C) in protein-coated wells in a 50-μl volume. Aliquots (5 μl) were removed and analyzed by gelatin substrate zymography (designated "solution phase") (25). Wells were then washed five times with 50 μl of assay buffer and bound MMP eluted by incubation with 50 μl of nonreducing Laemmli sample dilution buffer (24) for 1 h at 25°C (designated "bound") prior to evaluation by gelatin zymography. The presence of bound MMP-2 was confirmed by incubating wells overnight at 4°C with 10% DMSO to disrupt MMP-2–type I collagen interactions (16). In control experiments, wells were incubated with 50 μl of assay buffer in place of Laemmli buffer.

MMP activity assays. To quantify the activity of bound MMP-2, wells were coated with collagen (100 μg) as described above and incubated overnight (4°C) with a 100-μl volume of APMA-activated MMP-2 (10 nM) containing 1% DMSO. Unbound enzyme was removed and wells washed twice with 100 μl of assay buffer before adding 100 μl of fresh assay buffer. The plate was then switched to 37°C, the quenched fluorescent peptide substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (26) was added to wells (10 μM), and substrate cleavage was evaluated using an excitation wavelength of 326 nm and monitoring emission at 396 nm with a fluorescent plate reader (Molecular Devices, SpectraMAX Gemini). A 1% concentration of DMSO was used to ensure the solubility of the synthetic substrate without affecting the ability of MMP-2 to bind collagen (data not shown; Ref. (16)). Steady-state initial velocities of substrate cleavage were established for solution phase and bound MMP-2 over a period of 15 min. Standard curves were generated by measuring the relative fluorescence emission of the cleavage product Mca-Pro-Leu-OH in solution containing 1% DMSO. Plots were constructed of velocity (fmol/s) vs substrate (μM) using SigmaPlot software, and regression values calculated to produce kinetic constants (V_{max}, k_{cat}, and K_m). Inhibition of MMP-2 was evaluated by adding increasing amounts of TIMP-2 and monitoring initial velocity as described above. Analyses were carried out in triplicate.

Enzyme stability. The stability of bound MMP-2 was determined by incubating APMA-activated MMP-2 in protein-coated microtiter wells overnight (4°C) in the presence of 1 μg/ml aprotinin (although no contaminating serine proteases were detected in the enzyme or matrix protein preparations). The temperature was raised to 37°C for 18 h to induce MMP-2 autolysis. Following incubation, MMP-2 activity in aliquots was analyzed using the quenched fluorescent peptide substrate and by gelatin zymography as described above.

RESULTS AND DISCUSSION

Interaction of MMP-2 with Type I Collagen

To evaluate the role of type I collagen binding in regulation of MMP-2 proteolytic potential, the func-

