Dynamic behaviors of vimentin induced by interaction with GlcNAc molecules

Kenta Komura, Hirohiko Ise1, and Toshihiro Akaike

Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan

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The cytoskeleton protein vimentin is dramatically altered following pathological events such as fibrosis and tumorigenesis. Vimentin binds to multivalent N-acetylglucosamine (GlcNAc) molecules at the cell surface and interacts with O-linked β-GlcNAc proteins. Moreover, dying cells can be engulfed by neighboring cells through surface interactions between vimentin and many O-GlcNAc proteins in cell debris. Here, we show that vimentin was altered by its interaction with GlcNAc-bearing molecules such as GlcNAc-bearing polymers. The interaction with GlcNAc-bearing polymers promoted the cell surface recruitment of vimentin followed by the phosphorylation of vimentin serine 71 and the increase in tetrameric vimentin disassembled from vimentin filaments in HeLa cells. Moreover, it was found that GlcNAc-bearing polymers and O-GlcNAc proteins from dying cells promoted vimentin expression and cell migration in the Madin–Darby canine kidney and Michigan Cancer Foundation-7 cells. These results suggest that interactions between surface vimentin and GlcNAc molecules, including the O-GlcNAc proteins from dying cells, may play a pivotal role in vimentin expression and the migration of cancer cells. We propose new mechanisms of vimentin expression in cancer cells.

Keywords: cancer cells / N-acetylglucosamine / O-GlcNAc protein / vimentin

Introduction

Vimentin is the major intermediate filament protein in mesenchymal cells and is important for the stabilization of the cellular structure and architecture. Moreover, many studies have revealed that vimentin has a number of critical functions that relate to the organization of proteins involved in adhesion, migration and cell signaling (Homan et al. 1998; Gonzales et al. 2001). In addition, the expression of vimentin is associated with malignant progression and invasion of cancer cells (Gabbiani et al. 1981, 1982; Caselitz et al. 1983). Recently, vimentin was recognized as a marker for the epithelial-mesenchymal transition (EMT) associated with several tumorigenic events (Gilles et al. 1997; Grille et al. 2003). EMT is characterized by the expression of vimentin in epithelial cells that normally express only keratin. The overexpression of vimentin has been reported in various tumor cell lines, including breast cancers, tumors of the central nervous system, malignant melanoma and hepatic cancers (Ben-Ze’ev and Raz 1985; Grille et al. 2003; Hu et al. 2004; Korschning et al. 2005; Trog et al. 2008). There is growing evidence of an association between the expression of vimentin and tumor progression. Vimentin is thought to be crucial for EMT and a hallmark of tumor invasiveness and aggressiveness (Bae et al. 1993; Gilles et al. 1997; Grille et al. 2003; Yokoyama et al. 2003). The role of vimentin in the growth and invasiveness of cancer cells has been indicated in a number of cancers. Indeed, the majority of cancers overexpress vimentin, and this overexpression has been associated with an increased migratory/invasive capacity of the cancer cells (Vuurduin et al. 2011); vimentin is thus used as an indicator of poor prognosis for cancer patients. Therefore, vimentin is an attractive potential target for cancer therapy. Moreover, several reports have shown that vimentin is expressed on the cell surface of various cancer cells (Cutrera et al. 2011; Kim et al. 2011; Steinmetz et al. 2011). Some recent studies have reported the discovery of a vimentin-binding minipeptide and virus nanoparticles for surface vimentin-targeted tumor-specific therapy (Cutrera et al. 2011). Vimentin is considered important in a variety of fundamental cellular processes; however, the physiological roles of vimentin in these processes remain elusive because the phenotypes of vimentin-deficient mice are rather mild, and these mice develop normally and are fertile (Colucci-Guyon et al. 1994). Therefore, it is important to elucidate the behaviors and molecular mechanisms of vimentin to reveal the physiological roles of this protein. Recently, we used artificial bio-mimicking glycopolymers and found that vimentin possesses N-acetylglucosamine (GlcNAc)-binding lectin-like properties at the cell surface (Ise et al. 2010). We also observed that many vimentin-expressing cells, including vascular smooth muscle cells and tumor cells, adhere to GlcNAc-bearing polymer-coated dishes. In addition, we found that the rod II domain of vimentin is localized at the cell surface and is directly bound to GlcNAc-bearing...
Fig. 1. Behavior of HeLa cells cultured on PV-GlcNAc-coated and collagen-coated dishes. (A) Number of adherent HeLa cells on the PV-GlcNAc-coated, collagen-coated and BSA-coated dishes after 4 h. The numbers of adherent cells were estimated by the methylthiazol tetrazolium assay. **P < 0.01. Results are expressed as the mean (SD) (n = 5). (B) Detection of the phosphorylation of FAK and vimentin S71 in HeLa cells cultured on PV-GlcNAc-coated and
polymers (Ise et al. 2010). Furthermore, we demonstrated that vimentin binds to O-linked β-GlcNAc (O-GlcNAc)-modified proteins as a physiological ligand and that many O-GlcNAc-modified proteins in cell debris released from dying cells interact with surface vimentin on neighboring cells (Ise et al. 2012). Our previous study suggested that the interaction between surface vimentin and O-GlcNAc-modified proteins is involved in clearing dying cells (Ise et al. 2012). These findings are expected to help identify the role of vimentin in various fundamental processes.

To elucidate the detailed physiological effects of vimentin induced by its GlcNAc-binding properties, we utilized an artificial GlcNAc-bearing polymer (poly[N-p-vinylbenzyl-2-acetoamido-2-deoxy-β-D-gluconosyl(1→4)]-2-acetoamido-2-deoxy-β-D-glucosamide] [PV-GlcNAc]). This polymer is classified as a multivalent glycoside ligand composed of GlcNAc as a hydrophilic moiety and a vinylbenzyl backbone as a hydrophobic moiety. This polymer mimics the glycoside structure of various O-GlcNAc-modified proteins and is therefore suitable for analyzing the behavior of vimentin.

In the present study, we utilized the interaction of PV-GlcNAc with HeLa cells, Madin–Darby canine kidney (MDCK) cells and Michigan Cancer Foundation-7 (MCF-7) cells to examine the dynamics of vimentin. HeLa cells strongly express vimentin and have mesenchymal properties. Therefore, we examined the phosphorylation, cell surface recruitment and polymeric structure of vimentin on the basis of the interaction between HeLa cells (transfected with vimentin mutants) and PV-GlcNAc. MDCK and MCF-7 cells have epithelial properties and therefore express E-cadherin. In addition, the expression of vimentin in these cells is remarkably altered by EMT and in vitro wound-healing systems (Gilles et al. 1999). We therefore examined whether cell migration and vimentin expression in MDCK-7 and MDCK cells are altered by interaction with PV-GlcNAc. In addition, we examined whether O-GlcNAc-modified proteins from dying cells, similar to PV-GlcNAc, induce vimentin expression and cell migration. Elucidation of the essential roles of surface vimentin based on its GlcNAc-binding properties will aid in revealing the involvement of vimentin in the malignancy and the invasion of cancer cells. Furthermore, these findings will be useful for developing new cancer-targeting therapies that use the interaction of vimentin with various GlcNAc-bearing materials (Aso et al. 2007; Ise et al. 2011; Kim et al. 2011).

