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Matrix Metalloproteinase-7 Facilitates Insulin-Like Growth Factor Bioavailability through Its Proteinase Activity on Insulin-Like Growth Factor Binding Protein 3

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ABSTRACT

Matrix metalloproteinase-7 (MMP-7) secreted by cancer cells has been implicated classically in the basement membrane destruction associated with tumor cell invasion and metastasis. Recent epidemiologic studies have established a correlation between high levels of circulating insulin-like growth factor (IGF) and low levels of IGF binding protein 3 (IGFBP-3), and relative risk of developing colon, breast, prostate, and lung cancer, which are known to produce MMP-7. In this study, IGFBP-3 was assessed as a candidate for the physiologic substrate of MMP-7. MMP-7 proteolysis generated four major fragments (26 kDa, 17 kDa, 15.5 kDa, and 15.5 kDa), and two cleavage sites were identified: one at the site of hydrolysis of the K^{144}-I^{145} peptide bond and one at the R^{95}-L^{96} peptide bond. The former site is different from the previously reported site of cleavage of IGFBP-3 by other proteases. Addition of IGFBP-3 inhibited IGF-I-mediated IGF type 1 receptor (IGF-IR) phosphorylation and activation of the downstream molecule Akt in BALB/c 3T3 fibroblasts overexpressing human IGF-IR (3T3-IGF-IR) and in two human colon cancer cell lines (COLO201 and HT29). Coincubation of the IGF-I/IGFBP-3 complex with MMP-7 restored IGF-I-mediated IGF-IR phosphorylation and activation of Akt in these cell lines. The IGF-I signal recovered by MMP-7 protected against apoptosis induced by anoikis in 3T3-IGF-IR cells. These results indicate that MMP-7 proteolysis of IGFBP-3 plays a crucial role in regulating IGF-I bioavailability, thereby promoting cell survival. This mechanism may contribute to the tumorigenesis of MMP-7-producing IGF-IR-expressing tumors in the primary site and to organ-specific metastasis in a paracrine manner.

INTRODUCTION

Proteolysis plays a central role in the regulation of a variety of physiologic and pathologic processes. The matrix metalloproteinases (MMPs) comprise an endopeptidase family that includes collagenases, gelatinases, stromelysins, and membrane-type MMP, and display a broad spectrum of proteolytic activities toward extracellular matrix components (1–3). MMPs are believed to mediate many biological processes in which tissue remodeling is implicated, such as embryo implantation and morphogenesis, cell migration, metastasis, tumor invasion, and wound healing (3). MMP-7 (matrilysin, pump-1) is a member of the MMP family and, when activated, displays broad proteolytic activity against a variety of extracellular matrix substrates, including collagens, proteoglycans, elastin, laminin, fibronectin, and casein (4–6). Unlike MMPs synthesized by stromal cells, MMP-7 is produced exclusively by cancer cells, and participates directly in the process of invasion and metastasis by various cancers, including squamous cell carcinomas of the head, neck, and lung (7); adenocarcinoma of the breast (8), prostate (9), stomach (10), and colon (10, 11); and hepatocellular carcinoma (12).

The insulin-like growth factors (IGFs) have been investigated widely for a possible role in cancer growth (13–16). They are expressed ubiquitously, and act as endocrine, paracrine, and autocrine growth factors. In most tissues, they are synthesized together with six molecular species of specific binding proteins [IGF binding protein (IGFBP) -1 to -6]. These IGFBPs have affinities for the IGFs that are either equal to or stronger than those of the IGF receptors, and they modulate IGF action in the cell environment, generally by inhibiting it (17). Limited proteolysis of IGFBPs is recognized as an essential mechanism in the regulation of IGF bioavailability in the bloodstream and at the cellular level (17, 18). IGFBP proteinases fall into three major classes. The first class comprises the kallikrein-like serine proteinases, including prostate-specific antigen, γ-nerve growth factor, plasmin, and thrombin. The second class consists of the cathepsins, which are activated under acidic conditions. The third class is the MMPs. Some evidence suggests that MMPs contribute to the initiation of growth by regulating access to growth factors in the extracellular matrix surrounding the tumor through a proteolytic cascade (19–21). However, the mechanisms of this growth regulation have not been characterized fully.

Several IGFBPs have been described as substrates of MMPs. MMP-1, MMP-2, MMP-3, and MMP-9 degrade IGFBP-3 (22, 23), and MMP-1 and MMP-2 degrade IGFBP-5 (24). IGFBP-1 is cleaved by MMP-2, MMP-3, MMP-7, and MMP-11 (25). Only MMP-9 has been reported to act as an IGFBP-3 proteinase that triggers an IGF autocrine response in cancer cells (23). Our hypothesis in the present study was that the MMP-7 produced by cancer cells could act as an IGFBP-3 proteinase to adjust the tissue environment by using IGF in an active paracrine manner. Consistent with this hypothesis, the data obtained in this study show that: (*a*) IGFBP-3 is a substrate for MMP-7 *in vitro*; (*b*) it produces two major IGFBP-3-specific cleavage sites (one reported here for the first time); (*c*) the proteolytic cleavage modifies the affinity of IGFBP-3 for IGF-I, which causes recovery of IGF-I signal transduction *in vitro*; and (*d*) the recovered IGF-I signal protects against apoptosis induced by anoikis. These findings support the idea that the MMP-7 produced by tumor cells controls IGF bioavailability in the surrounding tissue, which favors cell survival in a tissue microenvironment.

