Matrix Metalloproteinase-2 Cleavage of the $\beta_1$ Integrin Ectodomain Facilitates Colon Cancer Cell Motility*

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**Background:** Cancer cell invasion requires integrins for adhesion/de-adhesion and MMPs for focalized proteolysis.

**Results:** MMP-2 is up-regulated in invasive colorectal tumors and degrades $\beta_1$ integrins.

**Conclusion:** Shedding of the I-like domain from $\beta_1$ integrins results in decreased adhesion and enhanced cell motility.

**Significance:** MMP-2 amplifies the motility of cancer cells, not only degrading extracellular matrix but also reducing the $\beta_1$ integrin expression.

Cancer cell invasion is a key element in metastasis that requires integrins for adhesion/de-adhesion, as well as matrix metalloproteinases (MMPs) for focalized proteolysis. Herein we show that MMP-2 is up-regulated in resected colorectal tumors and degrades $\beta_1$ integrins with the release of fragments containing the $\beta_1$ I-domain. The $\beta_1$ cleavage pattern is similar to that produced by digestion of $\alpha_{5}\beta_1$ and $\alpha_2\beta_1$ with MMP-2. Two such fragments, at 25 and 75 kDa, were identified after immunoprecipitation, with monoclonal antibody BD610468 reacting with the NH$_2$-terminal I-like ectodomain followed by SDS-PAGE and microsequencing using electrospray (ISI-Q-TOF-Micromass) spectrometry. Cleavage of the $\beta_1$ integrin can be abolished by inhibition of MMP-2 activity; it can be induced by up-regulation of MMP-2 expression, as exemplified by HT29 colon cancer cells transfected with pCMV6-XL5-MMP-2. Co-immunoprecipitation studies of colon cancer cells showed that the $\beta_1$ integrin subunit is associated with MMP-2. The MMP-2-mediated shedding of the I-like domain from $\beta_1$ integrins resulted in decreased adhesion of colon cancer cells to collagen and fibronectin, thus abolishing their receptivity. Furthermore, such cells showed enhanced motility as evaluated by a “wound healing-like” assay and time-lapse microscopy, indicating their increased invasiveness. Altogether, our data demonstrate that MMP-2 amplifies the motility of colon cancer cells, not only by digesting the extracellular matrix components in the vicinity of cancer cells but also by inactivating their major $\beta_1$ integrin receptors.

As cancer cells undergo metastasis, they penetrate and attach to components of the extracellular matrix. The escape of the metastatic cells from a primary tumor results from the disruption of the tissue architecture and requires reversible modulation of cell-matrix and cell-cell contacts, cytoskeletal rearrangement, and acquisition of enhanced proteolytic potential (1, 2). It is currently generally accepted that the combined participation of integrins and matrix metalloproteinases (MMPs)$^3$ is essential for the invasion of tumor cells into the surrounding connective matrix, intravasation and extravasation from blood vessels, and metastasis to distant organs (3).

MMPs are a family of zinc-dependent endopeptidases that can be divided into two distinct groups: secreted MMPs, e.g. MMP-2, and membrane-type MMPs such as MT-MMP. Secreted MMP-2 may be stored in the extracellular depots as a precursor (zymogen) (4). It can be activated by specific proteases, many of which are localized at the cell membrane and help to spatially define regions of matrix breakdown, in the so-called invadopodial structures and at the tips of sprouting capillaries. MMP-2 is highly expressed in colon cancer cells and after secretion due to direct interaction with different proteins, including integrin receptors; it can also be found associated with cellular membranes (5). When it is bound to the cell surface, MMP-2 affects intracellular signaling, facilitates proenzyme localization and activation, and mediates cell motility by disrupting cell contacts with the extracellular matrix. Although MMP-2 degrades a wide variety of proteins, such as chemotactic molecules, adhesion molecules, proteinase inhibitors, cell-surface receptors, blood clotting factors, latent growth factors, and growth factor-binding proteins, there are no data to show that it cleaves integrins, thus down-regulating their expression and receptor activity. Therefore, in the present study we attempted to evaluate whether the structure and receptor activity of $\beta_1$ integrins, representing the major adhesive receptors in colon cancer cells, is modified because of direct interaction with MMP-2.

**MATERIALS AND METHODS**

**Reagents**—All standard tissue culture reagents, including Dulbecco’s modified Eagle’s medium, fetal bovine serum, and Lipofectamine 2000 reagent, were from Invitrogen. The Wizard Miniprep and Maxiprep kits for isolation of plasmid DNA were purchased from Promega Corp. Protein A/G-agarose, etc.

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$^3$The abbreviations used are: MMP, matrix metalloproteinase; ECM, extracellular matrix.
enhanced chemiluminescence (ECL) Western blotting substrate, and the BCA (bicinchoninic acid) protein assay kit were obtained from Pierce. The anti-β1 monoclonal antibodies BD610468, AB1952, and MAB1959, as well as anti-MMP-2 monoclonal antibody MAB13405 and horseradish peroxidase-conjugated avidin, were from Chemicon. The horseradish peroxidase-conjugated goat anti-mouse antibody was purchased from Jackson ImmunoResearch (West Grove, PA). The mixture of protease inhibitors was from Hoffmann-La Roche. All other reagents, except where noted, were from Sigma.

