Prometastatic Effect of N-Acetylglucosaminyltransferase V Is Due to Modification and Stabilization of Active Matriptase by Adding β1–6 GlcNAc Branching*

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Oligosaccharide moieties of glycoproteins are structurally altered during development, carcinogenesis, and malignant transformations. It is well known that β1–6 GlcNAc branching, a product of UDP-GlcNAc α-mannoside β1–6-N-acetylglucosaminyltransferase (GnT-V), is associated with malignant transformation as the results of such alterations. However, the mechanism by which β1–6 GlcNAc branching is linked to metastasis remains unclear, because the identification of specific glycoprotein(s) that are glycosylated by GnT-V and its biological function have not been examined. We herein report that matriptase, which activates both urokinase-type plasminogen activator and hepatocyte growth factor, is a target protein for GnT-V. The overexpression of GnT-V in gastric cancer cells leads to severe peritoneal dissemination in athymic mice, which can be attributed to the increased expression of matriptase. This increase was due to the acquired resistance of matriptase to degradation, since it is glycosylated by GnT-V and a corresponding increase in the active form. These results indicate that this process is a key element in malignant transformation, as the direct result of oligosaccharide modification.

N-Glycans are widely distributed on cell surfaces and secreted glycoproteins, where structural change is observed in development, carcinogenesis, and malignant transformation (1–3). Recent findings suggest that the structural changes in N-glycans are one of the critical steps for cellular transformation and are directly linked to malignant transformation. Previous studies have revealed that β1–6 GlcNAc branching on N-glycans, a product of UDP-GlcNAc α-mannoside β1–6-N-acetylglucosaminyltransferase (GnT-V; EC 2.4.1.155), is a key structure associated with tumor metastasis and malignant transformation (4–6). Since we reported on the purification and cDNA cloning of human GnT-V (7, 8), numerous studies have reported that β1–6 GlcNAc branching is associated with malignant transformation, including tumor invasion and metastasis (9–12). Gene transcription of GnT-V is regulated by proto-oncogenes such as the Ets family (13, 14), src (15) and erbB2 (16). The sequence analysis of the 5′-flanking region of GnT-V revealed the functional binding sites of the Ets family. In addition, certain transcription factors belonging to the Ets family are activated by the Ras-Raf-mitogen-activated protein kinase signaling pathway, which leads to cell proliferation and transformation (17). The Ras proto-oncogene sustains activating mutations in ~20% of all human tumors. Ras signaling is induced by other common mutations, such as the amplification of Neu/ErbB-2 in breast cancer (18). These findings suggest that elevated GnT-V activity in human tumors might commonly occur at the level of gene expression.

A recent study using GnT-V knockout mice demonstrated that the expression of GnT-V is essential for tumor growth and metastasis (12). The author reported that GnT-V stimulated membrane ruffling and phosphatidylinositol 3-kinase-protein kinase B activation. However, functional changes in specific glycoproteins that contain β1–6 GlcNAc branching have not been described in terms of tumor metastasis, and the biological significance of GnT-V appears to be different for each type of cancer. A high level of expression of GnT-V in human colorectal and breast cancer is correlated with distant or lymph node metastasis with a poor prognosis (9, 11, 19). In contrast, the expression of GnT-V is an early event in hepatocarcinogenesis, and the level of GnT-V expression in hepatoma does not correlate with the prognosis of the patient after an operation (10, 20). In fact, hepatoma cells, which express high levels of GnT-V, such as Huh7 and HepG2 cells, showed no metastasis in studies using athymic mice. This discrepancy between colon cancer and hepatoma might be due to the target glycoproteins of GnT-V. Although structural analyses of N-glycans on glycoprotein(s) that are glycosylated by GnT-V, such as integrins and LAMP-2 (lysosomal associated membrane protein 2), have been carried out, our biological knowledge of these proteins’ function by β1–6 GlcNAc branching is insufficient (6).