MATERIALS AND METHODS.

Proteins and Reagents. Recombinant human IGF-I was obtained from R&D Systems Inc. (Minneapolis, MN), and recombinant human glycosylated IGFBP-3 was obtained from Genzyme/Teche (Minneapolis, MN). Recombinant human active MMP-7, MMP-3, MMP-2, and tissue inhibitor of metalloproteinase (TIMP) -1 were obtained from Chemicon International Inc. (Temecula, CA). 1,10-Phenanthroline was purchased from Sigma (St. Louis, MO). Protease inhibitor mixture tablets (EDTA-free and complete) were purchased from Roche Diagnostic (Mannheim, Germany). Agarose was purchased from Wako (Osaka, Japan).

Cell Culture. BALB/c 3T3 fibroblasts overexpressing the human IGF type I receptor (3T3-IGF-IR; a gift of Drs. A. Ullrich and R. Lammers) were

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cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum (Sigma). COLO201 and HT29 cells (ATCC CCL-224 and HTB-38; American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum. Tissue culture plasticware was obtained from Corning Glass Works (Corning, NY).

Enzyme Cleavage Assays. Recombinant human glycosylated IGFBP-3 was cleaved by exposure to active MMP-7 (enzyme:substrate ratio ranging from 1:2 to 1:8) in a cleavage buffer [150 mM NaCl, 10 mM HEPES (pH 7.4), and 5 mM $CaCl_2$] for 30–180 min at 37°C. The amount of IGFBP-3 was kept constant at 400 ng (final concentration 10 μg/ml), and the amount of enzyme varied with the enzyme:substrate ratio used. Reactions were terminated by addition of sample buffer containing the reducing agent 2-mercaptoethanol. The reaction solution was boiled and then resolved by 15% SDS-PAGE. 1,10-Phenanthroline (0.1 mM and 1 mM), EDTA (10 mM and 50 mM), and TIMP-1 (3.5 μg/ml) were used as MMP inhibitors. EDTA-free protease inhibitor mixture (4% v/v, 1 tablet/ml in H_2O) was used as a negative control. According to the manufacturer's instructions for this reagent, serine/cysteine protease activity is inhibited fully at this dilution.

Detection of the Product of IGFBP-3 Degradation by MMP-7. Western blot analyses were performed after sample transfer (50 ng of IGFBP-3) to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). Nonspecific binding was blocked for 1 h with 5% nonfat dry milk and 1% BSA in PBS (pH 7.4) containing 0.1% Tween 20 at room temperature. The membrane was incubated overnight in the mouse anti-IGFBP-3 monoclonal antibody (mAb; 1 μg/ml; clone 84728.111; R&D Systems, Inc.) at 4°C and then for 1 h with peroxidase-labeled goat antimouse antibody (1:3000; Zymed Laboratories, Inc., San Francisco, CA). The IGFBP-3 bands were visualized with ECL chemiluminescent reagent (Amersham Corp., Arlington Heights, IL).