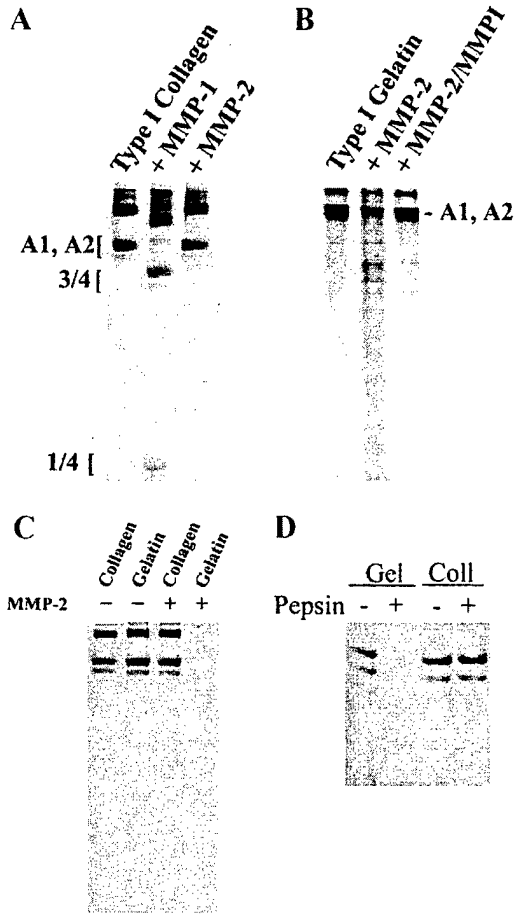


FIG. 1. MMP-2 does not cleave soluble or immobilized type I collagen. (A) Soluble human placental type I collagen (10 μg) was incubated at 25°C for 18 h in the presence of MMP-2 or MMP-1 (50 nM) as indicated. Reaction products were analyzed by electrophoresis on 8–15% gradient SDS–polyacrylamide gels followed by Coomassie blue staining. The migration positions of the $\frac{3}{4}$ and $\frac{1}{4}$ collagenase cleavage products are indicated. (B) Type I gelatin (10 μg) was incubated in the presence or absence of MMP-2 (50 nM) as indicated under conditions as described in (A). Control reactions contained the broad spectrum hydroxamic acid-based inhibitor MMPI (10 μM). (C) Collagen- or gelatin-coated microtiter wells were incubated with MMP-2 (~1 nM) for 18 h at 35°C followed by solubilization and analysis of bound protein by electrophoresis on 8% SDS–polyacrylamide gels and Coomassie blue staining. (D) Collagen- or gelatin-coated microtiter wells were incubated with pepsin (1 unit) for 15 min at 37°C followed by solubilization and analysis of bound protein by electrophoresis on 8% SDS–polyacrylamide gels and Coomassie blue staining.

tional consequences of the MMP-2–collagen interaction were evaluated. Although the gelatinolytic activity of MMP-2 is well established, evidence that MMP-2 can cleave soluble type I collagen is limited to a single report (18). To determine whether MMP-2 functions as an interstitial collagenase, type I collagen was incubated with catalytic amounts of TIMP-2-free MMP-2 or MMP-1 (collagenase-1). While MMP-1 cleaves native type I collagen into 3/4 and 1/4 alpha chain fragments, no collagen cleavage is catalyzed by MMP-2 (Fig. 1A). Equivalent concentrations of MMP-2 efficiently cleave type I gelatin (Fig. 1B), confirming that the purified enzyme is catalytically active. These results support previous conclusions that native type I collagen does not function as substrate for human MMP-2 (19–22). Thus, the interstitial collagenase activity previously attributed to MMP-2 (18) may reflect enzymatic properties unique to the avian enzyme.

As interaction of MMP-2 with type I collagen may function as a mechanism for posttranslational regulation of MMP-2 activity, association of MMP-2 with collagen immobilized on microtiter plates by passive adsorption was evaluated. To first assess the structural integrity of immobilized collagen, microtiter plates coated with collagen or gelatin were incubated with either MMP-2 (Fig. 1C) or pepsin (Fig. 1D). Whereas gelatin was susceptible to cleavage by both MMP-2 and pepsin (Fig. 1C, lane 4; Fig. 1D, lane 2), immobilized collagen was resistant to these gelatinolytic proteinases, indicating that immobilization does not result in collagen denaturation. Microtiter plates coated with type I collagen or BSA by passive adsorption were then incubated with MMP-2 overnight, and a qualitative analysis of collagen-bound MMP-2 obtained by gelatin zymography of solubilized proteins. Bound MMP-2 was detectable only in type I collagen-coated wells, but not in BSA-coated control samples (Fig. 2, lanes 2 and 4). Major autolytic fragments of MMP-2 (43/40K) lacking the hemopexin domain but retaining the FNII repeats (11) were also collagen-associated (Fig. 2). To evaluate the reversibility of binding, collagen-coated wells containing bound MMP-2 were

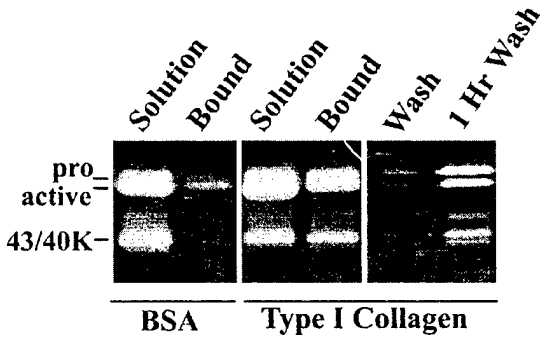


FIG. 2. MMP-2 binds to type I collagen. MMP-2 (50 nM) was preactivated with APMA as described under Materials and Methods and incubated overnight (4°C) in wells coated with type I collagen or BSA (150 μg) as indicated. Soluble enzyme was removed (designated "solution," lanes 1 and 3). After washing wells five times with assay buffer (100 mM Tris–HCl, pH 7.15, 100 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij-35), bound enzyme was eluted using nonreducing Laemmli sample dilution buffer (50 μl, designated "bound," lanes 2 and 4). To assess the reversibility of binding, collagen-coated wells containing bound MMP-2 were washed with assay buffer as described above (lane 5), incubated for 1 h in assay buffer, and the solubilized enzyme analyzed (designated "1 h wash," lane 6). All samples were evaluated by gelatin zymography on 9% SDS–polyacrylamide gels containing copolymerized gelatin as described under Materials and Methods.