The present study demonstrates a novel pathway of GnT-V-mediated metastasis via the up-regulation of matriptase (21, 22), an epithelium-derived, integral membrane serine protease that activates two important cancer invasion effectors, membrane-bound activator of urokinase-type plasminogen activator and hepatocyte growth factor (HGF), on the surface of cancer
cells (23, 24). In addition, we described biochemical analyses of this protease with reference to the significance of the extent of β1–6 GlcNAc branching.

**EXPERIMENTAL PROCEDURES**

*Establishment of GnT-V-transfected MKN45 Cells—*Human gastric cancer cell line MKN45 was obtained from the Japanese Cancer Research Resource (Tokyo, Japan). Cells were cultured in RPMI 1640 medium (Nikkon Kagaku, Kyoto, Japan) containing 10% fetal bovine serum (Invitrogen) and antibiotics. A human GnT-V cDNA (8) was inserted into a mammalian expression vector pCXN, which is regulated by the β-actin promoter (25), and 5 µg of the GnT-V expression vector was transfected into MKN45 cells by LipofectAMINE (Invitrogen). Selection was performed via the addition of 500 µg/ml G418 (Sigma). Positive and negative clones (vector alone) were randomly selected. Two positive clones (GnT-V-1 and GnT-V-2) and one negative clone (mock) were used for the experiments that are described herein, but the results using other clones were very similar. Matriptase transfecteds were established in a manner similar to that for Gn-T-V transfecteds, using a pDNA3.1/Neo vector (Invitrogen) containing the matriptase cDNA sequence.

**Assay of GnT-V and Northern Blot Analysis—**GnT-V activity was assayed as previously reported (26). Briefly, pyridylaminated biantennary sugar chain and 10–20 µg of sonicated cell lysates in phosphate-buffered saline containing 0.1% SDS were electrophoresed on 10% gel and then incubated with 125 nM MEs buffer (pH 6.25). 40 mM UDP-GlcNAc, 200 mM GlnAc, 0.5% Triton X-100, 10 mM EDTA in buffer for 2 h at 37°C. Enzymatic products were analyzed by high performance liquid chromatography. Protein concentrations were determined by a bicinchoninic acid kit (Pierce) using bovine serum albumin as the standard. Total RNAs were prepared from MKN45 cells according to the method previously reported (27). Twenty µg of RNAs were electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde and transferred onto a Nitrocellulose membrane (Bio-Rad). The membrane filter was prehybridized in a hybridization buffer for 3 h at 42°C and then hybridized with a [α-32P]CTP-labeled GnT-V cDNA fragment for 12 h at 42°C in a hybridization buffer (10). The membrane filter was washed with 2× standard saline citrate, pH 7.4, and 0.1% SDS twice at 55°C and then washed with 2× standard saline citrate, pH 7.4, and 0.1% SDS twice for 30 min. The filter was then exposed to an x-ray film (Eastman Kodak Co.) with an intensified screen at ~80°C for 1 day.

**Lectin Blot—**Lectin blot analysis was performed as described previously (28). Subconfluent cultures of mock and Gn-T-V transfecteds were washed three times with PBS and incubated in serum-free RPMI 1640 for 72 h. The resultant conditioned medium was concentrated 10-fold by Centricon-30. Twenty µl of concentrated conditioned medium from mock- and Gn-T-V-transfected MKN45 cells (GnT-V transfecteds) were electrophoresed on an 8% polyacrylamide gel and then transferred onto a nitrocellulose membrane (Bio-Rad). After blocking with PBS containing 3% bovine serum albumin overnight at 4°C, the filter was incubated with 1 µg/ml biotinylated leukoagglutinin (Lc-PHA, Seikagaku Corp., Tokyo, Japan) which preferentially recognizes β1–6 branches of tri- or tetra-antennary sugar chain (29) for 1 h at room temperature. The washing and developing procedures have been described previously (28). To verify that the total proteins were equally loaded, the gel was stained with Coomassie Brilliant Blue.

**Gelatin Zymography and Inhibitor Studies—**Gelatin zymography analysis of this protease with reference to the significance of the extent of β1–6 GlcNAc branching.