Determination of Cleavage Sites in IGFBP-3 Produced by Digestion with MMP-7. Recombinant human glycosylated IGFBP-3 (500 ng) was incubated with active MMP-7 (250 ng) for 10–300 min at 37°C in a final volume of 20 μl, and the reaction was terminated by adding reducing sample buffer. The proteolytic fragment patterns were evaluated with a silver staining kit (Daiichi Pure Chemicals, Tokyo, Japan). To determine the cleavage site, the proteolytic fragments were separated by SDS-PAGE, followed by blotting on a Problott membrane (Applied Biosystems, Foster City, CA); 50 ng of IGFBP-3 protein were run per lane. Proteins were visualized by staining with the SYPRO Ruby protein blot stain (Molecular Probes, Inc., Eugene, OR) according to the manufacturer's protocol. Amino acid sequences were determined by automated Edman degradation with a Procise cLC protein sequencer (Applied Biosystems). Each fragment was analyzed for the presence of the COOH-terminal of IGFBP-3 by Western blot analysis with an anti-IGFBP-3-COOH-terminal mAb (1:500; clone C-19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Phosphorylation of IGF-IR and Akt. Subconfluent 3T3-IGF-IR, COLO201, and HT29 cells were cultured in serum-free medium for 24 h and then pulsed with IGF-I (10 ng/ml) and/or IGFBP-3 (200 ng/ml) and/or MMP-7 (200 ng/ml) for 30 min at 37°C. When adding MMP-7 to IGF-I and IGFBP-3, the three proteins were preincubated in cleavage buffer (final concentrations of IGF-I, IGFBP-3, and MMP-7 of 1.5, 30, and 30 μg/ml, respectively) *in vitro* for 30 min at 37°C, and this mixture was added to the serum-free culture medium. We confirmed the loss of the IGFBP-3 band under these preincubation conditions by silver staining (data not shown). TIMP-1 (42 μg/ml) also was preincubated with MMP-7 *in vitro* for 30 min at room temperature. After washing twice with ice-cold PBS, cells were lysed at 4°C for 30 min with 200 μl of lysis buffer [20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM $MgCl_2$, 1% NP40, 10% glycerol, 8 μl of complete protease inhibitor mixture (1 tablet/ml H_2O), 1 mM sodium orthovanadate, and 10 mM NaF]. Lysates were centrifuged at 14,000 $\times$ *g* for 30 min at 4°C, and their protein concentration was determined by using the Bio-Rad protein assay dye reagents (Hercules, CA). Five hundred μg of cell lysate were immunoprecipitated with anti-IGF-IR mAb (2 μg/mg protein lysate; clone αIR3; Oncogene Research Products, Cambridge, MA) for 3 h at 4°C, and immunoprecipitates were collected using the IMMUNOcatcher kit (CytoSignal Research Product, Irvine, CA). Each sample (derived from 20 μg total cell lysate from 3T3-IGF-IR cells and 50 μg from COLO201 and HT29 cells) was fractionated by 7.5% SDS-PAGE under reducing conditions. Tyrosine-phosphorylated proteins were detected by Western blot analysis with an antiphosphotyrosine mAb (1:1000; clone 4G10; Upstate Biotechnology, Lake Placid, NY). Total IGF-IR protein levels were estimated with an anti-IGF-IR polyclonal antibody (1:500; clone C-20; Santa Cruz Biotechnology) using the same membrane. Cell lysates (20 μg of 3T3-IGF-IR and 50 μg of COLO201 and HT29) were fractionated by 10% SDS-PAGE under reducing conditions. The Akt and phospho-Akt levels were estimated with anti-Akt or anti-phospho-Akt mAb (1:1000 each; Cell Signaling Technology, Beverly MA).

Assay of Anoikis Protection of 3T3-IGF-IR Cells by IGF-I. 3T3-IGF-IR cells were seeded in 60-mm dishes in DMEM with 10% fetal bovine serum and grown to 70% confluence. The cells then were washed twice with PBS and switched to serum-free medium for 16 h. After the cells had been detached with trypsin/EDTA, they were plated onto 0.9% agarose-coated 60-mm dishes in serum-free medium for 9 h. The cells then were incubated with IGF axis component molecules (10 ng/ml IGF-I, 1 μg/ml IGFBP-3, and 1 μg/ml MMP-7) and harvested from dishes using pipettes to obtain single-cell suspensions. Double staining by the FITC-conjugated Annexin V and propidium iodide method was used to detect apoptosis and necrosis from the same cell samples (26). Cells (2×10^5/ml) were stained simultaneously with FITC Annexin V and propidium iodide as recommended by the rh Annexin/FITC kit (MedSystems Diagnostics, Vienna, Austria) and subjected to flow cytometric analyses on an FACSCalibur (Becton Dickinson, San Jose, CA) to detect the percentage of early apoptotic (FITC-stained and propidium iodide-unstained) cells. A minimum of 10,000 cells was examined for each sample.

RESULTS

In this study, we tested the ability of MMP-7 to catalyze the proteolysis of IGFBP-3 *in vitro*. Western blot analysis revealed that IGFBP-3 was cleaved by MMP-7 in a dose- and time-dependent manner (Fig. 1*A*). MMP-2 and MMP-3 did not exhibit this proteolytic activity under the same assay conditions (Fig. 1*A*, *Lanes 8* and *9*). Several MMP inhibitors (EDTA, 1,10-phenanthroline, and TIMP-1) and a serine/cysteine protease inhibitor mixture were used to demon-