**Tumor Experiments**—The colorectal tumors, after resection, were washed twice with cold PBS containing a mixture of protease inhibitors (Hoffmann-La Roche) and sodium azide (1 mg/ml) and then rapidly frozen and stored at −80°C. According to the TNM classification system made by the American Joint Committee on Cancer, tumors used in these studies were graded as pT2pN0, pT3pN1, and pT3pN2, where T assesses the primary tumor and N the regional lymph nodes. Twenty-one tumors that after histopathologic analyses corresponded to stages pT3pN2 (n = 8), pT3pN1 (n = 8), and pT2pN0 (n = 5) were used. Before the experiments, samples of the tumors were homogenized on ice in ProteoJET mammalian cell lysis reagent (Fermentas) containing a mixture of protease inhibitors. The homogenates were fractionated by centrifugation for 5 min at 5,000 g (F), for 15 min at 14,000 g (F), and then again for 15 min at 10,000 g (F). The supernatants were obtained for the immunoprecipitation and co-immunoprecipitation experiments.

**Carcinoma Cell Lines and Culture Conditions**—Human colon cancer cell lines (HT29 and LoVo) were obtained from the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wrocław, Poland). The colon carcinoma cells were cultured and maintained in MEM-supplemented as described above. Before some experiments, for example such as zymography, or FACS, the cells were transfected with pCMV-L5-MMP-2, pEGFP-N1-MMP-2, or control pEGFP-N1 vector, were lysed with a non-denaturing lysis buffer (1% CHAPS, 25 mM HEPES containing 150 mM NaCl, 5 mM MgCl2, 2 mM NaF, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin, pH 7.5) for 20 min on ice and centrifuged at 14,000 × g for 30 min at 4°C. The cytoskeletal debris was pelleted at 10,000 × g for 10 min. Next, the protein concentration was measured using the BCA method. 500 μg of protein from each lysate was incubated with 2.5 μg of the anti-β1 or anti-MMP-2 antibodies on a rotator overnight at 4°C. Subsequently, 100 μl of protein A/G-agarose bead slurry was added to each cell extract, and the incubation was continued for another 3 h. The captured immune complexes were washed eight times with a lysis buffer containing 0.1% CHAPS and dissolved using Laemmli sample buffer. SDS-PAGE and Western immunoblotting onto a nitrocellulose membrane were performed by means of standard procedures using monoclonal antibodies against the β1 integrin subunit or anti-MMP-2 (Table 1). The β1 integrin subunit was detected with the monoclonal antibody BD610468 or AB1952 (Santa Cruz Biotechnology), and β-actin was detected with the rabbit polyclonal antibody (Abcam) followed by the appropriate secondary antibodies conjugated with HRP (Santa Cruz Biotechnology). All incubations were done in a blocking buffer. Immunodetection was accomplished using the enhanced chemiluminescence kit and Kodak BioMax light film (Eastman Kodak). The developed films were scanned and the protein bands quantitated by the Gel Doc 2000 gel documentation system (Bio-Rad).

**Peptide Microsequencing and Data Processing**—Protein bands at 25 and 75 kDa reacting with the monoclonal antibody BD610468 were excised from the gel and subjected to in-gel
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digestion with trypsin. Proteins in each gel slice were subjected to reduction using 10 mM DTT, alkylation with 50 mM iodoacetamide, and trypptic digestion with modified trypsin (10 μg/ml, Promega) at 37 °C for 14 h. After in-gel digestion, the product peptides were extracted stepwise with three portions of 60 μl of 0.1% TFA, 2% acetonitrile and loaded on an RP-18 precolumn (LC Packings, Amsterdam, The Netherlands). Peptides were eluted to a nano-HPLC RP18 column (75 mm × 15 cm capillary; LC Packings) by an ACN gradient in the presence of formic acid and directly applied into an electrospray (ESI-Q-TOF; Micromass UK Ltd., Manchester, United Kingdom) spectrometer. The spectrometer was operated in data-dependent MS to MS/MS switch mode to give peptide sequencing data in addition to mass fingerprint data. The NCBI nonredundant protein database was searched with the MASCOT program (Matrix Science, London).

Surface Plasmon Resonance—The interaction of the β11 domain containing fragments present in tumor extracts with anti-β1 monoclonal antibody (BD610468) was evaluated by surface plasmon resonance (SPR). BD610468 monoclonal antibody or control mouse IgG were immobilized onto the CM5 sensor chip via amine coupling (Biacore AB, Uppsala, Sweden) according to the manufacturer’s instructions. Experiments were performed at 37 °C in 10 mM Hepes buffer, pH 7.4, containing 1 mM CaCl2. Before analysis, tumor extracts were centrifuged for 30 min at 10 000 × g and 15-μl aliquots containing 2 μg of tumor proteins were injected. The complex formation was observed at a flow rate of 5 μl/min. The same extracts were tested in parallel using the sensor chip coated with control mouse IgG. Experimental data were analyzed using BIAevaluation 3.2 software supplied by Biacore AB. The results were expressed in resonance units (RU), an arbitrary unit specific for the Biacore instrument (1000 RU corresponds to ~1 ng of bound protein/mm²). The association rate constant, k_on, and dissociation rate constant, k_off, were determined from individual association and dissociation phases, respectively, assuming one-to-one interactions.