**Results**

**Adhesion of HeLa cells to PV-GlcNAc-coated dishes**

Vimentin-expressing cells such as HeLa cells adhere to PV-GlcNAc-coated dishes via vimentin on the cell surface (Ise et al. 2010). We investigated what kind of molecule is stimulated by the adhesion of HeLa cells to PV-GlcNAc-coated dishes. The phosphorylation of focal adhesion kinase (FAK) and vimentin in HeLa cells cultured on PV-GlcNAc-coated and collagen-coated dishes was examined by western blot analysis. HeLa cells were observed to adhere to the PV-GlcNAc-coated dishes, similar to how HeLa cells adhere to collagen-coated dishes (Figure 1A). It was considered that the adhesion of HeLa cells to PV-GlcNAc-coated dishes was 30% lower than that of HeLa cells to collagen-coated dishes because the surface vimentin on the HeLa cells was partly degraded by trypsinization. On the PV-GlcNAc-coated dishes, the phosphorylation of vimentin serine 71 (S71) was observed, whereas that of FAK (Figure 1B) was not observed. On the collagen-coated dishes, FAK was phosphorylated, whereas vimentin S71 was not phosphorylated (Figure 1B). These results showed that vimentin S71 phosphorylation was induced by the interaction with the PV-GlcNAc-coated dishes. Next, we examined the localization of vimentin following the adhesion of the HeLa cells to the PV-GlcNAc-coated dishes by biotinylating the total cell surface proteins and immunoprecipitating the biotinylated vimentin with anti-vimentin antibodies. At 15 min of incubation, the biotinylated vimentin in the HeLa cells that adhered to the PV-GlcNAc-coated dishes was detected by western blot analysis (Figure 1C). Confocal laser scanning microscopy (CLSM) revealed that vimentin was localized to the center of the HeLa cells on the collagen-coated dishes and to the entire underside of the HeLa cells on the PV-GlcNAc-coated dishes (Figure 1D). These results indicated that the cell surface recruitment of vimentin was immediately induced by the adhesion to the PV-GlcNAc-coated dishes.

Adhesion of vimentin-knockdown HeLa cells to PV-GlcNAc-coated dishes

To determine whether the HeLa cells adhered to the PV-GlcNAc-coated dishes through vimentin directly on the cell surface, we examined the adhesion of vimentin-knockdown cells to the PV-GlcNAc-coated dishes. Vimentin-knockdown and negative-knockdown HeLa cells were produced by transfection of vimentin small hairpin RNA (shRNA) and negative shRNA vectors. Western blot analysis revealed that the vimentin-knockdown cells expressed less vimentin than the negative-knockdown HeLa cells (mock cells; Figure 1E). The cells were incubated on PV-GlcNAc-coated and collagen-coated dishes for 4 h. The adhesion of the vimentin-knockdown HeLa cells to the PV-GlcNAc-coated dishes was significantly lower than that of the mock cells and normal HeLa cells (Figure 1E).

Next, the adhesion of these cells to the collagen-coated dishes was examined. It was observed that the down-regulation of vimentin induced a decreased adhesion to the collagen-coated dishes. Because vimentin plays a key role in adhesion by regulating integrin functions, vimentin-knockdown cells do not adhere to collagen-coated dishes (Tsuruta and Jones 2003;...
Adhesion of vimentin-mutated HeLa cells to PV-GlcNAc-coated dishes

The rod II domain of vimentin is a GlcNAc-binding site, and E382 and E396 within this site are important for the interaction with PV-GlcNAc (Ise et al. 2010). Therefore, we examined the architecture of vimentin possessing point mutations (E374A, E375A, E382A, D385A, D394A and E396A) in HeLa cells. The mutated vimentin was fused to the Azami green (AG) protein and transfected into HeLa cells. Western blot analysis confirmed that the proteins were normally expressed (Figure 2B). CLSM showed that vimentins containing the E374A, E375A, E382A, D385A and D394A mutations each formed a filamentous structure in the transfected HeLa cells. However, vimentin E396A aggregated without forming a filamentous structure. Moreover, to examine the structure of the AG-vimentin mutants, embryonic fibroblasts derived from vimentin-knockout mice [vimentin-ko mouse embryonic fibroblasts (MEFs)] were transfected with these mutants. The vimentin-ko MEFs expressed desmin but not vimentin (Supplementary data, Figure S1A) and therefore adhered to both the PV-GlcNAc-coated and collagen-coated dishes (Supplementary data, Figure S1B). These mutant points were normally expressed in the vimentin-ko MEFs (Supplementary data, Figure S1C), as observed with the HeLa cells. The E396A mutant aggregated without forming a filamentous structure in the vimentin-ko MEFs (Supplementary data, Figure S1D).

Next, to examine whether the HeLa cells that expressed the mutated vimentin could adhere to the PV-GlcNAc-coated dishes, vimentin-knockdown HeLa cells were transfected with the vimentin point mutants. Because the vimentin-knockdown HeLa cells expressed green fluorescent protein (GFP), DsRed-fused vimentin mutants were transfected into the vimentin-knockdown HeLa cells. Western blot analysis confirmed that these mutants were normally expressed in the vimentin-knockdown HeLa cells (Figure 2C). The vimentin-knockdown HeLa cells were transfected with the DsRed mutants and the DsRed-vimentin (wild-type) were then cultured on the PV-GlcNAc-coated and collagen-coated dishes for 4 h. The wild-type-transfected cells adhered to the PV-GlcNAc and collagen-coated dishes, whereas the expression of the E382A and E396A mutants did not (Figure 2D, left). It was assumed that these point mutants did not influence the recruitment of integrin to the cell surface. Recombinant vimentin rod II domains containing the E382A or E396A point mutations are incapable of binding to PV-GlcNAc (Ise et al. 2010). This is consistent with our results in which both the E382A and E396A vimentins lost the GlcNAc-binding property at the cell surface (Figure 2D). Interestingly, E382A vimentin but not E396A vimentin formed an intracellular filamentous structure. This suggested that the filamentous structure of vimentin might not be related to GlcNAc binding.

Behavior of vimentin following the addition of PV-GlcNAc to the medium

To investigate the behavior of vimentin following its interaction with PV-GlcNAc, we examined the phosphorylation and structure of vimentin filaments in HeLa cells cultured on collagen-coated dishes supplemented with PV-GlcNAc. The HeLa cells were incubated for 2 h with glycoside-bearing polymers (200 µg/mL) such as glucose-bearing polymers (PV-MA), galactose-bearing polymers (PV-LA) and PV-GlcNAc. Vimentin S71 was phosphorylated following the addition of PV-GlcNAc but not following the addition of the other glycoside-bearing polymers (Figure 3A). It was observed that HeLa cells could adhere to the PV-GlcNAc-coated dishes at both 37 and 4°C; however, HeLa cells could adhere to the PV-MA-coated dishes at 37°C but not at 4°C (Supplementary data, Figure S4A). These results suggested that the HeLa cells interacted with PV-MA through pathways that are independent of the interaction of vimentin with PV-GlcNAc. In addition, we determined that >50 µg/mL of PV-GlcNAc induced the phosphorylation of vimentin S71 (Figure 3B); this phosphorylation was detected 15 min after the addition of PV-GlcNAc and then gradually increased for 2 h (Figure 3C).