**Western Blot Analysis—**Twenty µl of a 10-fold concentrated conditioned medium from mock and Gn-T-V transfecteds was electrophoresed on an 8% polyacrylamide gel, and then transferred onto a nitrocellulose membrane. After blocking with PBS containing 5% skim milk for 2 h at room temperature, the membrane filter was incubated with 1:2000 diluted anti-human matriptase, mAb 21-9 (32), for 2 h at room temperature. The filter was washed three times with Tris-buffered saline containing 0.1% SDS and then incubated with peroxidase-conjugated anti- rat IgG (Toyobo Co., Ltd., Osaka, Japan) for 1 h. After the membrane had been washed with Tris-buffered saline containing 0.05% Tween 20, it was developed by ECL (Amersham Biosciences), according to the manufacturer’s protocol.

**Matriptase Immunodepletion from MKN45-GnT-V Cell Conditioned Medium—**Fifty µl of mAb 21-9 was incubated with 100 µl of protein G-Sepharose CL-4B (Amersham Biosciences) for 2 h at 4°C with constant rotating, followed by sedimentation of the agarose beads by low speed centrifugation. The beads were washed five times with Tris-buffered saline containing 0.05% Tween 20. For immunodepletion of matriptase from GnT-V-transfected MKN45 cells, the conditioned medium was incubated overnight at 4°C with protein G-Sepharose CL-4B bound with mAb 21-9. The beads were pelleted by low speed centrifugation, and the supernatant was then concentrated 10-fold using a Centricon 30 concentrator (Millipore Corp., Bedford, MA). The concentrated supernatants were used for gelatin zymography and immunoblot analysis of matriptase.

**Flow Cytometric Analysis—**Cells in subconfluent and confluent conditions were removed from 10-cm culture dishes using PBS plus 0.2% trypsin (0.02% EDTA) and were centrifuged at 1000 rpm for 5 min. The pellets were resuspended in 200 µl of 1× trypsin inhibitor. The cells were then washed three times with 1 ml of PBS, resuspended in 100 µl of fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulins (Toyobo) diluted 1:50, and incubated for 30 min on ice. The cells were then washed twice with 1 ml of PBS, incubated with a primary antibody (mAb 21-9 diluted 1:100) for 30 min on ice. Cells were then washed three times with 1 ml of PBS, resuspended in 100 µl of fluorescein isothiocyanate-conjugated goat anti-rat immunoglobulins (Toyobo) diluted 1:50, and incubated for 30 min on ice. After washing three times, flow cytometry analyses were performed using a FACScan instrument (Becton Dickinson, Franklin Lakes, NJ) operating with CELLQuest software.

**Degradation Assay—**Cells were suspended in 100 mM Tris-HCl (pH 7.5) and 1% Triton X-100. After incubation on ice for 30 min, the solution was centrifuged at 15,000 rpm for 20 min, and the supernatants were used as cell lysates. Protein concentration was assayed by means of a BCA protein assay kit (Pierce). The cell lysates were incubated for the indicated times at 37°C, subjected to SDS-PAGE, and then transferred to nitrocellulose membrane, and analyzed by Western blot using mAb 21-9.

**Pulse-Chase Experiments—**Mock and Gn-T-V transfecteds of MKN45 cells in six-well tissue culture plates were preincubated for 2 h at 37°C with methionine- and cysteine-free medium containing 10% fetal calf serum. The cells were then washed once with fresh medium and were incubated with or without chase medium. After washing three times, flow cytometry analyses were performed using a FACScan instrument (Becton Dickinson, Franklin Lakes, NJ) operating with CELLQuest software.
Cancer Metastasis Mediated by GnT-V