Adhesion Assay—Wells of F8 Maxisorp loose Nunc-Immu-no™ modules (Nunc™ brand products) were coated with 50 μl of fibronectin or collagen type I used at a concentration of 10 μg/ml in TBS (0.02 M Tris/HCl, 0.15 m NaCl, pH 7.5). The proteins were allowed to bind for 2 h at 37 °C before the wells were rinsed twice with TBS and blocked for 1.5 h at 37 °C in a humidified 5% CO2 atmosphere with 200 μl of 1% heat-denatured BSA in TBS, pH 7.5, containing 0.1 mM CaCl2. The cells were harvested and added to the wells at 1.5 × 105 cells/0.15 ml of DMEM for 1.5 h at 37 °C in 5% CO2 atmosphere. The cells that did not adhere were removed by gentle washing with TBS containing 0.1 mM CaCl2, pH 7.5. The total cell-associated protein was determined by dissolving the attached cells directly in the microtiter wells with 200 μl of Pierce BCA protein assay reagent. The modules were incubated for 30 min at 37 °C, and the absorbance of each well was determined at 562 nm with a microplate reader (BioKinetics Reader EL340, Bio-Tek Instruments).

Invasion Assay—Invasion assays were conducted on polycarbonate filters, 8 μm (Costar® brand Transwell®). The filters were coated with basement membrane Matrigel™ (25 μg/filter). The Matrigel was spread onto the filters, dried under a hood, and reconstituted with serum-free medium. The cells were harvested with trypsin/EDTA and diluted, depending upon the type of cells tested, in MEM-α with 0.1% BSA to a final density of 2 × 105 cells/ml, and 50 μl of the cell suspension was added to the upper chamber. The conditioned medium was obtained by incubating mouse fibroblasts (3T3) for 24 h in serum-free medium in the presence of ascorbate (50 mg/liter). This medium, used as a source of chemoattractants, was placed in the lower compartment of the chambers. The assembled chambers were incubated for 6 h at 37 °C and 5% CO2. Then, the cells on the upper surface of the filter were completely removed by wiping with a cotton swab. The filters were fixed in methanol, and the cells were stained with Mayer’s hematoxylin and eosin and counted from the lower surface of the filter.

Wound Healing-like Cell Migration Assay—HT29 cells transfected with pCMV6-XL5 or pCMV6-XL5-MMP-2 were grown to confluence in the MEM-α supplemented with 10% fetal bovine serum, streptomycin (100 μg/ml), penicillin (100 units/ml), and t-glutamine (2 mM) using 24-well plates. Then, the cells were starved for 4 h in FBS-free medium and wounded across the cell monolayer by scraping away a swathe of cells with a 200 μl pipette tip. After scraping, cells were rinsed twice with MEM-α to remove wound-derived loose and dislodged cells and further cultured in the replaced MEM-α containing 1% BSA for 2 to 24 h. After the wounding, images were recorded immediately (time zero) and again 4 and 24 h later and then stored for analysis. The migration of cells into the denuded area was evaluated with an inverted Nikon phase-contrast microscope (Tokyo, Japan) at ×4006 magnification and photographed with a digital camera. Migration of cells was quantified using image analysis of a minimum of seven randomly selected fields of view of the denuded area as described previously (6). All experiments were performed in quadruplicate, and each experiment was repeated at least three times.

Time-lapse Microscopy—Spontaneous migration assays were performed by videomicroscopy as described previously (7). HT29 cells transfected with pCMV6-XL5 or with pCMV6-XL5-MMP-2 were seeded on uncoated wells in 12-well plates in 2 ml/well containing 1 × 104 cells). Twenty-four hours after seeding, cell migration analysis was performed using an inverted microscope (Axiovert 200 M; Zeiss, Oberkochen, Germany) equipped with a transparent environmental chamber (Climabox; Zeiss) under 5% (v/v) CO2 at 37 °C. The microscope was driven by Metamorph software (Roper Scientific, Evry, France), and images were recorded with a charge-coupled device camera (CoolsnapHQ; Roper Scientific) every 10 min during 14 h. Cell migration was characterized and quantified using an interactive tracking method as described (8).

Statistical Analysis—All values are expressed as the mean ± S.D. compared with the controls and among the separate experiments. Paired and unpaired Student’s t tests were employed to determine the significance of changes. A p value of <0.05 was considered statistically significant.