washed free of detectable enzyme, followed by incubation in assay buffer. Within 1 h significant MMP-2 is dissociated from collagen into the solution phase (Fig. 2, lane 6), indicating that MMP-2 interaction with immobilized type I collagen is reversible. These data are in good agreement with previous reports that have estimated that the dissociation constant for MMP-2 binding to type I collagen is in the low micromolar range (16, 17).

To quantify the catalytic activity of bound MMP-2, wells were coated with various matrix proteins and incubated with MMP-2 as described above. Laminin-1 and the fibrous proteins fibrinogen and elastin (27) were utilized to control for matrix specificity and nonspecific enzyme trapping within fibrous matrices. Following incubation, soluble enzyme was removed (solution phase), and the activity of bound MMP-2 evaluated by the addition of a quenched fluorescent peptide substrate (26). Type I collagen- and elastin-coated wells reproducibly retained ~20 and ~8% of total MMP-2 activity, respectively, relative to other control matrix protein surfaces upon which little enzymatic activity was detected (Fig. 3A). Gelatin zymography following solubilization of proteins confirmed the presence of collagen-bound MMP-2 activity (Fig. 3B). These data support previously published results (17) which demonstrate binding of both pro- and active MMP-2 to type I collagen and suggest that collagen binding does not negatively influence enzymatic activity.

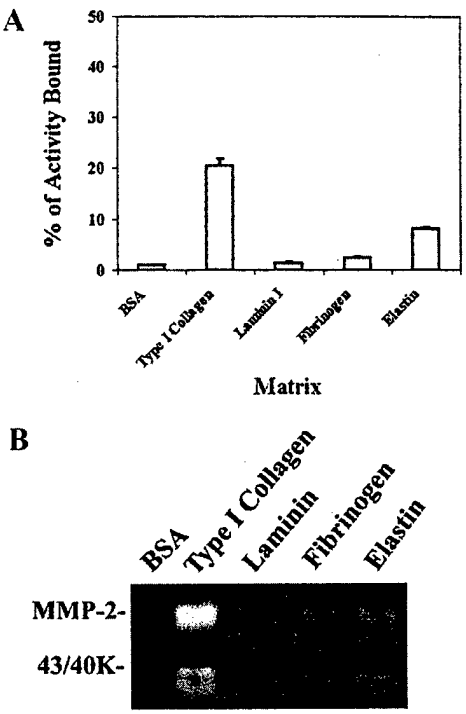
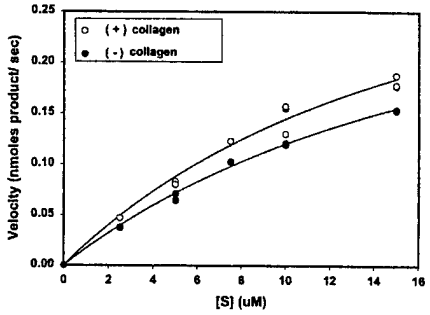


FIG. 3. Quantification of MMP-2 catalytic activity. (A) To quantify the activity of MMP-2 bound to collagen, MMP-2 (10 nM) was incubated overnight (4°C) in wells coated with type I collagen, Laminin-1, fibrinogen, elastin, or BSA as indicated. Unbound enzyme was removed, wells washed five times, and the activity of MMP-2 quantified using the quenched fluorescent peptide substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2 (10 μM) and monitoring emission at 396 nm using an excitation wavelength of 326 nm. Steady-state velocities were established and compared with the activity of 10 nM soluble MMP-2 (designated 100%) (n = 3). (B) Following quantitative analysis, MMP-2 was eluted from wells (coated with specific proteins, as indicated) using nonreducing Laemmli sample buffer and analyzed by gelatin zymography.