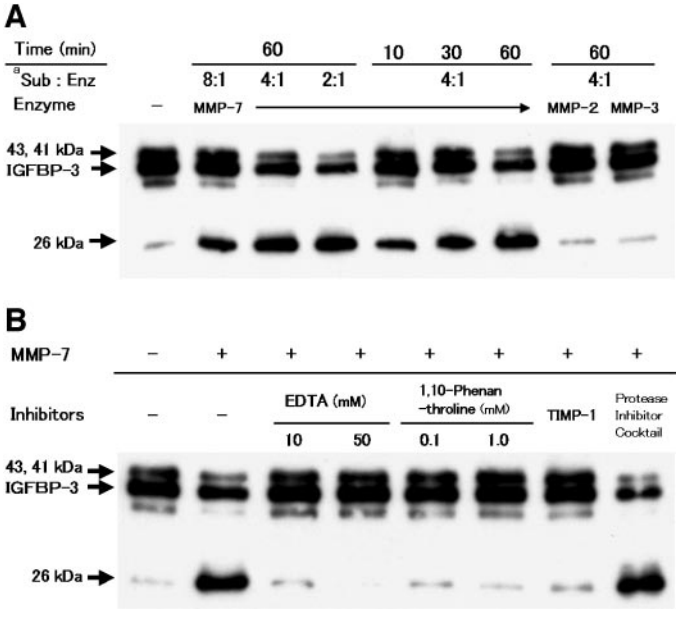

Fig. 1. Degradation of glycosylated insulin-like growth factor binding protein 3 (*IGFBP-3*) by active matrix metalloproteinase-7 (*MMP-7*). Recombinant IGFBP-3 protein migrates as a 43- and 41-kDa doublet, according to the level of its glycosylation. *A*, glycosylated IGFBP-3 (10 μg/ml) was incubated with active MMP-7 as described under "Materials and Methods." Samples then were separated by 15% SDS-PAGE under reducing conditions and transferred to a polyvinylidene difluoride membrane. IGFBP-3 proteolysis was detected by Western blot analysis with anti-IGFBP-3 monoclonal antibody (mAb; 1 μg/ml). *a*, Sub:Enz = substrate:enzyme ratio. *b*, glycosylated IGFBP-3 (10 μg/ml) was incubated with various protease inhibitors (1,10-phenanthroline, EDTA, recombinant human tissue inhibitor of metalloproteinase 1, or serine/cysteine protease inhibitor mixture) in the presence of MMP-7 (2.5 μg/ml) for 1 h at 37°C. Samples were analyzed by Western blot analysis with an anti-IGFBP-3 mAb (1 μg/ml).

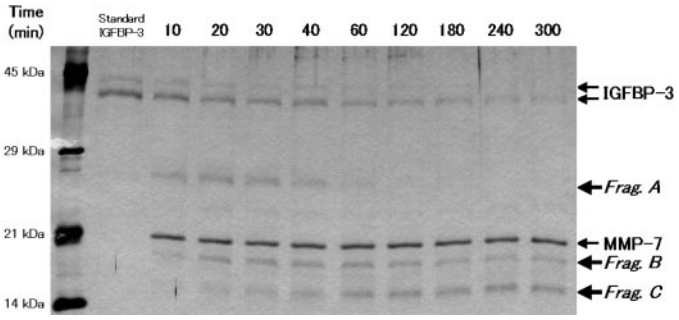

Fig. 2. Identification of matrix metalloproteinase-7 (*MMP-7*) cleavage fragments by using glycosylated insulin-like growth factor binding protein 3 (*IGFBP-3*). Time course analysis was performed. Glycosylated IGFBP-3 (25 μg/ml) was incubated with active MMP-7 (12.5 μg/ml) as described under "Materials and Methods." Samples then were separated by 15% SDS-PAGE under reducing conditions, and silver staining was performed.

strate that the MMP-7 cleavage of IGFBP-3 is specific. EDTA and 1,10-phenanthroline inhibited IGFBP-3 degradation in a dose-dependent manner; 3.5 μg/ml TIMP-1 inhibited completely MMP-7 proteolytic activity; and the serine/cysteine protease inhibitor mixture had no effect (Fig. 1*B*). These findings suggest that MMP-7, and not contamination by other proteases, is responsible for the IGFBP-3 cleavage.

To additionally characterize the proteolytic cleavage of IGFBP-3 by MMP-7, we performed a cleavage assay with recombinant human glycosylated IGFBP-3 and analyzed the products by the silver staining method (Fig. 2). MMP-7 degraded IGFBP-3 into three distinct fragments with apparent molecular masses of 26 kDa (Fragment A), 17 kDa (Fragment B), and 15.5 kDa (Fragment C; Fig. 2). To identify the cleavage site of MMP-7, the IGFBP-3 proteolysis fragments were NH_2-terminal sequenced directly from bands electrotransferred to nylon membranes. The NH_2-terminal sequences of each fragment are shown in Table 1. Fragment C consisted of two fragments (Fragment C1 and C2). Western blot analysis showed that only Fragment B contained the COOH-terminal of IGFBP-3 (data not shown). Because the *N*-glycosylation sites of IGFBP-3 are located at N^{89}, N^{109}, and N^{172} in the nonconserved region (27), each fragment contained one glycosylated arginine residue, and the estimated molecular weight of each fragment matched the migration positions observed in SDS-PAGE. The amino acid sequence of IGFBP-3 is shown in Fig. 3 with the cleavage sites for MMP-7 indicated. The degradation time course suggested that the first cleavage site was a K-I bond (K^{144}-I^{145}), producing Fragment A and Fragment B, and that Fragment A was cleaved additionally at R^{95}-L^{96}, producing Fragment C1. The molecular weight of Fragment C2, on SDS-PAGE (15.5 kDa), suggested the presence of an unknown cleavage site on the COOH-terminal side.

A schematic diagram showing two possible cleavage patterns is shown in Fig. 4.