RESULTS

Cleavage of the β1 Integrin Subunit in Colorectal Tumors—To determine whether β1 integrins are susceptible to proteoly-
sis in colorectal tumor, samples of cancer collected during surgery were extracted and separated by SDS-PAGE followed by Western immunoblotting. We used two types of anti-β1 monoclonal antibodies: BD610468 reacting with the NH₂-terminal I-like ectodomain and AB1952 directed against the COOH-terminal cytoplasmic tail. Fig. 1A shows that the β1 integrin NH₂ terminus is subjected to proteolytic cleavage in the colorectal tumors and the extent of degradation depends on the stage of malignancy. When the same quantities of tumor extracts were subjected to Western blotting analysis, the most extensive cleavage of the β1 integrin subunit could be observed at cancer stage pT3pN2 (Fig. 1A, lanes a–c), and essentially no degradation products were detected at stage pT2pN0 (lanes g, h). In contrast to the HT29 cells giving a single band corresponding to the molecular mass of the intact β1 integrin subunit, in the colorectal tumor samples of stage pT3pN2 the BD610468 antibody detected two additional bands, namely at 75 and 25 kDa. There was only a 25-kDa fragment detected in tumor samples of stage pT3pN1 (Fig. 1A, lanes e, f, and i). To determine whether the level of the MMP-2 protein was elevated in the colorectal tumors, the same protein extracts were immunoblotted with anti-MMP-2 MAB13405. The data presented in Fig. 1A indicate that in most patient samples there is a large up-regulation of MMP-2 with a molecular mass of 72 kDa when compared with the HT29 cells. This conclusion was further supported by scanning immunoblots obtained after separation of large group of tumors differing in malignance stage (Fig. 1B). Up-regulation of MMP-2 was associated with an increased degradation of β1 integrin subunit as evaluated based on the intensity of the 25-kDa band stained with BD610468 antibody (Fig. 1C). When tested by zymography (Fig. 1D), tumor samples classified as pT3pN2, the same as those presented in Fig. 1A (lanes a–c), showed a much higher activity level of MMP-2 when compared with those of lower malignancy grade tumors described as pT2pN0 (Fig. 1A, lanes g and h). There was no significant difference in MMP-9 activity, which can be used as an internal control of loading. It explains why the latter tumor samples contain much less degraded β1 integrin subunit than the most malignant tumors (pT3pN2 stage) (Fig. 1C).

To further evaluate the extent of β1 integrin subunit cleavage in different tumor samples, surface plasmon resonance experiments were performed. Fig. 1E shows the binding of peptide fragments containing the β1 I-domain present in tumor extracts to the BD610468 antibody immobilized on the sensor chips. In control experiments, the same tumor extracts were passed through the sensor chip containing mouse IgG (Fig. 1F). In the case of the most malignant tumors (pT3pN2), the maximum response monitored at the end of the protein injection phase ranged from 3822 to 4755 resonance units. Tumors with earlier malignancy stages gave a strikingly weaker response, indicating significantly lower concentration of β1 cleavage fragments.

Proteolytic degradation of the β1 integrins was further evidenced when we used the antibody AB1952 against the COOH-terminal cytoplasmic tail of the β1 subunit. In addition to a 130-kDa band corresponding to the full-length β1 integrin, a series of lower molecular weight bands could be seen. These results confirmed that the NH₂ terminus of the β1 integrin is proteolytically degraded, thus reducing the size of the protein from 130 to 70, 35, and 25 kDa (Fig. 1G, lane a). To prove that the β1 integrin subunit is a direct MMP-2 target in colorectal carcinoma, we next searched whether the two proteins could associate with one another. Co-immunoprecipitation experiments were thus performed using the protein extracts of the colorectal tumor samples with the use of the anti-β1 integrin antibody AB1952 or anti-MMP-2 antibody (Fig. 1G). After separation by SDS-PAGE, the β1 or MMP-2 immunoprecipitate was blotted with the antibody specific to the β1 NH₂-terminal I-like domain (Fig. 1G, lane b) or with anti-β1 reacting with the cytoplasmic tail, respectively (Fig. 1G, lane c). The β1 immunoprecipitate showed the full-length 130-kDa protein together with a 19-kDa fragment corresponding to the I-like domain (Fig. 1G, lane b). The MMP-2 immunoprecipitate contained, in addition to a full-length β1 integrin, several cleavage fragments, indicating extensive degradation of the β1 subunit. This demonstrates that the association between the β1 integrin ectodomain and MMP-2 during immunoprecipitation induced degradation of the β1 integrin.