MMP-2 Kinetic Activity in the Presence of Type I Collagen

As the FNII-like modules reside within the catalytic domain of MMP-2, enzymatic activity may be influenced by collagen binding. To test this hypothesis, the kinetics of substrate cleavage by MMP-2 were measured in the absence or presence of type I collagen using a quenched fluorescent peptide substrate. Although steady-state velocity was reproducibly higher in the presence of collagen, no significant difference in catalytic efficiency (k_{cat}/K_m) was observed (Fig. 4), indicating that overall enzyme activity is not altered by collagen binding. As MMP-2-collagen binding is of relatively low affinity (16, 17) and likely involves continual exchange of enzyme between the insoluble (bound) and solution phase (Fig. 2), these data demonstrate that transient collagen interactions do not decrease MMP-2 proteolytic potential. It should be noted, however, that minor variations in the activity of bound MMP-2 (relative to solution phase) may not be detected in this system, which evaluates the equilibrium effects of collagen binding on catalytic activity.

In addition to substrate cleavage, interaction of MMP-2 with its primary inhibitor TIMP-2 may also be influenced by collagen binding (28, 29). To address this possibility, MMP-2 was incubated with increasing molar amounts of TIMP-2 in the presence or absence of collagen, and the remaining enzymatic activity evaluated (Fig. 5). TIMP-2 effectively inhibited MMP-2 activity at a MMP-2:TIMP-2 molar ratio of approximately 1.2:1 and this ratio was unaltered by collagen. Further, no differences were observed in the rate of inhibition (not shown), indicating that collagen binding does not restrict access of the inhibitor to the enzyme active site. However, as indicated above, the dissociation of MMP-2 from collagen fibers during the time course of the assay may mask subtle differences in inhibition kinetics. In this regard, it is interesting to note that inhibition of MMP-2 by membrane-localized TIMP-2 is slowed in the presence of soluble type I collagen or gelatin (10). These differences may reflect collagen-induced changes in enzyme stability during the time course of the assay, as stabilization against autolysis would contribute to overall higher levels of catalytic activity.



Condition	K_m	K_{cat}	k_{cat}/K_m
	μM	s^{-1}	$M^{-1}s^{-1}$
1nM MMP-2 + BSA	22.9	3.14	1.37×10^5
1nM MMP-2 + Collagen	21.2	3.54	1.67×10^5

FIG. 4. MMP-2 kinetic activity is not altered by type I collagen. Microtiter plates were coated with type I collagen or BSA (100 μg) as indicated. Wells were loaded with increasing concentrations of quenched fluorescent substrate (0–15 μM, as indicated) and substrate cleavage initiated by the addition of APMA-activated MMP-2 (10 nM). Substrate cleavage was monitored as described in Fig. 3, and kinetic parameters analyzed by nonlinear regression of the velocity data. Kinetic constants of substrate cleavage in type I collagen- or BSA-coated wells are summarized in the inset.

Type I Collagen Stabilizes MMP-2 against Autolysis

Previous studies have demonstrated that MMP-2 undergoes autolytic processing in solution phase, resulting in a truncated enzyme with reduced or abrogated catalytic activity (10, 11). To evaluate the effect of collagen binding on enzyme stability, MMP-2 autolysis was examined in the presence of collagen or control matrix proteins. Binding of activated MMP-2 to immobilized proteins was carried out at 4°C, followed by incubation at 37°C to promote enzyme autolysis. In solution phase, a significant decrease in activity is observed following incubation for 16 h at 37°C (Fig. 6A). However, in the presence of type I collagen, MMP-2 activity is stabilized (Fig. 6B). This effect is specific to type I collagen, as neither laminin-1, fibrinogen, nor elastin enhanced MMP-2 stability (Fig. 6B). This result was confirmed using the quenched fluorescent peptide substrate, demonstrating a statistically significant retention of MMP-2 catalytic activity (Table I). Together these data indicate that although collagen binding has no effect on MMP-2 catalytic activity or TIMP-2 inhibition, proteolytic potential may be en-