The consequences of IGFBP-3 proteolysis on IGF-induced signal transduction were analyzed. After 24-h serum-starved 3T3-IGF-IR cells had been incubated with IGF-I, IGFBP-3, and MMP-7 for 30 min, anti-IGF-IR mAb-immunoprecipitated lysates were obtained. Fig. 5*A* shows that IGF-I phosphorylated markedly the IGF-IR (Fig. 5*A*, *Lane 2*) but that IGFBP-3 had no effect on IGF-IR phosphorylation (Fig. 5*A*, *Lane 3*). MMP-7 alone had a slight stimulatory effect on IGF-IR (Fig. 5*A*, *Lane 4*). Fig. 5*A* also demonstrates that IGFBP-3 inhibited completely IGF-I stimulation to the baseline level (Fig. 5*A*, *Lane 5*). However, adding MMP-7 to IGF-I and IGFBP-3 restored completely the stimulatory effect on the IGF-IR (Fig. 5*A*, *Lane 6*). To investigate IGF-I-mediated postreceptor signal transduction, we evaluated the phosphorylation of Akt, which is one of the downstream molecules in this pathway (28–31). The results showed that Akt was phosphorylated through IGF-IR activation in this cell line and that proteolysis of IGFBP-3 by MMP-7 reversed fully the IGF-I-mediated signal transduction (Fig. 5*A*, *Lane 6*). A similar phosphoprotein banding pattern was obtained in the human colon cancer cell line COLO201 (Fig. 5*B*). We selected this cell line because no pro-MMP-7 protein was detected in COLO201 culture medium, regardless of its mRNA expression (data not shown). We also examined HT29, another human colon cancer cell line with a high protein level of pro-MMP-7, and the same results were obtained (Fig. 5*C*). In this cell line, we confirmed that TIMP-1 inhibited the phosphorylation of IGF-IR and Akt mediated by active IGF from IGFBP-3 cleavage by MMP-7 (Fig. 5*C*, *Lane 7*).

To confirm that the IGF-I-mediated antiapoptotic effect is restored by MMP-7 proteolysis of IGFBP-3 *in vitro*, we performed the anoikis protection assay with 3T3-IGF-IR cells. Apoptosis was induced by anoikis for 9 h in the absence of serum after 16 h of serum starvation. Early apoptotic cells were evaluated by the percentage of cells in

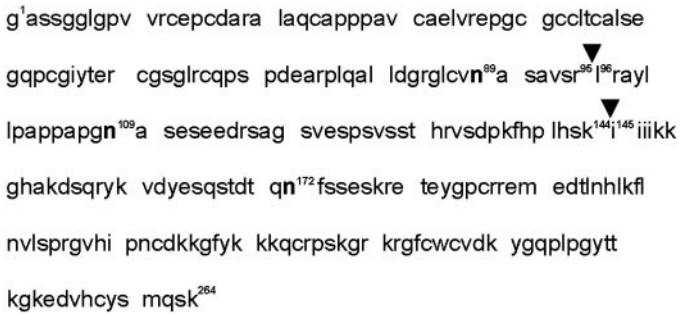

Fig. 3. Amino acid sequence of human insulin-like growth factor binding protein 3. *Arrowheads* indicate the cleavage sites for matrix metalloproteinase-7. *n* = *N*-linked glycosylation site.

Table 1 *Cleavage sites in IGFBP-3 produced by MMP-7*

Cleavage sites in insulin-like growth factor binding protein 3 (IGFBP-3) produced by matrix metalloproteinase-7 (MMP-7) were determined by *N*-terminal sequence analysis as described under "Materials and Methods." The NH_2-terminal sequence of each fragment is shown with its apparent and calculated molecular weight. Each fragment was analyzed for the presence of the COOH terminal of IGFBP-3 by Western blot analysis with the anti-carboxy terminal peptides of IGFBP-3 monoclonal antibody (0.4 μg/ml). Because the *N*-glycosylation sites of IGFBP-3 are located at N^{89}, N^{109}, and N^{172}, each fragment contained one glycosylated arginine residue.

	Molecular weight (apparent, kDa)	NH_2-terminal sequence	N-G site[a]	Molecular weight (calculated, kDa)	COOH-terminal[b]
Fragment A[c]	26	G^1ASSGGL	1	25.3	−
Fragment B[c]	17	I^{145}IIIKKG	1	17.7	+
Fragment C[c]	15.5 (Fragment C1)	G^1ASSGGL	1	16.3	
	15.5 (Fragment C2)	L^{96}RXXLLP	1 or 2	Unknown	−

[a] N-G site = *N*-linked glycosylation site.
[b] COOH-terminal = presence (+) or absence (−) of COOH terminal of IGFBP-3.
[c] The letters correspond to the cleavage products represented in Fig. 2.