Cleavage of the β1 Integrin Subunit in HT29 Colon Cancer Cells with Elevated MMP-2 Expression—To evaluate the consequences of β1 integrin cleavage to the adhesive properties of the colon cancer cells, we next switched to model cell lines that correspond to the different stages of cancer. For this purpose we used undifferentiated HT29 and LoVo cells having a phenotype characteristic of Duke’s stage C. As a first step to identifying MMP-2 as an enzyme that degrades β1 integrins in colon cancer cells, we transfected the HT29 cells with control pCMV6 vector and the same vector carrying MMP-2 or pCMV6-XL5-MMP-2 for 24 or 48 h. Then the cells were solubilized with 1% Nonidet P-40 and used to investigate the effect of MMP-2 on the expression of the β1 integrin subunit. Fig. 2A shows the presence of MMP-2 and the β1 integrin subunit in the co-precipitating proteins by the anti-β1 antibody, AB1952. As evidenced by immunoblotting with the anti-MMP-2 (MAB13405) and anti-β1 (BD610468) antibodies, both proteins can form a complex also in HT29 cells. Up-regulation of MMP-2 in HT29 after transfection with pCMV6-XL5-MMP-2 resulted in enhanced degradation of the β1 integrin subunit, as evidenced by the appearance of the 25-kDa fragment containing the β1 I-like domain. The same was observed when LoVo cells were transfected with pCMV6-XL5-MMP-2 (not shown). Consistently, the increased expression of MMP-2 in HT29 cells transfected with pCMV6-XL5-MMP-2 resulted in enhanced proteolytic activity as shown by zymography (Fig. 2B). Fig. 2C shows adhesion of the transfected HT29 and LoVo cells to collagen and fibronectin. Transfection of the HT29 cells with pCMV6-XL5-MMP-2 significantly reduced adhesion to both proteins when compared with cells transfected with the empty vector pCMV, due to partial degradation of their α2β1 and α5β1 receptors, respectively. Similarly, up-regulation of MMP-2 expression in the LoVo cells resulted in significant inhibition of their adhesion to collagen and fibronectin. Consistently, incubation of HT29 and LoVo with exogenously added MMP-2 significantly reduced their adhesion to collagen (Fig. 2D). In this experiment, both types of cells (2.5 × 10⁵ cells/ml) were incubated with 10 μg of human recombinant MMP-2 for 2 h. Then
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FIGURE 1. **Extended cleavage of the β1 integrin subunit in colorectal tumors.** Samples of cancer cells collected from patients with colorectal tumors were extracted with the Fermentas mammalian cell lysis reagent. A, the tumor extracts (lanes a–c and e–i) and HT29 lysate (lane d) were separated by SDS-PAGE and subjected to Western immunoblotting using the anti-β1 antibody BD610468. Shown are representative samples of tumors classified as pT3pN2 (lanes a–c), pT3pN1 (lanes e, f, and i), and pT2pN0 (lanes g and h). MMP-2 and β-actin were detected with MAB13405 and the polyclonal anti-β-actin antibodies, respectively. After immunodetection, accomplished by using an enhanced chemiluminescence kit, the films were scanned and the protein bands were quantitated using the Gel Doc 2000 gel documentation system (Bio-Rad). To quantify the densitometric scans, the background was subtracted and the area for each protein peak was determined. Data obtained from three separate experiments in which five to eight different tumor samples corresponding to grades pT3pN2, pT3pN1, and pT2pN0, respectively, were analyzed and normalized to β-actin expression. B and C, relative expression of MMP-2 and the 25-kDa β1 fragment, respectively, expressed as the percentage of that found in the most malignant tumors, i.e., pT3pN2. **p < 0.001.** D, tumor samples classified as pT3pN2 showed significantly higher activity of MMP-2 than those described as pT2pN0, as evaluated by gelatin zymography performed using a 10% polyacrylamide gel copolymerized with 1 mg/ml gelatin as a substrate. E, the level of the β1 I-domain containing peptide fragments present in tumor extracts as evaluated by surface plasmon resonance using BD610468 monoclonal antibody immobilized on a sensor chip, CM5. For this purpose, 2 μg of proteins present in 15 μl of tumor extracts were injected, and the complex formation was observed at a flow rate of 5 μl/min. F, the same extracts were tested in parallel using the sensor chips coated with control mouse IgG. In G, the protein extracts of colorectal tumors were analyzed by Western immunoblotting (IB) using the anti-β1 antibody AB1952 (lane d) or subjected to immunoprecipitation (IP) with anti-β1 AB1952 and blotted with anti-β1 BD610468 (lane b) and control IgG (lane c), immunoprecipitated with anti-MMP-2 MAB13405, and then blotted with anti-β1 AB1952 (lane d) or anti-mouse IgG (lane e). In parallel, control experiments in which nonimmune IgG was used for immunoprecipitation and anti-β1 BD610468 or anti-anti-β1 AB1952 for blotting were done (lanes f and g). The data are representative of 3–5 independent experiments.
MMP-2 was inhibited by adding GM6001, and the 200-μl aliquots were placed in plate wells coated with collagen. The adhering cells were quantitated by means of CyQUANT (Molecular Probes) using the Wallac VICTOR 1420 multilabel counter (PerkinElmer Life Sciences). Fig. 2, E and F, shows that up-regulation of MMP-2 expression in HT29 cells resulting from transfection of cells with pCMV6-XL5-MMP-2 did not influence their viability or ability to proliferate.

Next, we assessed the similarity of the β1 subunit fragments found in the colorectal tumors and the pCMV6-XL5-MMP-2-transfected HT29 cells with those produced by MMP-2 digestion of purified α2β1 or α5β1 integrins. Recombinant human α2β1 or α5β1 was mixed with recombinant human MMP-2 in a stoichiometric molar ratio of 10:1 and incubated at 37 °C. Twenty-μl aliquots were withdrawn at 0 and 24 h; next, digestion was stopped by adding EDTA along with the sample buffer, and the mixture was separated by SDS-PAGE. The β1 integrin subunit and its fragments were blotted using anti-β1 antibody AB1952 or BD610468 (Fig. 3). Recombinant α2β1 or α5β1 showed the presence of high molecular degradation products including 75 kDa containing the I-like domain detected by the BD610468 antibody. After a 1-h digestion with MMP-2, both integrins released a 50-kDa fragment, which, after 24 h digestion of α2β1 or α5β1, was further cleaved to a 35- or 25-kDa fragment, respectively. The 75- and 25-kDa fragments corresponded well to the β1 cleavage products detected in the HT29 cells transfected with MMP-2 (Fig. 2) and in the colorectal tumors. To identify the peptide fragments reacting with BD610468, protein bands with molecular masses of 75 and 25 kDa were excised and subjected to microsequencing using an
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**TABLE 2**
The identity of a 19-kDa fragment detected by BD610468 with the β1 I-domain

Samples of cancer collected during surgery were extracted and separated by SDS-PAGE followed by Western immunoblotting using anti-β1 monoclonal antibody BD610468 reacting with the NH2-terminal I-like ectodomain. Protein bands at 75 and 19 kDa identified with BD610468 were analyzed by sequencing as described under “Materials and Methods.” Annotations in the databases for assigned proteins: Mass, predicted molecular mass of the identified protein; Peptide matches, number of peptides identified from the amino acid sequences of assigned proteins; Score, total probability score from the Mascot search for the identified protein.