RESULTS

Establishment of GnT-V Transfectants—To determine the molecular mechanisms of GnT-V in cancer metastasis through oligosaccharide modification, we used a gastric cancer MKN45 cell, because this cell line does not express detectable levels of GnT-V. We established two positive clones of MKN45 cells lines that express high levels of GnT-V (GnT-V transfectants) and one negative clone (mock transfectant). Levels of other glycosyltransferases, such as N-acetylgalactosaminyltransferase III and α1–6 fucosyltransferase were not affected by GnT-V gene transfection (Table I). Northern blot analysis showed a high expression of GnT-V mRNA in the GnT-V transfectants, which is consistent with the observed protein expression (Fig. 1, a and b), indicating that the high levels of GnT-V activity in MKN45-GnT-V-1 and -GnT-V-2 were due to the high transcriptional level.

\[ L_4 \text{-PHA Lectin Blot} - \] To better understand the change in oligosaccharide structure in GnT-V transfectants, lectin blotting was performed using \( L_4 \)-PHA, which binds preferentially to GlcNAc residues on β1–6 branches of tri- or tetra-antennary sugar chains (29). Secretory proteins from mock and GnT-V transfectants of MKN45 cells were highly reactive to \( L_4 \)-PHA in reducing conditions. Under reducing condition, numerous bands of 40–220 kDa in molecular mass were strongly stained with \( L_4 \)-PHA (Fig. 1c, left panel). SDS-polyacrylamide gel electrophoresis analysis showed no changes in total secretory proteins from mock and GnT-V transfectants (Fig. 1c, right panel). These results suggest that the overexpression of GnT-V in MKN45 cells leads to an increase in the frequency of β1–6 branches on N-glycans of their glycoproteins.

Experimental Metastasis—To evaluate the metastatic potential of GnT-V transfectants, we investigated tumor formation in the various organs after injection into the peritoneum of athymic nude mice. A marked dissemination of metastatic cancer cells was observed in the GnT-V transfectants compared with parental cells or negative transfectants (mock). The incidence of tumor metastasis in liver and lymph nodes was significantly higher in the GnT-V transfectants than that in parent or mock cells. The expression of GnT-V mRNA in these metastatic lesions was dramatically increased (Fig. 2b). All data relating to experimental metastasis are summarized in Table II. These results suggest that glycoprotein(s) containing β1–6 GlcNAc branching might be involved in cancer metastasis to visceral organs and lymph nodes.

Analysis of Proteases Secreted by GnT-V-transfected MKN45 Cells—To understand the mechanisms underlying the increases in the metastatic potential of GnT-V transfectants, the gelatinolytic activity in the conditioned medium of mock and GnT-V transfectants was assayed by gelatin zymography (Fig. 3). Gelatin zymography revealed an increase in the gelatinase activity of an ~80-kDa protease in the GnT-V transfectants, which was not observed in the mock transfectants (Fig. 3a). The intensity of this band of GnT-V transfectants was significantly higher than that of Mock-transfected MKN45 cells. To characterize this protease of 80 kDa, the gelatin gel was incubated with various types of protease inhibitors. The gelatinolytic activities of all proteases were eliminated by the addition of EDTA, an inhibitor of metal-dependent proteases (Fig. 3b), suggesting that these proteases require metal ions in their activity. Matrix metalloproteinase-specific inhibitor BE16627B (31) failed to inhibit the activity of the 80-kDa protease. However, the gelatinolytic activities of the ~92- and 72-kDa pro-

| Table I | Enzymatic activities of GnT-V, N-acetylgalactosaminyltransferase III (GnT-III), and α1–6 fucosyltransferase (α1–6 Fuc-T) in GnT-V gene-transfected MKN45 cells |
|--------|--------------------------------------------------|
| Cells  | GnT-V | GnT-III | α1-6Fuc-T |
| Parent | ND    | ND      | 384 ± 8   |
| Mock   | ND    | ND      | 398 ± 12  |
| MKN45-GnT-V-1 | 592 ± 12 | ND | 409 ± 14 |
| MKN45-GnT-V-2 | 891 ± 29 | ND | 374 ± 11 |