Next, the cell surface recruitment of vimentin following the addition of PV-GlcNAc was examined by the biotinylation of surface proteins. Thirty minutes after the addition of PV-GlcNAc, biotinylated vimentin was detected. These results indicated that the cell surface localization of vimentin was promoted by the addition of PV-GlcNAc (Figure 3D). The behavior of vimentin after the addition of PV-GlcNAc was examined by immunocytchemistry. CLSM revealed that vimentin was expressed in the peripheral area of the HeLa cells and that the vimentin filaments were slightly disassembled at 1 and 2 h after the addition of PV-GlcNAc (200 µg/mL; Figure 3E). Similar results were obtained with HeLa cells on PV-GlcNAc-coated dishes (Figure 1D). The disassembly of the vimentin filaments is likely due to the presence of PV-GlcNAc. It has been reported that vimentin can exist in cells in both a detergent-soluble form (disassembled vimentin) and a detergent-insoluble form (vimentin filaments; Eriksson et al. 2004; Helfand et al. 2011). Therefore, the polymeric structure of vimentin in the detergent-soluble and detergent-insoluble lysate fractions was analyzed by blue native polyacrylamide gel electrophoresis (BN-PAGE) and western blot analysis. In the detergent-soluble fraction, only tetrameric vimentin was observed, whereas in the detergent-insoluble fraction, large vimentin filaments were detected. Moreover, the addition of PV-GlcNAc augmented the formation of tetrameric vimentin in the detergent-soluble fraction (Figure 3F). Ligand blotting with fluorescein isothiocyanate-conjugated PV-GlcNAc (FITC-PV-GlcNAc) was
Fig. 2. Architecture of point-mutated vimentin in HeLa cells and adhesion of these cells to PV-GlcNAc-coated and collagen-coated dishes. (A) Detection of various point-mutated AG-vimentins (E374A, E375A, E382A, D385A, D394A and E396A) transfected into HeLa cells. (B) Fluorescent images of the point-mutated AG-vimentin-expressing HeLa cells. The cell nucleus was stained with DAPI (blue). Scale bar = 5 µm. (C) Detection of point-mutated DsRed-vimentins (E374A, E375A, E382A, D385A, D394A and E396A) expression in vimentin-knockdown HeLa cells. (D) Percentage of HeLa cells adhered to the PV-GlcNAc-coated and collagen-coated dishes after 4 h. The percentages were estimated from the number of adherent cells relative to the number of adherent vimentin-knockdown HeLa cells transfected with DsRed wild-type vimentin. **P < 0.01. Results are expressed as the mean (SD) (n = 5).
used to investigate which tetrameric or filamentous form of vimentin bound to GlcNAc. Ligand blotting studies showed tetrameric vimentin in the detergent-soluble fraction that possessed GlcNAc-binding properties (Figure 3G). This ligand blotting was performed in the same fractions analyzed in Figure 3F. Next, by using surface biotinylation, we examined whether the cell surface-localized vimentin was in the tetrameric or filamentous form. The western blot analysis showed that the biotinylated vimentin was only detected in the detergent-soluble fraction (Figure 3H). Therefore, the disassembly of vimentin was promoted by the presence of PV-GlcNAc, and the augmented tetrameric vimentin in the detergent-soluble
fraction was recruited to the cell surface, where it was capable of binding GlcNAc.

The role of Rho-associated kinase and protein kinase C in the phosphorylation of vimentin S71 and its interaction with PV-GlcNAc

The phosphorylation of vimentin S71 was observed following the incubation of the HeLa cells with PV-GlcNAc (Figure 3). Vimentin S71 is phosphorylated by Rho-associated kinase (ROCK; Goto et al. 1998; Izawa and Inagaki 2006), and we therefore used a ROCK inhibitor [1-(5-isoquinoline sulfonyl) homopiperazine dihydrochloride, HA-1077] to examine the behavior of vimentin as well as the adhesion of HA-1077-treated HeLa cells to PV-GlcNAc-coated dishes. It has been reported that the cell surface recruitment of vimentin is regulated in a protein kinase C (PKC)-dependent manner (Mor-Vaknin et al. 2003; Ise et al. 2010). Therefore, the effect of PKC on the adhesion of the HeLa cells to the PV-GlcNAc-coated dishes was also examined by the inhibition of PKC with GF109203X. At 2 h after the addition of PV-GlcNAc to the HA-1077-treated or GF109203X-treated HeLa cells, the phosphorylation of vimentin S71 was examined by western blot analysis. The phosphorylation of vimentin S71 by PV-GlcNAc was slightly inhibited by the treatment with HA-1077; however, these inhibitors had no effect on the phosphorylation of vimentin S55 (Figure 4A). The ROCK inhibition with HA-1077 was not complete. The ROCK inhibition of HA-1077 might not be strong and specific, and HA-1077 might inhibit some phosphatases. In the GF109203X-treated HeLa cells, the phosphorylation of vimentin S71 by PV-GlcNAc was not inhibited (Figure 4A). Surface biotinylation revealed that the cell surface recruitment of vimentin was inhibited in the HeLa cells by treatment with HA-1077 and GF109203X (Figure 4B). Next, the disassembled vimentin (tetrameric vimentin in the detergent-soluble fraction) in the inhibitor-treated HeLa cells was examined by BN-PAGE. BN-PAGE analysis showed that the augmentation of disassembled vimentin induced by PV-GlcNAc was reduced by treatment with the inhibitors (Figure 4C). In addition, the HeLa cells treated with the inhibitors adhered less to the PV-GlcNAc-coated dishes than did the non-treated HeLa cells. Comparatively, the adhesion of the HA-1077-treated HeLa cells to the collagen-coated dishes did not decrease compared with the control, but the adhesion of the GF109203X-treated HeLa cells slightly decreased (Figure 4D). Because PKC is involved in integrin-mediated cell adhesion, the inhibition might induce a decreased adhesion to the collagen-coated dishes. The down-regulation of RhoA down-regulated the function of ROCK, and we therefore generated RhoA- and PKCe-knockdown HeLa cells using RhoA and PKCe shRNA (Supplementary data, Figure S2A and B). The phosphorylation of vimentin S71, the cell surface recruitment and the interactions of PV-GlcNAc were examined in the RhoA- and PKCe-knockdown HeLa cells. The phosphorylation of vimentin S71 was suppressed in the RhoA-knockdown HeLa cells but not in the PKCe-knockdown HeLa cells (Supplementary data, Figure S2C). Surface biotinylation showed that the cell surface localization of vimentin was suppressed in both of these knockdown cells (Supplementary data, Figure S2C). Moreover, the adhesion of the knockdown HeLa cells to the PV-GlcNAc-coated dishes was inhibited, but the adhesion of these cells to the collagen-coated dishes was not inhibited (Supplementary data, Figure S2D). These results are similar to those obtained with the ROCK and PKC inhibitor-treated HeLa cells. The adhesion of the PKCe-knockdown HeLa cells to the collagen-coated dishes was not inhibited because of the function of the other PKC family members. These results demonstrate that the activation of ROCK and PKC are important in the disassembly of vimentin filaments and the recruitment of vimentin tetramers to the cell surface.