| Peptide cleavage fragment | Score | Peptide matches | Sequences | β1 fragment |
|---------------------------|-------|----------------|-----------|------------|
| 25 kDa                    | 513   | 8              | OCPPPDEIENPR | 78–84 |
|                           |       |                | LGREDITQSQPOQLVLR | 106–122 |
|                           |       |                | SGEQPTFLTK    | 125–134 |
|                           |       |                | DDLINNVK      | 157–163 |
|                           |       |                | SLTGLDNNMNR   | 164–174 |
|                           |       |                | IGFQSFVEK     | 182–190 |
|                           |       |                | TVWMYSTTPAK   | 191–202 |
|                           |       |                | GEVPNELVGK    | 229–238 |
|                           |       |                |                |          |
| 75 kDa                    | 513   | 9              | LKREDITQSQPOQLVLR | 106–122 |
|                           |       |                | GEPQFTFLKL    | 125–134 |
|                           |       |                | SLTGLDNNMNR   | 164–174 |
|                           |       |                | IGFQSFVEK     | 182–190 |
|                           |       |                | TVWMYSTTPAK   | 191–202 |
|                           |       |                | GEVPNELVGK    | 229–238 |
|                           |       |                | DNTHEIYSGK    | 542–551 |
|                           |       |                | FCCECDFNCDR   | 552–562 |
|                           |       |                | GICRCUVC      | 611–619 |

![Figure 3](https://example.com) MMP-2 releases the NH2-terminal I-like domain from β1 and α5β1. Cleavage fragments (fragments) of the β1 integrin subunit were produced after digestion of α2β1 and α5β1 with MMP-2. In this experiment, MMP-2 was added to a 10–μg sample of α2β1 or α5β1 dissolved in 100 μl of 20 mM Tris buffer containing 2 mM CaCl2 and 1 mM MgCl2, pH 7.3, to a molar ratio of 1:10 and incubated for 0–24 h at 37 °C. Then, 25 μl aliquots were separated by SDS-PAGE followed by Western immunoblotting using anti-β1 monoclonal antibodies, AB1952 and BD610468. These recognizably different epitopes are located in the COOH-terminal cytoplasmic domain and the NH2-terminal I-like domain, respectively. In the last two lanes (a and b), two samples of the CRC cells were analyzed in parallel. The gels presented here are representative of three independent experiments.

Electrospray spectrometer. Peptidic analysis showed that both protein fragments contained the NH2-terminal region of β1 integrin subunit as evidenced by peptide fragments covering almost 50% of the I-domain sequence (Table 2).

**MMP-2 Secreted from Colon Cancer Cells Cleaves Surface-exposed β1 Integrins**—To further investigate whether the increased level of MMP-2 affects β1 integrin expression, we compared the two types of colon cancer cells, HT29 and LoVo, which differ in invasiveness (9). We tested the expression of MMP-2 and its secretion from these cells upon activation with thymosin β4. The data shown in Fig. 4, A and B, indicate that the more invasive LoVo cells exhibit higher MMP-2 expression when compared with HT29, as measured at the level of protein antigen by Western immunoblotting. Their treatment with 160 nm thymosin β4 for 24 h significantly up-regulated both the expression and secretion of MMP-2 as analyzed by real-time RT-PCR and zymography, respectively (Fig. 4, C and D). To identify the β1 cleavage fragments associated with cellular membranes, both control and thymosin β4-activated HT29 cells were biotinylated using the membrane-impermeable reagent EZ-Link® NHS-SS-biotin (sulcinimidyl-2-(bionamidoethyl)-1,3-dithiopropionate) according to the manufacturer’s protocol. Next, the membrane proteins that bound biotin were analyzed by SDS-PAGE and blotting or were used in immuno-precipitation experiments. For this purpose, after being washed with serum-free DMEM, the cells were either solubilized in Laemmli sample buffer or extracted with a nondenaturing lysis buffer containing 1% CHAPS. After separation by SDS-PAGE and electrophoretic transfer to nitrocellulose, the proteins containing biotin were visualized by avidin conjugated with horseradish peroxidase. Fig. 4E shows that thymosin β4 pointedly augmented the level of the membrane-associated proteins labeled with biotin. When extracts of the same cells were immunoprecipitated with the anti-β1 antibody BD610468 and stained with avidin conjugated with horseradish peroxidase, there was only a single band of 50 kDa on the immunoblots (Fig. 4F). This band represented the β1 cleavage fragment containing the I-like domain, and its concentration was much higher in the thymosin β4-activated HT-29 cells when compared with the control cells. Fig. 4G shows that there are some other fragments of the β1 integrin subunit among the biotinylated membrane proteins as well. In this experiment, the anti-β1 antibody AB1952 specific to the COOH-terminal tail was used to obtain the immunoprecipitate, and the cleavage fragments were blotted with the anti-β1 antibody MAB1959 reacting with an unknown epitope of the β1 integrin.