FIG. 1. Establishment of GnT-V transfectants of MKN45 cells. a, Northern blot analysis of GnT-V-transfected MKN45 cells. Twenty μg of total RNAs extracted from parental MKN45 cells (lane 1), mock transfectants (lane 2), and GnT-V transfectants MKN45-GnT-V1 (lane 3) and MKN45-GnT-V2 (lane 4) were electrophoresed on 1.0% agarose gel containing formaldehyde and then analyzed by Northern blot hybridization using 32P-labeled GnT-V cDNA (top). Comparable amounts of RNAs were confirmed by ethidium bromide staining (bottom). b, Western blot of GnT-V. Twenty μg of total cellular proteins were electrophoresed on SDS-PAGE. After blotting onto a nitrocellulose filter, Western blot analysis was performed using anti-GnT-V antibody. c, L4-PHA lectin blot analysis of the conditioned media from mock (lane 1) and GnT-V transfectants (lane 2). Detailed procedures are described under “Experimental Procedures.” Right, Coomassie Brilliant Blue staining of gels to show comparable amounts of proteins in each lane.
teins in the conditioned medium were completely inhibited by BE16627B (Fig. 3c). These results strongly suggest that the 92- and 72-kDa proteases correspond to matrix metalloproteinase 9 and 2, respectively. Treatment with pepstatin, an inhibitor of aspartate protease, had no detectable effects on the gelatinolytic activities of the 80-kDa protease (data not shown). The proteolytic activity of the 80-kDa protease in the conditioned medium was completely blocked by aprotinin, a serine protease inhibitor (Fig. 3d). These results indicate that the 80-kDa protease is a metal-dependent serine protease.

Characterization of a Serine Protease Observed in the Conditioned Media of GnT-V Transfectants—The results of gelatin zymography suggest that an 80-kDa protease from the GnT-V transfectants of MKN45 cells was a divalent cation-dependent serine protease. A search of the literature for such a protease indicated that it is very similar to a protease secreted from a human breast cancer cell, T47D (30). This protease was purified and identified as matriptase (21) and independently cloned as the membrane-type serine protease-1 by another group (22). Matriptase is a type II, integral membrane, trypsin-like serine protease, which may be involved in tissue remodeling, cell growth, and cancer metastasis (23, 24). Western blot analysis using the anti-matriptase antibody mAb 21-9 showed dramatic increases in both the cleaved 80-kDa (noncomplexed) and 95- and 110-kDa (complexed) forms of matriptase with fragments of hepatocyte growth factor activator inhibitor-1 (32, 33) in the conditioned media of GnT-V transfectants, compared with that of mock cells (Fig. 4a). In contrast, the expression of matriptase in the total cellular proteins was nearly equivalent for the GnT-V and mock transfectants. Three forms of matriptase were observed in the cell lysate, including cleaved (noncomplexed) 80-kDa, full-length 90-kDa, and 125-kDa forms, complexed with the 55-kDa full-length hepatocyte growth factor activator inhibitor-1. Interestingly, the activated form of matriptase-hepatocyte growth factor activator inhibitor-1 complexes was

### Table II

| Cells         | Liver | Lymph node |
|---------------|-------|------------|
| Parent        | 0/4   | 1/4        |
| Mock          | 0/12  | 4/12       |
| Clones 1 and 2| 0/7, 0/5 | 2/7, 2/5   |
| MKN45-GnT-V   | 8/13a,b | 13/13a,b   |
| Clones 1 and 2| 4/6, 4/7 | 6/6, 7/7   |
| MKN45-matriptase| 2/10c | 8/10c      |
| Clones 1 and 2| 1/5, 1/5 | 4/5, 4/5   |

* p < 0.005, compared with mock (clones 1 and 2) by χ² test.
* * p < 0.05, compared with MKN45 matriptase (clones 1 and 2) by χ² test.
* * Not significant compared with mock (clones 1 and 2) by χ² test.
* * d p < 0.03, compared with mock (clones 1 and 2), by χ² test.