Next, we examined vimentin’s behavior in vimentin-knockdown MEFs transfected with S71A- and S71E-mutated AG-vimentin. S71E-mutated AG-vimentin was constitutively phosphorylated, whereas S71A-mutated AG-vimentin was not (Figure 5A). A point mutation from serine to glutamic acid mimics phosphorylation because the serine site is altered to a negatively charged amino acid by this point mutation. Because of this mutation, the point mutation can also be detected by anti-phosphorylated protein antibodies. CLSM showed that S71E AG-vimentin was expressed in the peripheral area of the vimentin-ko MEFs and was slightly disassembled, similar to vimentin in PV-GlcNAc-treated HeLa cells, whereas S71A AG-vimentin accumulated at the nuclear region and formed filamentous structures similar to normal HeLa cells (Figure 5B). In addition, BN-PAGE demonstrated that the AG-vimentin tetramer (the detergent-soluble fraction) was augmented in the vimentin-ko MEFs transfected with S71E AG-vimentin (Figure 5C) and that S71E AG-vimentin was constitutively disassembled. The right panel of Figure 5C indicates the same amount of total AG-vimentin expression in these cells.

Next, the cell surface recruitment of these mutated AG-vimentins was examined using surface biotinylation. S71E AG-vimentin was constitutively exposed at the cell surface without the treatment with PV-GlcNAc (Figure 5D), whereas S71A AG-vimentin was recruited to the cell surface following the treatment with PV-GlcNAc, similarly what was observed with the wild-type cells (Figure 5D). Because other serine residues of vimentin, including S38, are phosphorylated by ROCK and PKC (Ando et al. 1989; Goto et al. 1998), we presume that PV-GlcNAc induces the recruitment of S71A AG-vimentin to the cell surface. The phosphorylation of vimentin sites S71 and S38 are involved in the disassembly of vimentin filaments (Eriksson et al. 2004; Helfand et al. 2011). Our results demonstrated that PV-GlcNAc induced the phosphorylation of vimentin S71 by ROCK, followed by the disassembly and cell surface recruitment of vimentin. These finding suggest that the disassembly of vimentin filaments (tetrameric vimentin) is important for the recruitment of vimentin to the cell surface and its ability to interact with PV-GlcNAc.

Behavior of vimentin following the addition of PV-GlcNAc to MDCK and MCF-7 cells

MDCK and MCF-7 cells express vimentin by EMT, and the expression of vimentin enhances the migration and invasion capabilities of these cell lines (Korsching et al. 2005; Cufí et al. 2010). Because these cells express a small amount of
Fig. 4. Structure of vimentin and PV-GlcNAc recognition of HeLa cells treated with ROCK and PKC inhibitors following PV-GlcNAc treatment. (A) Detection of vimentin phosphorylated at S71 or S55 in ROCK and PKC inhibitor-treated HeLa cells following 200 µg/mL of PV-GlcNAc treatment. (B) Detection of the cell surface localization of vimentin in ROCK or PKC inhibitor-treated HeLa cells following 200 µg/mL of PV-GlcNAc treatment using surface biotinylation. (C) BN-PAGE and western blot analysis of the polymeric structure of vimentin complexes in the detergent-soluble and detergent-insoluble fractions of the inhibitor-treated HeLa cells following 200 µg/mL of PV-GlcNAc treatment. Upper panel: immunoblot with anti-vimentin antibodies. Middle panel: CBB staining. Bottom panel: total vimentin and β-actin expression in the HeLa cells. (D) Percentage of inhibitor-treated HeLa cells adhered to PV-GlcNAc-coated and collagen-coated dishes after 4 h. The percentages were calculated from the number of adherent treated cells relative to the number of adherent normal HeLa cells. **P < 0.01. Results are expressed as the mean (SD) (n = 5).
Fig. 5. Architecture of S71E-AG-vimentin and S71A-AG-vimentin and surface localization in the vimentin-ko MEFs expressing these mutants following PV-GlcNAc treatment. (A) Constitutive phosphorylation of S71E-mutated and S71A-mutated AG-vimentin in the vimentin-ko MEFs expressing the mutants. (B) CLSM images of vimentin-ko MEFs that express S71E-AG-vimentin and S71A-AG-vimentin (green). The cell nucleus was stained with DAPI (blue). Scale bar = 50 µm. (C) BN-PAGE and western blot analysis of the polymeric structures of S71E-mutated and S71A-mutated vimentin complexes in the detergent-soluble and detergent-insoluble fractions of the vimentin-ko MEFs expressing the mutants (left panel) as well as the total AG-vimentin expression in the mutants (right panel). (D) Detection of the cell surface localization of AG-vimentin in the mutant vimentin-ko MEFs subjected to 200 µg/mL of PV-GlcNAc treatment using surface biotinylation.
Fig. 6. Expression of vimentin and cell migration of the MCF-7 cells following PV-GlcNAc treatment. (A) Detection of vimentin expression in the MCF-7 cells with various glycoside-bearing polymers (200 µg/mL). (B) Detection of vimentin expression in the MCF-7 cells exposed to 200 µg/mL of PV-GlcNAc. (C) Detection of vimentin, Snail and Slug mRNA expression in the 200 µg/mL of PV-GlcNAc-treated MCF-7 cells using real-time RT–PCR. Results are expressed as the mean (SD) (n = 5). (D) CLSM images of immunostaining with anti-vimentin antibodies (green) and anti-E-cadherin antibodies (red) of the 200 µg/mL of PV-GlcNAc-treated MCF-7 cells. The cell nucleus was stained with DAPI (blue). Scale bar = 20 µm. (E) BN-PAGE and western blot analysis of the polymeric structure of the vimentin complexes in the detergent-soluble and detergent-insoluble fractions of the 200 µg/mL of PV-GlcNAc-treated MCF-7 cells.

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vimentin (Ise et al. 2010), they may be able to interact with PV-GlcNAc. Therefore, we examined whether the interactions of PV-GlcNAc with vimentin regulate the expression of vimentin in epithelial-like cells such as MDCK and MCF-7 cells. The MCF-7 cells were incubated with PV-MA (200 μg/mL) and PV-GlcNAc (200 μg/mL) for 24 h, and the expression of vimentin and E-cadherin was then examined. Our results revealed that the expression of vimentin was enhanced by incubation with PV-GlcNAc but not PV-MA (Figure 6A). Next, the time dependency of vimentin expression induced by PV-GlcNAc was examined by western blot analysis and real-time polymerase chain reaction (PCR). Vimentin was observed at 24 h (Figure 6B), and vimentin mRNA was observed at 12 h (Figure 6C). In addition, the vimentin expression was assumed to be regulated by EMT because it increased in the presence of PV-GlcNAc. Therefore, the expression of E-cadherin and the EMT-regulating transcription factors Snail and Slug was examined by western blot analysis and real-time PCR. However, the E-cadherin protein expression and the Snail and Slug mRNA expression were unaltered by the presence of PV-GlcNAc (Figure 6B and C). Immunocytochemistry indicated that the vimentin induced by PV-GlcNAc was not filamentous (Figure 6D). Indeed, BN-PAGE revealed that vimentin was almost completely tetrameric and not filamentous (Figure 6E).

The expression of vimentin enhances cell migration and invasion (Gilles et al. 1999). Therefore, we examined the migration of the MCF-7 cells following the addition of PV-GlcNAc using an in vitro wound-healing model. The migration of the MCF-7 cells was enhanced by PV-GlcNAc in a dose-dependent manner (Figure 6F). The expression of vimentin and phosphorylated vimentin following the addition of PV-GlcNAc to the MDCK cells was also examined. The expression and the phosphorylation level of vimentin, induced by PV-GlcNAc, increased after 15 min (Figure 7A–C). Cell surface recruitment of vimentin after treatment with PV-GlcNAc was also observed in the MDCK cells at 1 h (Figure 7D). However, the expression of E-cadherin was not altered by PV-GlcNAc (Figure 7B). Immunocytochemistry showed that the vimentin structure was hardly altered by PV-GlcNAc at 24 h (Figure 7E). Similar to the MCF-7 cells, the migration of the MDCK cells was also enhanced by PV-GlcNAc in a dose-dependent manner (Figure 7F and G). These results demonstrated that stimulation with PV-GlcNAc induced the expression of vimentin and the migration of MCF-7 and MDCK cells without EMT. Therefore, treatment with GlcNAc-containing molecules may increase the vimentin expression level and the migratory and invasive capacity of cancer cells.