**Increased Motility of HT29 Cells Transfected with MMP-2**—Finally, to reinforce the theory that reduced adhesiveness supports increased motility of cancer cells, we used HT29 cells transfected with pCMV6-XL5-MMP-2 and analyzed their migration by a “wound healing-like” assay and time-lapse microscopy. The extent of the β1 integrin cleavage was evaluated in parallel experiments. These assays characterize directional cell migration and random cell migration, respectively. Although, both motilities involve distinct mechanisms (10), integrins contribute equally to cell migration. As expected, transfection of HT29 with pCMV6-XL5-MMP-2 resulted in markedly increased cellular migration when analyzed by both the wound healing-like assay and time-lapse microscopy. There was more rapid migration across the wound in cells over-expressing MMP-2 than in controls (HT29 transfected with pCMV6) (*p < 0.01; Fig. 5A and B*). Migration was completely abolished when HT29 cells were treated with 10 μM APR101, a specific MMP-2 inhibitor. Interestingly, treatment of HT29 cells with siRNA specific to β1 mRNA induced cell migration to the same extent as their transfection with pCMV6-XL5-MMP-2. Similarly, control HT29 cells did not migrate during the 24 h of videomicroscopy (Fig. 5C). In contrast, HT29-MMP-2 showed enhanced random migration, indicating the significantly higher migration speed (Fig. 5D) and average translocation (Fig. 5E) when compared with control HT29 cells. In contrast to control cells, HT29-MMP-2 always showed the
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FIGURE 4. Increased secretion of MMP-2 from colon cancer cells leads to more extended cleavage of β1 integrins. A, HT29 and LoVo cells differing in invasiveness were used in these experiments. B, human colon carcinoma cells were allowed to invade MatrigelTM placed on 8 μm polycarbonate filters (Transwell® Costar®) and to migrate into the lower part of the filter. The cells on the lower side of the filter were counted. The protein extracts of the HT29 and LoVo cells were separated by SDS-PAGE and immunoblotted with the anti-MMP-2 monoclonal antibody MAB13405. Blotting with anti-β-actin was used as a loading control. C and D, when incubated with 160 nM Tβ4 for 24 h, both the HT29 and LoVo cells were enriched in mRNA MMP-2, as analyzed by real-time RT-PCR (C), and showed significantly higher secretion of MMP-2, as evaluated by gelatin zymography performed using 10% polyacrylamide gel copolymerized with 1 mg/ml gelatin as a substrate. s, standard MMP-2. The gels were stained in 0.5% Coomassie Brilliant Blue R-250 (D). S, standard MMP-2; C, control cells. To detect the β1 cleavage fragments associated with the cell membranes, the control cells (C) or Tβ4-treated HT29 cells were biotinylated using succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate; then the cell extracts were separated by SDS-PAGE. E, the cellular proteins of the control HT29 cells and cells were activated with Tβ4 stained with Coomassie Brilliant Blue (Coom. (first two lanes)) or visualized after electrophoretic transfer to nitrocellulose by staining with avidin conjugated with horseradish peroxidase (A-HRP (last two lanes)). F and G, the immunoprecipitates (IP) obtained from the control and Tβ4-activated HT29 using the anti-β1 antibodies BD610468 and AB1952 and blotted (IB) with avidin-horseradish peroxidase or the anti-β1 antibody (MAB1959), respectively. The same immunoprecipitates were also blotted with goat anti-mouse IgG. The arrows show an intact β1 integrin subunit (F) and its cleavage fragments (fragm. (G)). Data were obtained from three to five separate experiments and expressed as the mean ± S.D. **, p < 0.001.

DISCUSSION

The combined cooperation of integrins and MMPs is required for cancer cell motility and invasiveness within the ECM (11, 12). It is generally believed that the interaction of MMPs and integrins may occur either directly (e.g. via binding of MMPs to adhesion sites (5)) or indirectly (e.g. via proteolysis-dependent unmasking of ECM integrin binding sites (13)). Such interactions have been detected in cell membranes, particularly in invadopodia, in caveolae, and at the leading edge of migrating cells, where directed proteolytic activity is needed (14). Dysregulated proteolysis has been implicated in tumor invasion and metastasis in multiple model systems, including double MMP-2/MMP-9-deficient mice, thus demonstrating that these enzymes cooperate in promoting the invasive phenotype of malignant cells in vivo (15).

The data presented here, obtained using in vivo and in vitro cancer cell sources, show the following. (a) MMP-2 expression is elevated in colorectal tumors resected during surgery. Obviously, in human cancer, tumor cells are not the only source of MMP-2, which is produced predominantly by stromal cells, e.g. immune, inflammatory, and vascular cells. Therefore, we next repeated most experiments using the colorectal carcinoma cell lines HT29 and LoVo. (b) The ectodomain of the β1 integrin subunit is lost both from the colorectal tumors and colorectal carcinoma HT29 cells in which MMP-2 is up-regulated. (c) MMP-2 associates with and degrades the β1 integrin subunit. (d) MMP-2-mediated β1 integrin cleavage decreases the adhesive properties of colorectal carcinoma cells, thus potentially enhancing their motility. (e) HT29 cells with up-regulated MMP-2 and a partially cleaved β1 integrin subunit showed enhanced motility as evaluated by a wound healing-like assay and time-lapse microscopy, indicating their increased invasiveness.

However, one has to keep in mind that there are a number of other substrates of MMP-2 that can be degraded extensively when this enzyme is up-regulated and cleavage of some of them may affect cell motility as well. In this work we focused on cleavage of the β1 integrin subunit because the role of this group of receptors in cancer cell migration is so well documented.