Figure 2: Experimental metastasis assay in athymic mice. a, 1 × 10⁶ cells of mock and GnT-V transfectants were injected into the peritoneum of athymic mice. After 1 month, the mice were sacrificed, and tumor formations were examined. The arrowheads indicate metastatic lesion of the tumor. b, 20 μg of total RNAs extracted from metastasis of lymph node in mock (lane 1) and GnT-V transfectants (lane 2) were analyzed by Northern blot hybridization (top). Comparable amounts of RNAs were confirmed by ethidium bromide staining (bottom).

Figure 3: Gelatin zymography of proteases secreted from mock transfectants and GnT-V transfectants. Twenty μl of a 10-fold concentrated conditioned medium from mock transfectants (lane 1) and GnT-V transfectants (lane 2) was subjected to gelatin zymography. After SDS-PAGE, the gel was incubated with different protease inhibitors as described under “Experimental Procedures.” a, control; b, indicates treatment with 10 mM EDTA; c, treatment with 10 μM of BE16627B, matrix metalloproteinase inhibitor; d, treatment with 10 μM aprotinin. After overnight incubation at pH 7.5, proteolytic activities were visualized by Coomassie Brilliant Blue staining.
and Western blot analysis using mAb 21-9 (lane 2), or with mAb 21-9-conjugated protein A-Sepharose complex (lane 3). After low speed centrifugation, the supernatant was subjected to gelatin zymography (c) and Western blot analysis using mAb 21-9 (d).

FIG. 4. Identification of a protease in the conditioned media from GnT-V transfectants. a, Western blot analysis of matriptase in conditioned media (CM) and cell lysates. Lanes 1 and 3 indicate mock transfectants. Lanes 2 and 4 indicate GnT-V transfectants. b, Northern blot analysis of matriptase on mock (lane 1) and GnT-V transfectants (lane 2). c and d, immunodepletion of matriptase. Lane 1 indicates no treatment. Conditioned medium from GnT-V transfectants was incubated with protein A-Sepharose alone (lane 2), or with mAb 21-9-conjugated protein A-Sepharose complex (lane 3). After low speed centrifugation, the supernatant was subjected to gelatin zymography (c) and Western blot analysis using mAb 21-9 (d).

observed only in the conditioned media of GnT-V transfectants, as evidenced by Western blot using anti-(total) matriptase mAb 21-9 (Fig. 4a) or anti-two-chain matriptase mAb M69 (data not shown). The mRNA expression of matriptase for the GnT-V and mock transfectants remained unchanged (Fig. 4b). In order to investigate the issue of whether or not the enhanced the protease expression in the conditioned media of GnT-V transfectants was, in fact, due to matriptase, we immunodepleted matriptase using the anti-matriptase antibody, mAb 21-9. The gelatinolytic activity of matriptase disappeared after immunodepletion (Fig. 4c). The immunodepletion of matriptase in cell conditioned media was confirmed by Western blot (Fig. 4d). These results indicate that the 80-kDa protease secreted from GnT-V transfectants of MKX45 cells was, in fact, matriptase.

To investigate matriptase activity per se between GnT-V transfectant and mock, gelatinolytic activity of these cells was evaluated after correction by the density of matriptase bands of Western blot. However, the relative gelatinolytic activity of matriptase on the basis of staining intensity of Western blot was almost the same between GnT-V transfectant and mock cells. Therefore, the apparent specific activity would not be too different. We have a plan to purify matriptases from the medium of GnT-V transfectant and mock cells and compare their gelatinolytic activity and their extent of degradation.

FIG. 5. Detection of β1–6 GlcNAc branching on matriptase. L4-PHA precipitation followed by Western blot of matriptase. Both pellets of L4-PHA precipitation (lane 1, mock transfectant; lane 2, GnT-V transfectant) were incubated in 1× SDS sample buffer in the absence of reducing agents at room temperature (−boiling) or 95 °C (+ boiling) for 5 min prior to SDS-PAGE and then subjected to Western blot analysis using mAb 21-9.

Attachment of β1–6 GlcNAc Branching to N-Glycan of Matriptase—Northern blot analysis showed that the expression of matriptase mRNA in the mock and GnT-V transfectants remained unchanged, suggesting that enhanced expression of matriptase did not occur at the transcriptional level. We next investigated the attachment of β1–6 GlcNAc branching oligosaccharides to matriptase in the GnT-V transfectants.