**Induction of vimentin expression and cell migration by the addition of dying cells to MCF-7 and MDCK cells**

PV-GlcNAc can be regarded as an artificial glycopolymer that mimics multimerized O-GlcNAc-modified proteins because of its multivalency. Moreover, O-GlcNAc-modified proteins in the extracellular debris released from dying cells are recognized and internalized through vimentin on the surface of neighboring cells (Ise et al. 2012). We hypothesized that this interaction is involved in the clearance of dying cells (Ise et al. 2012). Therefore, we examined whether O-GlcNAc-modified proteins released from dying cells enhanced vimentin expression and cell migration. The cell death of vimentin−ko MEFs was induced by ultraviolet C (UVC) irradiation for 30 min, and the UV-irradiated cells were cultured for 1 h after the irradiation. To avoid detecting vimentin derived from the added UV-irradiated cells, vimentin−ko MEFs were used. Cell death was observed 1 h after the irradiation (Supplementary data, Figure S3). The UV-irradiated cells were cocultured with the MCF-7 and MDCK cells. In the MCF-7 cells, the protein and mRNA expression of vimentin were augmented at 24 h following the addition of the UV-irradiated cells (Figure 8A–C). Similar to when PV-GlcNAc was added, the E-cadherin, Snail and Slug expression levels were unaltered after the addition of the UV-irradiated cells (Figure 8A and B). The migration of the MCF-7 cells was also observed at 24 h (Figure 8D). In the MDCK cells, the expression of vimentin was enhanced at 3 h (Figure 8E and F), and the migration was observed at 24 h (Figure 8G).

Next, to investigate whether the up-regulation of vimentin in the MCF-7 and MDCK cells was induced by the O-GlcNAc-modified protein in the cell debris, we examined whether the up-regulation of vimentin decreased when the O-GlcNAc modification level in the UV-irradiated cells decreased. We reduced the level of O-GlcNAc modification of the UV-irradiated cells by knockdown of O-β-N-acetylglucosamine transferase (OGT) with small interfering RNA (siRNA). Using this knockdown strategy, the level of OGT and O-GlcNAc modification in vimentin−ko MEFs was reduced (Figure 9A). The up-regulation of vimentin in the MCF-7 and MDCK cells following the addition of the UV-irradiated cells treated with OGT siRNA was slower than the up-regulation following the addition of the normal UV-irradiated cells (Figure 9B–D). These results demonstrated that the up-regulation of vimentin weakened with the reduction in the O-GlcNAc modification level of the UV-irradiated cells. Next, to confirm whether the up-regulation of vimentin depended upon the presence of the GlcNAc molecules, the effect of GlcNAc-grafted poly-L-lysine such as O-GlcNAc-modified polypeptides and cell lysates containing O-GlcNAc-modified proteins from vimentin−ko MEFs was examined in MCF-7 cells (Supplementary data, Figure S4B–E). The cell surface recruitment of vimentin in HeLa cells was induced by the addition of 1 μg/mL of GlcNAc-grafted poly-L-lysine (Supplementary data, Figure S4C). Moreover, the up-regulation of vimentin in MCF-7 cells was induced by 1 μg/mL of GlcNAc-grafted poly-L-lysine, induced surface localization and up-regulation of vimentin.

(left panel), CBβ staining of these fractions (middle panel) and vimentin expression in the total protein in these cells (right panel). (F) Cell migration of PV-GlcNAc-treated MCF-7 cells using the in vitro wound-healing assay. The MCF-7 cells were incubated with 0–200 μg/mL of PV-GlcNAc in DMEM containing 10% FBS for 10 h. Left panel: phase-contrast images of 0, 50 and 200 μg/mL of PV-GlcNAc-treated MCF-7 cells. Scale bar = 100 μm. Right panel: quantification of the invaded area of the PV-GlcNAc-treated MCF-7 cells. **P < 0.01. Results are expressed as the mean (SD) (n = 5).
This polymer is likely more effective than PV-GlcNAc because the valency of GlcNAc-grafted poly-l-lysine is higher than the valency of PV-GlcNAc. In addition, the cell lysate containing O-GlcNAc-modified proteins from vimentin-ko MEFs induced the up-regulation of vimentin in MCF-7 cells (Supplementary data, Figure S4E). Our findings...

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suggest that a multitude of GlcNAc molecules, including O-GlcNAc-modified proteins, can induce the expression of vimentin and cell migration. We propose that the O-GlcNAc-modified proteins released from cells with damaged cell membranes such as dying cells regulate the expression of vimentin and cell migration.

**Discussion**

The present study demonstrated that GlcNAc molecules such as PV-GlcNAc interact with vimentin, promoting the phosphorylation of vimentin by ROCK and PKC, the subsequent disassembly of vimentin filaments and the rapid recruitment of vimentin to the cell surface. These results are consistent with our previous study in which O-GlcNAc-modified proteins released from dying cells induced the formation of tetrameric vimentin, cell surface recruitment and engulfment of O-GlcNAc-modified proteins in cell debris through interactions with surface-localized vimentin (Ise et al. 2012). This study provided details on the behavior of vimentin based on the GlcNAc-binding properties of vimentin, using PV-GlcNAc as an artificial bio-mimicking glycopolymer. We also used S71E-mutated vimentin that is constitutively phosphorylated to demonstrate that the augmentation and the cell surface recruitment of tetrameric vimentin are promoted by the phosphorylation of vimentin S71. Moreover, S71A-mutated vimentin, which is unable to undergo phosphorylation at S71, is also recruited to the cell surface by PV-GlcNAc. Previous reports showed that vimentin S38 is phosphorylated by ROCK and PKC (Ando et al. 1989; Goto 2004; PV-GlcNAc). Previous reports showed that vimentin S38 is mutated vimentin, which is unable to undergo phosphorylation of GlcNAc-binding properties of vimentin, using PV-GlcNAc. Studies released from dying cells induced the formation of tetrameric vimentin, cell surface recruitment and engulfment of O-GlcNAc-modified proteins in cell debris through interactions with surface-localized vimentin (Ise et al. 2012). This study provided details on the behavior of vimentin based on the GlcNAc-binding properties of vimentin, using PV-GlcNAc as an artificial bio-mimicking glycopolymer. We also used S71E-mutated vimentin that is constitutively phosphorylated to demonstrate that the augmentation and the cell surface recruitment of tetrameric vimentin are promoted by the phosphorylation of vimentin S71. Moreover, S71A-mutated vimentin, which is unable to undergo phosphorylation at S71, is also recruited to the cell surface by PV-GlcNAc. Previous reports showed that vimentin S38 is phosphorylated by ROCK and PKC (Ando et al. 1989; Goto et al. 1998) and that this phosphorylation induces the disassembly of vimentin filaments (Helfand et al. 2011). Therefore, the cell surface recruitment of S71A-mutated vimentin by PV-GlcNAc might be induced by the phosphorylation of S38-vimentin by ROCK and PKC. These results suggest that the formation of tetrameric vimentin (vimentin disassembly) via the phosphorylation of S71 or S38 by ROCK and PKC is necessary for promoting cell surface recruitment and binding to GlcNAc derivatives. Although the mechanisms of the activation of these kinases remain unclear, we suggest that the accumulation of tetrameric vimentin at the cell surface following the treatment with PV-GlcNAc induces the activation of ROCK and PKC. The vimentin rod II domain, the GlcNAc-binding site of vimentin, may have affinity for lipid bilayers, including transmembrane domains (Perides et al. 1987; Nishimura and Balch 1997). Therefore, tetrameric vimentin may easily move from the cytoplasm to the outside of the membrane.