The fact that some of the MMP-2 expressed in the colorectal cancer cells associates with the β1 integrin, and that they co-immunoprecipitate, indicates that interaction of these two proteins does not always result in cleavage of the integrin. MMP-2
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as an extracellular protease binds to the ectodomain of the β1 integrin, and if MMP-2 were activated within a MMP-2/β1 integrin complex, it would completely degrade the integrin. Our results suggest that limited proteolysis of β1 integrins is another mechanism by which expression of active integrins can be controlled in colorectal carcinoma cells. It remains unclear how MMP-2 makes it to the correct location at the cell surface and how the proteolytic activity is controlled at the pericellular space. Several observations suggest that secreted MMPs can localize especially at the cell surface because of their interaction with “docking” molecules (16, 17). The presence of such MMP-binding proteins allows cells to concentrate and activate MMPs at the close interface between their plasma membrane and the extracellular matrix, and in cancer cells, this may be a key event in promoting tumor invasion and angiogenesis (18, 19). Recent observations suggest that integrins can accumulate MMPs, particularly gelatinases, at the surface of cell membranes. MMP-9 is described as associating with β1 integrins (20) and MMP-2 with α5β1 and αV integrins (21–24). The mechanism by which they might be involved in activating specific MMPs is known only for MMP-2. The latter, which is activated on the cell surface, is in a multimeric complex composed of MMP-2, membrane type 1 MMP (MT1-MMP), and tissue inhibitor of metalloproteinase 2 (TIMP2). This complex also includes integrin αVβ3, to which MMP-2 binds via the COOH-terminal hemopexin-like domain (5). Initially, this domain binds to TIMP2 (25) which, in turn, associates with the membrane-bound MT1-MMP, and the NH2-terminal propeptide of MMP-2 is released by MT1-MMP. The resulting intermediate form is capable of binding integrin αVβ3 (26). This interaction activates MMP-2, thereby localizing its proteolytic activity to the invasive front of cells (27). Such interactions have been detected in caveolae and invadopodia and at the leading edge of migrating cells, where directed proteolytic activity is needed. So, during invasion, integrin αVβ3 functions not only as an adhesion/migration receptor but also in activating and localizing the proteases that are required for ECM degradation. Negative feedback regulation of integrin/protease binding is provided to prevent excessive degradation of the ECM by the MMP-2-dependent generation of hemopexin fragments. Such fragments block protease activation by competing with MMP-2 for binding to αVβ3. The binding of MMP-2 to integrin αVβ3 via its hemopexin domain is crucial for mesenchymal cell invasive activity (28). Likewise, high local concentrations of active MMP-14 on the cell membrane of metastatic cancer cells play important roles in cell migration (29–31). Interestingly, the interaction of MMP-2 with α5β1 induces apoptosis of rat myocytes after stimulation by the β-adrenergic receptor (21). In colon carcinoma cells, de novo expression of α5β1 results in growth arrest and reduced cellular proliferation in vitro (32).

Earlier studies have described various changes in the expression of integrins observed during cancer progression, for instance, a dramatic increase in their expression levels (33–37). In some cells, carcinogenesis led to a switch in integrin expression, e.g. the major integrins α5β1 and α6β1 on normal hepatocytes are replaced by α1β1, α2β1, and α3β1, which are expressed in abundance in cancer cells (37–39). This exchange of integrin expression is coupled with the gaining of migratory

FIGURE 5. Increased expression of MMP-2 and the β1 integrin ectodomain cleavage enhances colon cancer cell motility. Confluent HT29 culture transfected with pCMV vector (●—●) or pCMV-XL5-MMP-2 (○—○) was starved for 4 h. In some experiments, HT29 cells were treated with siRNAβ1 (▲—▲) or siRNAα (△—△) (150 pmol each) for 24 h or with 10 μM APR101 (■—■), a specific inhibitor of MMP-2. After the wounding, the cells were maintained in M199 containing 1% bovine serum albumin. A, cell culture images were recorded immediately (0 h) and after 20 h. B, migration of wounded cells was estimated by quantification of percent recovery as described under “Materials and Methods.” C–E, migration of HT29 cells tested by time-lapse microscopy. HT29 cells transfected with pCMV vector, or pCMV-XL5-MMP-2 (25 × 10³/well) were seeded on collagen or plastic. Cell migration was monitored for 24 h using a single cell tracking software. The trajectory of 10 representative cells was analyzed. Scale bar, 180 μm. The average translocation and velocity displayed on the 1 diagrams were determined by means of computer-assisted phase contrast videomicroscopy for a period of 24 h as described under “Materials and Methods.” Data are shown as the mean ± S.D. of at least three determinations. *** p < 0.01.
capacity, thus suggesting an important role for β1 integrins in cancer cell motility. Consistently, our studies have shown that the in vitro and in vivo blocking of β1 integrin expression in endothelial cells and colorectal carcinoma cells, for example with siRNA or DNAzymes, abolishes their proangiogenic properties and inhibit the growth of tumors (40, 41).

Taken together, our data presented here suggest that integrin β1 undergoes MMP-2-mediated cleavage in colorectal carcinoma cells in a time-dependent manner, thus decreasing cell adhesion and potentially enhancing cell motility. A further understanding of the mechanism regulating integrin cleavage may provide new therapeutic approaches to either modulating or detecting early tumor cell invasion or migration. Future work will be focused on revealing the regulatory features that control cleavage of the integrin β1 on the tumor cell surface.

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