Matriptase contains four potential sites for Asn-linked oligosaccharides (22). To determine the addition of β1–6 branching on the oligosaccharides of matriptase, L4-PHA precipitation followed by Western blot of matriptase revealed the strong binding of matriptase to L4-PHA lectin precipitation pellets in GnT-V transfectants (Fig. 5). The result indicates that N-glycans of matriptase were glycosylated by GnT-V.

Molecular Mechanism Underlying the Enhanced Matriptase Expression in GnT-V Transfectants—Flow cytometric analysis indicated almost the same level of matriptase expression on the cell surface of GnT-V transfectants, when the cells were grown under subconfluent conditions, compared with control cells. When grown under confluent conditions, however, the expression of matriptase on the cell surface was higher than that of control cells (Fig. 6a) despite no change in mRNA expression of matriptase (data not shown). Western blot of matriptase also showed the similar results of flow cytometric analysis (Fig. 6b). To determine the reason for this discrepancy, the degradation of matriptase in a cell lysate was analyzed. As expected, the degradation of matriptase was dramatically delayed in cell lysates in 100 mM Tris-HCl (pH 7.5) and 1% Triton X-100 buffer from GnT-V transfectants. Even after 300 min, ~80% of the matriptase was not degraded in the case of GnT-V transfectants (Fig. 7a). Degradation resistance of matriptase was also observed in GnT-V-transfected colon cancer cells, WiDr (Fig. 7b) and DLD (data not shown). The addition of β1–6 GlcNAc branching on matriptase was also confirmed by L4-PHA precipitation (data not shown).

Interestingly, the matriptase bands that were resistant to degradation coincided with those containing β1–6 GlcNAc branching. When a lysis buffer containing EDTA was used in this assay, no difference between mock and GnT-V transfectants was observed, and incubation for 30 min resulted in the complete disappearance of the matriptase band (data not shown). Pulse-chase studies of matriptase showed that the half-life of matriptase was not changed in total cell lysates but was markedly prolonged in the conditioned media of GnT-V.
Fig. 6. Increases in the cell surface expression of matriptase in confluent conditions. a, flow cytometry analysis of matriptase was performed as described under “Experimental Procedures.” The shaded histogram indicates the autofluorescence of cells (no first antibody), the unbroken line indicates mock transfectant, and the broken line indicates GnT-V transfectant. b, Western blot of matriptase was performed in the same condition of flow cytometry. Lane 1, mock transfectant; lane 2, GnT-V transfectant.
transfectants (Fig. 7c). When we investigated the time-dependent accumulation of matriptase in the conditioned media, an additional accumulation of matriptase in the cell culture of the GnT-V transfectants was observed after 60 h of culture (Fig. 8). These results strongly suggest that the up-regulation of matriptase in the GnT-V transfectants is due to the resistance of matriptase to degradation, as a result of \( ^{-} H^{1} \text{Nac}^{6} \) GlcNAc branching.

**Effect of Elevated Expression of Matriptase on Tumor Metastasis**—To investigate the issue of whether or not the elevated expression of matriptase is directly involved in tumor metastasis, we established matriptase transfectants of MKN45 cells, using the same method that was used for the GnT-V transfectants. These cells secreted high levels of matriptase into the conditioned media, including the active form (data not shown). When matriptase transfectants were injected peritoneally into athymic mice, an increase in metastasis to the lymph nodes was observed, similar to the data obtained for the GnT-V transfectants (Table II). However, matriptase transfectants did not promote liver metastasis, which was observed in GnT-V transfectants.