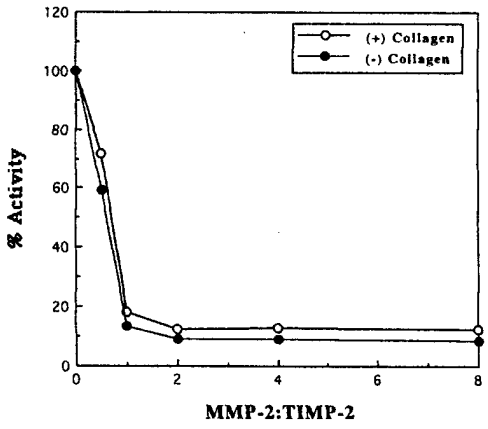


FIG. 5. Effect of type I collagen on MMP-2 inhibition by TIMP-2. MMP-2 (10 nM) was allowed to bind to collagen-coated wells overnight (4°C) before addition of the quenched fluorescent peptide substrate (10 μM) and the indicated molar ratio of TIMP-2. Steady-state velocity was calculated as described under Materials and Methods. Data are plotted as percentage activity, with MMP-2 activity in the absence of TIMP-2 designated as 100%.

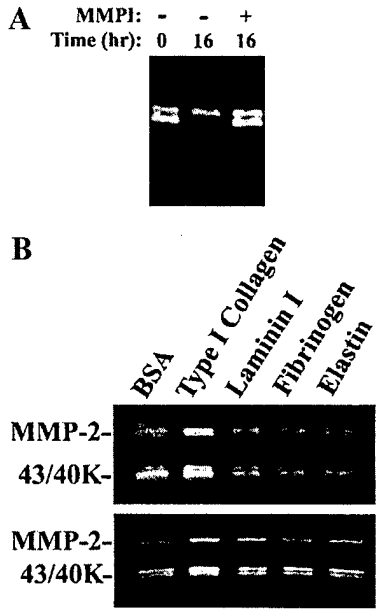


FIG. 6. Collagen stabilizes MMP-2 against autolysis. (A) APMA-activated MMP-2 (10 nM) was incubated for 16 h at 37°C in the absence or presence of the MMP inhibitor MMPI (10 μM) as indicated to prevent autolysis. (B) Microtiter wells were coated with the indicated matrix proteins (100 μg), incubated with MMP-2 (10 nM) overnight (4°C), and shifted to 37°C for 18 h to promote enzyme autolysis. Dimethyl sulfoxide (10% final concentration) was added to wells to elute bound MMP-2 and duplicate samples were analyzed by gelatin zymography (top and bottom).

TABLE I

Type I Collagen Stabilizes MMP-2 Activity[a]

Condition	Velocity (fmol/s)	Product generated (pmol ± SD)
Bovine serum albumin	66.9 ± 1.1	206.9 ± 9.0
Type I collagen	118.5 ± 2.1	340.9 ± 4.3
Laminin I	86.7 ± 3.6	242.8 ± 5.1
Fibrinogen	80.6 ± 2.9	224.1 ± 9.6
Elastin	90.4 ± 3.9	241.5 ± 7.4

[a] Microtiter wells were coated with the indicated matrix proteins (100 μg), incubated with MMP-2 (10 nM) overnight (4°C), and shifted to 37°C for 18 h to promote enzyme autolysis. Substrate (7.5 μM) was added and steady-state reaction velocities and product generation were evaluated for 45 min (N = 3).

hanced in the presence of collagen due to protection from autolytic inactivation.

It has previously been reported that high concentrations (0.5 mg/ml) of soluble type I collagen stabilize MMP-2 activity (10). Our data support these findings and demonstrate that immobilized collagen, which may mimic more closely the insoluble collagen structures found *in vivo,* can also potentiate MMP-2 activity. We hypothesize that as MMP-2 binds to immobilized type I collagen, the concentration of solution-phase enzyme is decreased, subsequently reducing the extent of soluble enzyme autolytic turnover. Further, these data demonstrate that type I collagen may play a dual role in regulation of MMP-2-mediated pericellular proteolysis. Initially, cellular binding to collagen or experimental engagement of collagen binding integrins using immobilized antibodies induces MT1-MMP-mediated activation of proMMP-2 in a variety of cell types (5, 30–35), thus increasing the concentration of active pericellular MMP-2. Subsequently, binding of the active proteinase to this integrin-associated collagen can further potentiate substrate cleavage by stabilizing active MMP-2 against autolytic inactivation. Based on these data, it is interesting to speculate that binding to type I collagen may function as an additional post-translational mechanism for regulation of MMP-2 activity by enhancing enzyme stability as well as increasing the proximity of active proteinase to potential extracellular matrix substrates.

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