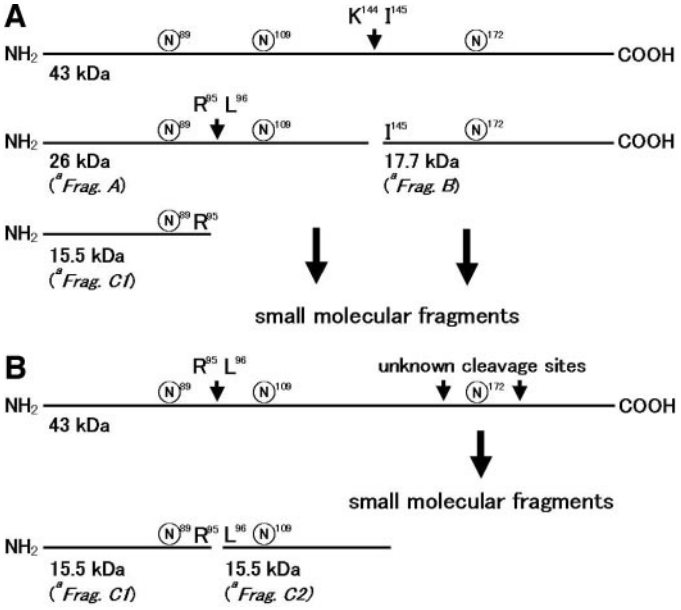

Fig. 4. Schematic diagram of the insulin-like growth factor binding protein 3 cleavage pathway for matrix metalloproteinase-7. *A*, the degradation time course suggested that the first cleavage site was a K-I bond (K^{144}-I^{145}), producing Fragment A and Fragment B, and that Fragment A was cleaved additionally at R^{95}-L^{96}, producing Fragment C1. *B*, the molecular weight of Fragment C2 on SDS-PAGE (*15.5 kDa*) suggested the presence of at least one unknown cleavage site on the COOH-terminal side. *a*, the letters correspond to the cleavage products in Table 1.

quadrant R2 in Fig. 6 (Annexin-positive/propidium iodide-negative cells). Thirty-five percent of cells were detected in quadrant R2 after the induction of apoptosis (Fig. 6*B*). This population was reduced to 9.3% by addition of 10 ng/ml IGF-I (Fig. 6*C*), and addition of 1 μg/ml IGFBP-3 inhibited this survival effect (Fig. 6*D*; quadrant R2 = 24%). The antiapoptotic effect of IGF-I was restored fully by 1 μg/ml MMP-7 through its IGFBP-3 proteinase activity (Fig. 6*E*; quadrant R2 = 9.3%). This protective effect of IGF-I against anoikis was inhibited partially by a phosphatidylinositol 3′-kinase inhibitor (20 μM LY294002) and a mitogen-activated protein kinase kinase inhibitor (50 μM PD98059; data not shown).

DISCUSSION

MMP-7 is considered generally to degrade extracellular matrix macromolecules, but it has been reported to act on substrates other than matrix components, such as α1-antitrypsin/α1-proteinase inhibitor (32, 33), tumor necrosis factor-α (34), pro-urokinase plasminogen activator-1 and urokinase plasminogen activator-1 (35), myelin basic protein (36), Fas ligand (37), E-cadherin (38), osteopontin (39), and connective tissue growth factor (40). Cleavage of some of these proteins by MMP-7 may be involved in modulating cell behavior.

We performed the cleavage assay at substrate:enzyme ratios of 8:1 to 1:1 (MMP-7, 1.25–10 μg/ml; IGFBP-3, 10 μg/ml). To predict the concentration of active MMP-7 around the tumor, we tried *in situ* carboxymethyl transferrin zymography using human colorectal cancers. According to the sensitivity of this method, concentration of carboxymethyl transferrin proteinase including MMP-7 around the tumor is considered to be $>$0.1 μM ($\geq$2.0 μg/ml of MMP-7). Conversely, serum concentration of IGFBP-3 is $\sim$3.0 μg/ml; therefore, we believe that the MMP-7:IGFBP-3 ratio we used in the present study is in line with the *in vivo* condition, although some problems remain about the substrate specificity of carboxymethyl transferrin for MMP-7.

The IGF-I/IGFBP-3 mixture was preincubated with MMP-7 *in vitro* in our assay because COLO201 and HT29 expressed high levels of TIMP-1 and -2 (data not shown). Physiologically, MMPs are secreted generally in an inactive form (pro-MMP) and must be processed by other proteases and overcome TIMP activity to display biological activity. Plasmin and MMP-3 have been found to activate MMP-7 by cleaving pro-MMP-7 *in vitro* (41); however, little is known about the mechanism of MMP-7 activation *in vivo*. We tried but failed to detect the IGFBP-3 proteinase activity of MMP-7 *in vivo*, although we confirmed the expression of active form of MMP-7 and IGF-IR in several human colorectal cancers using Western blot analysis (data not shown). Development of mAbs recognizing the truncated form of IGFBP and phospho-IGF-IR will be mandatory for confirming the activation of IGF signal pathway in human cancers.