**DISCUSSION**

While increases in \( ^{-} H^{1} \text{Nac}^{6} \) GlcNAc branching on glycoproteins were observed by GnT-V gene transfection, key molecules directly linked to tumor metastasis seem to be limited. The present study demonstrated the identification of such key molecules in cancer metastasis mediated by GnT-V. We found that the addition of \( ^{-} H^{1} \text{Nac}^{6} \) GlcNAc branching on matriptase inhibited its degradation, resulting in the up-regulation of matriptase expression in the conditioned media and on the cell surface despite the fact that no changes in matriptase mRNA expression were observed. Treatment with EDTA caused instability in matriptase, which contains a \( ^{2+} \text{Ca} \) binding site in both the low density lipoprotein receptor domain and CUB domain and shows no difference of degradation in cell lysates between mock and GnT-V transfectants. Whereas a protease that degrades matriptase has not yet been identified, these data suggest no change in matriptase degradation proteases by GnT-V gene transfection. Moreover, this increase in matriptase expression is directly linked to tumor metastasis, because the overexpres-
sion of matriptase in gastric cancer cells led to lymph node metastasis in athymic mice. Recently, a large-peptide inhibitor of matriptase, ecotin (34), has been shown to retard the growth of PC-3 prostate tumors in nude mice (22). These data suggest that matriptase might be a central regulator of cell migration and cancer invasion.

We investigated the co-localization of immunohistochemical staining of GnT-V and matriptase using human colorectal cancer tissues. Matriptase was found to be expressed in most cancer tissues as well as in the surrounding normal epithelial tissues. Positive staining for GnT-V was observed in eight cases of 19 cancer tissues. A dramatic increase in matriptase staining in a limited area of tumors was observed in three cases of colon cancer and metastatic lymph nodes, and all of these cases showed positive staining for GnT-V. Therefore, the results observed in the in vitro experiments vis à vis matriptase stability may explain the increased immunostaining of matriptase that is observed in human colon cancers.

The expression of GnT-V was correlated with tumor metastasis or poor prognosis in colon cancers (9, 11, 19) and the mammary gland (35), which show a high expression of matriptase, but not in cancers of the liver (20) and lung (2), which show no expression or very low expression of matriptase. The poor prognosis of GnT-V in certain types of cancer might have a similar profile of organs that express matriptase. Therefore, the acquired resistance to degradation, as the result of the addition of β1–6 GlcNAc branching in their cancer cells, is a more important factor in the up-regulation of matriptase, leading to tumor metastasis and malignant transformation.

Breast cancers developed in GnT-V knockout mice are quite small and show low incidences of metastases (12). These data suggest that GnT-V might be essential for tumor growth as well as metastasis. Matriptase may play an important role in cell growth via the activation of HGF. Mature HGF, activated by matriptase, activates c-Met, leading to changes in cellular proliferation, cell motility, and cell morphology in a cell type-dependent manner. It was reported that GnT-V-transfected Madin-Darby canine kidney cells showed a 5-fold increase in motility after HGF treatment (6). Because matriptase bearing β1–6 GlcNAc branching is resistant to degradation, it might serve as an activator of HGF for a much longer period of time. In addition to its gelatinolytic activity, matriptase can activate urokinase-type plasminogen activator (23, 24), which in turn activates plasminogen, leading to a proteolytic activation cascade of several extracellular matrix-degrading protease systems (36). This enhanced proliferation, cellular motility, and extracellular matrix degradation may contribute to cancer growth and metastasis (37, 38). Matriptase is also involved in the activation of protease-activated receptor-2 (24), which plays a pivotal role in cell adhesion through G protein-coupled receptors (39). While a dramatic elevation in cell growth and adhesion to the extracellular matrix was not markedly changed in the in vitro experiments using the GnT-V transfectant of MKN45 cells (data not shown), the athymic mice experiments showed that matriptase may be involved in the invasive phenotype of cancer cells, as described above.

GnT-V plays an important role in T cell activation by modification of oligosaccharides on the T cell receptor (40). In our experiments, T cell systems are not involved in GnT-V-mediated metastasis, since we used athymic mice, which are devoid of these cells. The present study outlines a novel pathway of tumor metas-

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2 S. Ihara, E. Miyoshi, K. Murata, S. Nakahara, K. Honke, R. B. Dickson, C.-Y. Lin, and N. Taniguchi, unpublished data.