Intriguingly, it was observed that the expression of vimentin and the migration of the MDCK and MCF-7 cells were augmented by the treatment with PV-GlcNAc. However, the expression level of E-cadherin in the PV-GlcNAc-treated MCF-7 and MDCK cells was not altered. In addition, the Snail and Slug mRNA expression levels were unaltered in the PV-GlcNAc-treated MCF-7 cells. These results suggest that the treatment with PV-GlcNAc promote the augmentation of vimentin expression without EMT. It was observed that the coculture with the dying cells (UV-irradiated cells) and MCF-7 or MDCK cells induced vimentin expression and cell migration without EMT, in a similar fashion to that of PV-GlcNAc. Moreover, it was observed that GlcNAc-grafted poly-l-lysine and cell lysates containing O-GlcNAc-modified proteins induced the up-regulation of vimentin in MCF-7 cells. The up-regulation of vimentin decreased when the O-GlcNAc modification level in the dying cells decreased. From these results, we suggest that GlcNAc molecules, especially O-GlcNAc-modified proteins released from dying cells, induce these phenomena.

The GlcNAc-binding property of surface-localized vimentin was recently reported to be involved in the clearance of O-GlcNAc-modified proteins as extracellular debris released by cell death (Ise et al. 2012). Interestingly, massive cell death caused by ischemia has been observed in the center of various malignant tumor tissues (Fukasawa et al. 1994; Lundgren et al. 2009), and vimentin expression is greatly increased in various malignant tumor tissues (Ben-Ze’ev and Raz 1985; Grille et al. 2003; Hu et al. 2004; Korsching et al. 2005; Trog et al. 2008). In addition, vimentin expression in cancer cells is promoted by the treatment with anticancer drugs and hypoxia (Sommers et al. 1992; Lundgren et al. 2009). The vimentin level may thus be induced in the presence of O-GlcNAc-modified proteins from dead tumor cells from anticancer drug treatment or hypoxia, and the increased surface-localized vimentin of neighboring and living tumor cells may subsequently lead to the internalization of the dead cells via interactions with O-GlcNAc proteins. Interestingly, although the overexpression of vimentin may be involved in tumor progression in various cancers (Korsching et al. 2005), vimentin-deficient embryonic stem cell-derived tumors showed a cellular composition similar to that of normal embryonic stem cell-derived tumors induced from wild-type cells, suggesting that vimentin is not essential for efficient tumor growth and differentiation during malignancy (Langa et al. 2000). Consequently, the up-regulation of vimentin expression in malignant tumor cells may be due to the interaction and internalization of O-GlcNAc-modified proteins from dying cells, enhancing cell migration and invasion, engulfing and clearing the dying cells through interactions with O-GlcNAc-modified proteins.

In this study, it was revealed that vimentin was dramatically altered in cells following the interaction with GlcNAc-containing molecules such as PV-GlcNAc- and O-GlcNAc-modified proteins from dying cells. This result implies that interactions between surface-localized vimentin and GlcNAc molecules are important for the migration and invasion of cancer cells. Therefore, our findings using GlcNAc-bearing polymers suggest that the inhibition of the recognition of these O-GlcNAc-modified proteins with vimentin is a useful strategy for suppressing the migration and invasion capacity of cancer cells and for targeting cancer tissues. Moreover, we previously reported that GlcNAc-conjugated liposomes and polymers are useful gene carriers for drug delivery (Aso et al. 2007; Kim et al. 2011) and may thus be advantageous for vimentin-targeted tumor-specific therapy.

In conclusion, this study suggests novel roles of vimentin in the migration and invasion of cancer cells, which may aid the development of new cancer therapies utilizing vimentin.
Fig. 8. Vimentin expression in the MCF-7 and MDCK cells following the addition of dying UV-irradiated vimentin-ko MEFs. (A) Detection of vimentin in the MCF-7 cells exposed to the UV-irradiated cells for 48 h. (B) Real-time RT–PCR detection of vimentin, Snail and Slug mRNA expression in MCF-7 cells exposed to UV-irradiated cells. Results are expressed as the mean (SD) (n = 5). (C) CLSM images of the MCF-7 cells exposed to UV-irradiated cells and immunostained with anti-vimentin antibodies (green) or anti-E-cadherin antibodies (red). The cell nucleus was stained with DAPI (blue). Scale bar = 20 µm.
Materials and methods

Cell culture
HeLa cells, MCF-7 cells, MDCK cells and MEFs from the vimentin-ko mice (6.25 × 10^4 cells/cm^2) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. After washing with phosphate-buffered saline (PBS), the cells were trypsinized with a 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (EDTA) solution for 5 min at 37°C. The cell assays were performed without serum. The MDCK cells (RCB0995) were provided by RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. The MCF-7 cells (JCRB0134) were provided by the Health Science Research Resources Bank. The MEFs from the vimentin-ko mice were kindly donated by Dr John E. Eriksson (Turku Centre for Biotechnology, University of Turku and Åbo Akademi, Finland). In the assays to determine the inhibition of ROCK and PKC, the HeLa cells were cultured in serum-free DMEM and treated with the ROCK inhibitor HA-1077 (10 μM; Enzo Life Sciences Inc., Farmingdale, NY) or the PKC inhibitor GF109203X (100 nM; Enzo Life Sciences Inc.) at 37°C in 5% CO_2 for 4 or 2 h, respectively. PV-GlcNAc (Cellagix Research Ltd, Yokohama, Japan) was dissolved in sterile water at various concentrations. GlcNAc-grafted poly-L-lysine was synthesized by the reductive amination of poly-L-lysine (Peptide Institute, Inc., Osaka, Japan) and chitobiose (Yaizu Suisankagaku Industry Co. Ltd, Shizuoka, Japan). Chitobiose was kindly donated by Yaizu Suisankagaku Industry Co. Ltd. The composition of this polymer was confirmed by nuclear magnetic resonance spectroscopy. HeLa cells expressing fusion proteins (AG-vimentin and DsRed-vimentin) were produced by transfecting HeLa cells with phsnAG1-MC1-rat vimentin (MBL Co. Ltd, Nagoya, Japan) or pDsRed-monomer-Hyg-C1-rat vimentin (Clontech Laboratories, Inc., Mountain View, CA) using the Neon Transfection System (Invitrogen, Carlsbad, CA). The vectors for various point mutants were prepared using the TaKaRa PrimeSTAR Mutagenesis Basal Kit (TaKaRa Bio Inc., Shiga, Japan) according to the manufacturer’s instructions. The dying cells were prepared by the UV irradiation of vimentin-ko MEFs. For UV irradiation, the cells were incubated in DMEM, seeded onto the PV-GlcNAc-coated wells at a density of 1 × 10^4 cells/well and incubated for 3 h at 37°C in 5% CO_2. After incubation, the unbound cells were removed by washing with PBS. The number of adhered cells was estimated using the methylthiazol tetrazolium assay (Ise et al. 2010).