Cleavage by MMP-7 shows a preference for hydrophobic residues such as Leu and Ile in the P1′ position (42), and the two major cleavage sites (K^{144}-I^{145} and R^{95}-L^{96}) we identified are consistent with this preference. The K^{144}-I^{145} bond has not been reported as a cleavage site for other proteases, but the R^{95}-L^{96} site was identified

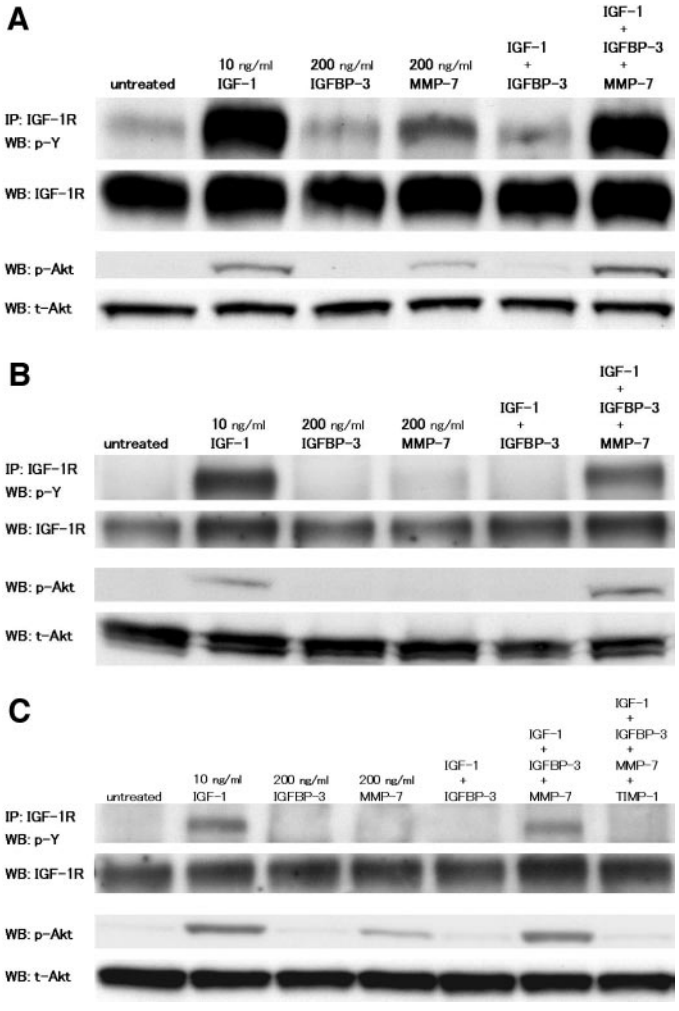

Fig. 5. Recovery of insulin-like growth factor (*IGF*) -I-mediated signal transduction through insulin-like growth factor binding protein 3 (*IGFBP-3*) degradation by matrix metalloproteinase-7 (*MMP-7*). Cell lysates from three cell lines exposed to IGF axis component molecules (IGF-I/IGFBP-3/MMP-7/tissue inhibitor of metalloproteinase-1, preincubation conditions; see "Materials and Methods") were immunoprecipitated with αIR3 monoclonal antibody (mAb) and separated by 7.5% SDS-PAGE. The blotted proteins were detected with an antiphosphotyrosine mAb. Total levels of IGF-I receptor (*IGF-IR*) are indicated in parallel. Cell lysates [20 μg in 3T3 fibroblasts overexpressing human IGF-IR (3T3-IGF-IR), 50 μg of COLO201 and HT29 per lane] were separated by 10% SDS-PAGE and analyzed for phospho-Akt levels using a phosphospecific mAb. Total levels of Akt are indicated in parallel. *p-* = phosphorylated; *t-* = total. *A*, 3T3-IGF-IR cells. *B*, COLO201 cells. *C*, HT29 cells.

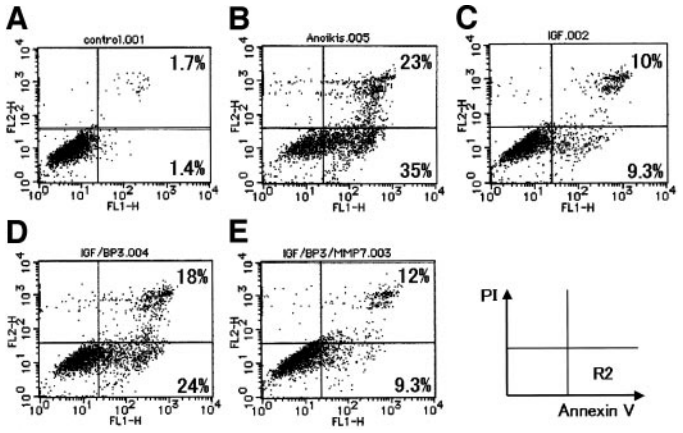

Fig. 6. Apoptosis induced by anoikis was prevented by the insulin-like growth factor (*IGF*) -I signal recovered through insulin-like growth factor binding protein 3 (IGFBP-3) degradation by matrix metalloproteinase-7 (*MMP-7*) in 3T3 fibroblasts overexpressing human IGF-IR cells. Apoptosis was induced by anoikis for 9 h in the absence of serum after 16 h of serum starvation. Early apoptotic cells were evaluated by the percentage of cells in quadrant R2 (Annexin-positive/propidium iodide-negative cells). *A*, adherent cells in DMEM with 10% fetal bovine serum; R2 = 1.4%. *B*, apoptosis induced by anoikis; R2 = 35%. *C*, anoikis with 10 ng/ml IGF-I; R2 = 9.3%. *D*, anoikis with 10 ng/ml IGF-I + 1 μg/ml IGFBP-3; R2 = 24%. *E*, anoikis with 10 ng/ml IGF-I + 1 μg/ml IGFBP-3 + 1 μg/ml MMP-7; R2 = 9.3%.

when IGFBP-3 was cleaved by plasmin (43). Our results were obtained with a glycosylated form of IGFBP-3, and the two cleavage sites were specific for IGFBP-3 and not other IGFBPs. On the basis of these findings, IGFBP-3 is a strong candidate for the physiologic substrate for MMP-7, although we did not confirm whether other IGFBPs are cleaved by MMP-7. Because MMP-7 is produced by cancer cells rather than by stromal cells, these cleavage site findings may be capable of being applied to a cancer-specific drug-targeting strategy and the development of cancer-targeted MMP inhibitors.

It is interesting whether serum from cancer patients contained IGFBP-3 cleavage products. IGFBP-3 fragments with several molecular weights have been identified in human serum, especially in the pregnant state (44), and a few reports are available about the cancer patients (45–47). If the fragment comprising residues 145–264 from specific cleavage by MMP-7 (Fragment B in the present study) can be detected in the serum, it may become a tumor marker for several human cancers.

Phosphorylation of Akt is induced by IGF-I in several tumors (28–31), suggesting a role for this growth factor as an antiapoptotic survival factor. In the tissue environment, tumor survival may depend on receptor expression on the tumor, the ligand present around the tumor, and how the tumors activate this ligand. Because IGF is a ubiquitous growth factor, whether a tumor possesses IGFBP proteinase activity and expresses the IGF-IR, it is not surprising that it uses this growth factor actively. For example, liver is the main source of endocrine IGF, and the secreted IGF forms a ternary complex with IGFBP-3 and an acid labile subunit, and circulates in an inactive form (17). Clinically, liver metastases develop frequently in MMP-7-producing cancers, especially colorectal cancer. Although the role of the IGF paracrine signal in liver metastasis has not been clarified, reports suggest that hepatocyte-derived IGF is a key molecule in metastasis to the liver (48, 49). High IGF-IR expression has been detected in liver metastasis by human colorectal cancer (50), and down-regulation of the IGF-IR, either by antisense strategies (51) or by dominant-negative mutants (52), causes loss of the metastatic phenotype. Conversely, liver metastasis by human colon cancer is inhibited by MMP-7-specific antisense oligonucleotides (53) in a nude mouse model, and the level of expression of MMP-7 is highest in the metastatic liver lesions of human colon cancers (54–56). The validity of our hypothesis is supported by these earlier reports.

IGFBP-3 is the most abundant circulating IGFBP. Recent epidemiologic studies have established a correlation between high circulating levels of IGF and low levels of IGFBP-3 and the relative risk of developing colon, breast, prostate, and lung cancer (57–61), suggesting that a systemic increase in the level of IGFBP-3 proteinase activity may contribute to cancer growth by increasing the bioavailability of IGFs in specific tissues. However, no direct causative mechanism has been established. Newell *et al.* (11) showed that MMP-7 is expressed focally in the epithelial component of benign colorectal adenomas, suggesting that it also might participate in the early stage of colorectal tumorigenesis. On the basis of this finding, Wilson *et al.* (62) confirmed that intestinal tumorigenesis in the *Min* (multiple intestinal neoplasia) mouse is prevented by the absence of MMP-7. In addition, Hassan *et al.* (63) reported that local IGF-2 supply is a modifier of intestinal adenoma growth in the *Min* mouse. These results can be better understood because of our new finding that MMP-7 can facilitate IGF bioavailability through its IGFBP-3 proteinase activity.

Anoikis is a form of apoptosis induced in cells because of loss of their adhesion to substrate. The present study failed to demonstrate an anoikis-protecting effect of IGF in cancer cell lines. This may be attributable to the low level of IGF-IR expression under conventional culture conditions. Rubini *et al.* (64) reported that 30,000 receptors per cell seem to be the minimum required for growth in soft agar. Conversely, there is an interesting report that the hypoxic and acidic conditions found within the tumor microenvironment can induce an increase in IGF-IR promoter activity (65), suggesting that IGF-IR expression can be altered by the tissue microenvironment.

In conclusion, MMP-7 functions as an IGFBP-3 proteinase. Our new findings will serve as an attractive molecular model to explain not only primary tumor growth but also organ-specific metastasis. The next step requires studies concerning the mechanism of MMP-7 activation and the regulation of IGF-IR expression *in vivo*.

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