Immunocytochemistry
Cells were cultured on 24 × 24 mm glass coverslips. The cells were fixed in 4% paraformaldehyde (PFA) in PBS for 15 min. The fixed cells were then washed with 50 mM glycine in PBS, permeabilized with 0.1% Triton X-100 in PBS for 5 min and blocked with 1% BSA in PBS for 15 min. Subsequently, vimentin and the E-cadherin were stained by incubating the cells with mouse monoclonal anti-vimentin antibodies (1:100; cloneV9; Sigma-Aldrich, St Louis, MO) and rat monoclonal anti-E-cadherin antibodies (1:100; clone DECM-A-1; Sigma-Aldrich) for 1 h at room temperature. The cells were then incubated with CF488A goat anti-mouse IgG (1:1000; Biotium, Inc., Hayward, CA) and Alexa Fluor 546 goat anti-rat IgG (1:1000; Invitrogen) for 1 h at room temperature. To detect cell death, cells were incubated for 15 min with Annexin V-Fluos (Roche Diagnostics GmbH, Mannheim, Germany) diluted 1:50 in Hank’s balanced salt solution. After the staining, cells were fixed with 4% PFA in PBS for 15 min. The cell surface membrane was stained with 2.5 μg/mL of 1,1’-dioctadecyl-3,3,3’,3’-tetramethyl-indocarbocyanine perchlorate (DiI) for 5 min. The nucleus was stained with 0.1 μg/mL of 4’,6-diamidino-2-phenylindole (DAPI; Dojinbo Laboratories, Kumamoto, Japan).

Confocal laser scanning microscopy
All of the cells and sections were observed by CLSM (A1/ AIR system; Nikon Instruments, Inc., Tokyo, Japan) with a 10×/0.45 lens or a 40×/0.95 lens (Plan Apo DIC M/N; or N1; Nikon Instruments, Inc.) at room temperature. Z stacks were formed from 10–20 optical sections per 0.5–1 μm. The fluorescent images were processed with the NIS-Elements Imaging Software (Nikon Instruments, Inc.).

Establishment of vimentin-, RhoA-, PKCe- and negative-knockdown HeLa cells
Human vimentin-, human RhoA-, PKCe- and negative-knockdown HeLa cells were established by transfecting cells incubated overnight at 37°C, as described previously (Ise et al. 2010). The surfaces of the polymer-coated wells were then blocked by incubation with bovine serum albumin (BSA; 0.1 mg/mL) in PBS for 1 h at 4°C. The cells were suspended in DMEM, seeded onto the PV-GlcNAc-coated wells at a density of 1 × 10^4 cells/well and incubated for 3 h at 37°C in 5% CO_2. After incubation, the unbound cells were removed by washing with PBS. The number of adhered cells was estimated using the methylthiazol tetrazolium assay (Ise et al. 2010).

Carbohydrate adhesion assay
Aliquots (100 μL) of 100 μg/mL of PV-GlcNAc aqueous solutions were added to 96-well suspension culture plates and incubated overnight at 37°C, as described previously (Ise et al. 2010). The surfaces of the polymer-coated wells were then blocked by incubation with bovine serum albumin (BSA; 0.1 mg/mL) in PBS for 1 h at 4°C. The cells were suspended in DMEM, seeded onto the PV-GlcNAc-coated wells at a density of 1 × 10^4 cells/well and incubated for 3 h at 37°C in 5% CO_2. After incubation, the unbound cells were removed by washing with PBS. The number of adhered cells was estimated using the methylthiazol tetrazolium assay (Ise et al. 2010).
with pcDNA 6.2-GW/EmGFP-miR-human vimentin, RhoA, PKCε (vimentin-, RhoA- and PKCε-miRNA vectors; Invitrogen) and pcDNA 6.2-GW/EmGFP-miR-neg control plasmid (negative-miRNA vector; Invitrogen), respectively. The vimentin-, RhoA- and PKCε-miRNA vectors targeted the sequences 5′-CACACTTTCA TA TTGCTGACG-3′ (nucleotides 1230–1250 of human vimentin mRNA, GenBank Accession No. NM_003380), 5′-GACA TGCTTGCTCA TAGTCT-3′ (nucleotides 330–349 of human RhoA mRNA, GenBank Accession No. NM_001664.2) and 5′-AGAAGGAAGAG TGTA TGTGA T-3′ (nucleotides 560–580 of human PKCε mRNA, GenBank Accession No. NM_001664.2), respectively. The negative-miRNA vector was not predicted to target any known vertebrate gene and contained an insert, 5′-GAA A GT ACTGCGCGTGGAGACGTTTTGGCCACTGACTGAC GTCTCCACGACGTACATT-3′, which could form a hairpin structure. Each of the vectors encoded GFP, and the miRNA-containing cells therefore expressed GFP synchronously. These vectors were transfected into cells by the Lipofectamine LTX reagent (Invitrogen). The stable cell lines that constitutively expressed miRNAs and GFP were selected with blasticidin (Invitrogen).

Biotinylation of cell surface proteins

The biotinylation method has been described previously (Ise et al. 2010). Briefly, to biotinylate the surface proteins of HeLa cells, the cells were incubated with 100 µg/mL of sulfo-succinimidyl N-(p-biotinyl)-6-aminohexanoate (biotin-AC5-Sulfo-Osu; Dojindo Laboratories) in PBS at 4°C for 30 min. Biotin-AC5-Sulfo-Osu is water soluble and cannot enter the cytoplasm. Thus, only surface proteins of HeLa cells were biotinylated. After biotinylation, the cells were incubated with DMEM for 5 min to quench the unreacted biotin-AC5-Sulfo-Osu and then washed three times with PBS.

Immunoprecipitation and western blot analysis

The cells were lysed with a lysis buffer comprising 25 mM Tris–HCl, pH 7.5, 2.5 mM EDTA, 137 mM NaCl, 2.7 mM KCl, 1% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40 (NP-40), 2 mM phenylmethylsulfonyl fluoride (PMSF) and a protease and phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). The lysate was centrifuged at 20,000 × g for 30 min at 4°C, and the supernatant was collected. These proteins were resolved.
on a 5–20% SDS–PAGE gradient gel (DRC Co. Ltd, Tokyo, Japan). The cells were biotinylated and lysed in NP-40 lysis buffer comprising 150 mM aminocaproic acid, 1% NP-40, 50 mM Bis–Tris, pH 7.0, 2 mM PMSF and a protease inhibitor cocktail (Nacalai Tesque). The lysate for BN-PAGE was prepared in a lysis buffer comprising 500 mM aminocaproic acid, 1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM Bis–Tris, pH 7.0, 2 mM PMSF and a protease inhibitor cocktail (Nacalai Tesque). The lysate was centrifuged at 20,000 × g for 30 min at 4°C. The supernatant (detergent-soluble fraction) and the pellet (detergent-insoluble fraction) were collected. Next, the pellets were dissolved in the CHAPS lysis buffer and sonicated. The detergent-soluble and detergent-insoluble fractions were isolated and incubated at 4°C for 1 h with protein G magnetic beads (Millipore Corp., Billerica, MA) bound to mouse monoclonal anti-vimentin antibodies (V9; Sigma-Aldrich).

After incubation, the proteins bound to these beads were resolved on a 10% SDS–PAGE gel (DRC). The proteins in the gel were electrophoblated onto a polyvinylidene fluoride (PVDF) membrane (Millipore Corp.). Western blot analysis was performed by incubation with horseradish peroxidase (HRP)-conjugated NeutrAvidin (1:5000; Thermo Fisher Scientific Inc., Rockford, IL), V9 (1:10,000; Sigma-Aldrich), mouse monoclonal anti-β-actin antibodies (1:10,000; Sigma-Aldrich), mouse monoclonal anti-RhoA antibodies (1:5000; Millipore Corp.), mouse monoclonal anti-FAK antibodies (1:2000; Millipore Corp.), mouse monoclonal anti-phospho FAK antibodies (1:2000; Millipore Corp.), rat monoclonal anti-phospho vimentin (S71) antibodies (1:5000; Enzo Life Sciences Inc.), mouse monoclonal anti-phospho vimentin (S55) antibodies (1:5000; Enzo Life Sciences Inc.), mouse monoclonal anti-E-cadherin antibodies (1:5000; BD, Franklin Lakes, NJ), anti-OGT antibodies (1:2000; GeneTex, Inc., Irvine, CA) or anti-O-GlcNAc antibodies (1:1000; Covance, Emeryville, CA). The ligand blotting was performed by incubation with FITC-PV-GlcNAc (1:1000; Celagix Research Ltd) and then with goat anti-FITC antibodies (1:2500; Bethyl Laboratories, Inc., Montgomery, TX). Subsequently, the PVDF membrane was incubated with HRP-conjugated anti-rat IgG, mouse IgG or mouse IgM antibodies (1:20,000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) as secondary antibodies.

Blue native polyacrylamide gel electrophoresis
BN-PAGE was performed according to the method by Valentijn et al. (2008). Briefly, the detergent-soluble and detergent-insoluble fractions were dissolved in a loading buffer comprising 0.5% Coomassie brilliant blue (CBB) G-250, 50 mM aminocaproic acid, 10 mM Bis–Tris, pH 7.0, and 10% glycerol and were then incubated for 30 min at 4°C. Subsequently, these solutions were resolved on a 5–20 or 5–10% gradient gel, followed by electrophoresis at 150 V for 1 h at room temperature using a DRC BN-PAGE system. The gel was stained using CBB Stain One (Nacalai Tesque). Western blotting and ligand blotting were performed.

Real-time reverse transcriptase–PCR
Total RNA was isolated from the cells using the TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. First-strand cDNAs were synthesized using PrimeScript RT master mix (TaKaRa Bio Inc.). For real-time reverse transcriptase–PCR (RT–PCR), the following primers were used: human vimentin sense primer 5′-ATTGGCT CGTCACTTCTGTGAAT-3′ and antisense primer 5′-CAG ATTAGTTCTCCACGGTTAC-3′; human Snail sense primer 5′-AAGGCCCTTCTCTAGGCCCT-3′ and antisense primer 5′-CGGAGGTTGGAGCGGTC-3′; human Slug sense primer 5′-GGGAATAATACCTCTACGTTGTTG-3′ and antisense primer 5′-CTTTCCTGGTACAAGAC-3′; and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense primer 5′-CGAGATCCCTCAAACTC-3′ and antisense primer 5′-TTCAACCCATGACGAC-3′. To detect the mRNA expression of human vimentin, Snail, Slug and GAPDH, real-time RT–PCR analysis was performed using the Fast EvaGreen qPCR Master Mix Kit (Biotium, Inc.) and the Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). The expression level of each target gene was normalized by subtracting the corresponding GAPDH cycle threshold (CT) values; this was accomplished using the ΔCT comparative method. Each value was obtained from three independent experiments.

Reduction of O-GlcNAc modification levels in vimentin-ko MEFs
The reduction in O-GlcNAc modification levels in vimentin-ko MEFs was obtained by the siRNA knockdown of OGT. The knockdown of OGT was performed for 48 h by transfection with OGT siRNAs (final concentration: 100 nM). The transfection was performed by the Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s instructions. Mouse OGT and negative control siRNAs were purchased as Stealth RNAi siRNA Duplex Oligoribonucleotides (Invitrogen). The targeting sequences of the mouse OGT siRNAs were as follows (nucleotides 749–773 of mouse OGT, GenBank Accession No. NM_139144): sense, 5′-CCAAGCAGUUCUAAUUGAGCAUU-3′; antisense, 5′-UUUGCUCUUUAAUAAACUCGGCUUGG-3′. The sequences of the negative control siRNA were as follows: sense, 5′-CCAUACGAGUUGGUGAAAGCAAC-3′; antisense, 5′-UUUCGUUUCACAAACUCGUUUGG-3′.

In vitro wound-healing assay
The in vitro wound-healing assay was performed using a culture insert (ibidi GmbH, Martinsried, Germany). The MCF-7 or MDCK cells were seeded at 7 × 10⁴ cells/well and cultured in DMEM containing 10% FBS for 24 h. After 24 h, the culture insert was removed, and the MCF-7 or MDCK cells were cultured in DMEM containing 10% FBS or 0.1% FBS, respectively.

Statistical analyses
All data are expressed as the mean (SD) of at least three independent experiments. Statistically significant differences between two groups have been determined using an unpaired Student’s t-test. P-values of <0.01 or <0.05 are considered statistically significant.
Supplementary Data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest

None declared.

Abbreviations

AG, Azami green; BN-PAGE, blue native polyacylamide gel electrophoresis; BSA, bovine serum albumin; CBB, Coomassie brilliant blue; CHAPS, 3-[{(3-cholamidopropyl)-N,N,N′,N′-tetramethylammonio]1-propanesulfonic acid; CLSM, confocal laser scanning microscopy; CT, cycle threshold; DAPI, 4′,6-diamidino-2-phenylindole; DiI, 1′,1′-diiodotridecyl-3,3′,3′-tetramethyl-indocarbocyanine perchlorate; DMEM, Dulbecco’s modified Eagle’s medium; EDTA, ethylenediaminetetraacetic acid; EMT, epithelial-mesenchymal transition; FAK, focal adhesion kinase; FBS, fetal bovine serum; FITC-PV-GlcNAc, fluorescein isothiocyanate-conjugated-PV-GlcNAc; GFP, green fluorescent protein; GlcNAc, N-acetylgalactosamine; HA-1077, 1-(5-isoquinoline sulfonyl)homopiperazine dihydrochloride; HRP, horseradish peroxidase; ko, knockout; MCF-7, Michigan Cancer Foundation-7; MDCK, Madin-Darby canine kidney; MEFs, mouse embryonic fibroblasts; NP-40, Nonidet P-40; O-GlcNAc, O-linked-β-GlcNAc; OGT, O-β-N-acetylglucosaminyltransferase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PFA, parafomaldehyde; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene fluoride; PV-GlcNAc, poly[N-[5-vinylbenzyl]-O-2-acetamide-2-deoxy-β-D-glucopyrannosyl-(1→4)-2-acetamide-2-deoxy-β-D-glucosamid]; PV-λA, galactose-bearing polymers; PV-MA, glucose-bearing polymers; RT, reverse transcriptase; ROCK, Rho-associated kinase; SDS, sodium dodecyl sulfate; shRNA, small hairpin RNA; siRNA, small interfering RNA; UVC, ultraviolet C.

References


