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Human Natural Killer-1 Sulfotransferase (HNK-1ST)-induced Sulfate Transfer Regulates Laminin-binding Glycans on α-Dystroglycan*[S]

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Background: α-Dystroglycan undergoes extensive glycosylation required for the interaction between α-dystroglycan and its ligands such as laminin.
Results: HNK-1ST suppressed the glycosylation and reduced the ligand binding activity of α-dystroglycan.
Conclusion: The sulfotransferase activity of HNK-1ST is essential for the modulation of α-dystroglycan.
Significance: This study identifies a novel role for HNK-1ST as a regulator of the functional glycans on α-dystroglycan other than HNK-1 biosynthesis.

Retinoic acid (RA) is a well established anti-tumor agent inducing differentiation in various cancer cells. Recently, a robust up-regulation of human natural killer-1 sulfotransferase (HNK-1ST) was found in several subsets of melanoma cells during RA-mediated differentiation. However, the molecular mechanism underlying the tumor suppression mediated by HNK-1ST remains unclear. Here, we show that HNK-1ST changed the glycosylation state and reduced the ligand binding activity of α-dystroglycan (α-DG) in RA-treated S91 melanoma cells, which contributed to an attenuation of cell migration. Knockdown of HNK-1ST restored the glycosylation of α-DG and the migration of RA-treated S91 cells, indicating that HNK-1ST functions through glycans on α-DG. Using CHO-K1 cells, we provide direct evidence that HNK-1ST but not other homologous sulfotransferases (C4ST1 and GalNAc4ST1) suppresses the glycosylation of α-DG. The activity-abolished mutant of HNK-1ST did not show the α-DG-modulating function, indicating that the sulfotransferase activity of HNK-1ST is essential. Finally, the HNK-1ST-dependent incorporation of [^{35}S]sulfate groups was detected on α-DG. These findings suggest a novel role for HNK-1ST as a tumor suppressor controlling the functional glycans on α-DG and the importance of sulfate transfer in the glycosylation of α-DG.

Invasiveness is a hallmark of malignant tumors. In the initial phase of invasion, cell-cell and/or cell-extracellular matrix interactions are crucial (1). The external region of a cell membrane, known as the glycocalyx, is dominated by glycosylated molecules, which have important roles in these interactions (2). Therefore, the aberrant expression of various genes involved in glycan synthesis or degradation, which causes compositional changes of the glycocalyx, is frequently associated with malignant transformation (3–5). Recently, Zhao *et al.* (6) reported the expression of human natural killer-1 sulfotransferase (HNK-1ST) to be strongly up-regulated in several subsets of murine and human melanoma cells during retinoic acid (RA)[2]-mediated differentiation. The expression of HNK-1ST is activated via an RA receptor-γ pathway, and the invasiveness of melanoma cells is suppressed along with HNK-1ST induction (6). HNK-1ST is a sulfotransferase involved in the biosynthesis of the HNK-1 carbohydrate, a neural glyco-epitope exhibiting abundant expression during brain development (7). Although HNK-1ST has the potential to control the cell surface expression of the HNK-1 carbohydrate, it is not clear how HNK-1ST is associated with the tumor-suppressive function.

We have demonstrated that the HNK-1 carbohydrate is required for the structural and functional development of the mammalian nervous system, such as the maturation of dendritic spines and the acquisition of synaptic plasticity, respectively (8–10). The HNK-1 carbohydrate has a unique structural feature, *i.e.* a sulfated glucuronic acid is attached to the nonreducing terminal of an *N*-acetyllactosamine residue (11, 12). Because the *N*-acetyllactosamine structure is commonly found in various glycoproteins and glycolipids, two glucuronyltransferases (GlcAT-P and GlcAT-S) and a sulfotransferase (HNK-1ST) had been cloned and characterized as key enzymes for the biosynthesis (7). GlcAT-P or GlcAT-S and HNK-1ST interact closely as a functional complex, cooperatively synthesizing the HNK-1 carbohydrate (13). However, although GlcAT-P and GlcAT-S show a highly restricted tissue distribution (14, 15),

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[2] The abbreviations used are: RA, retinoic acid; α-DG, α-dystroglycan; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; CMD, congenital muscular dystrophy; EGFP, enhanced GFP.

HNK-1ST is more ubiquitous and exists in several tissues where neither GlcAT-P nor GlcAT-S is observed, including skeletal muscle, heart, spleen, and reproductive organs (16, 17). These findings suggest that HNK-1ST has another function, which might underlie the RA-mediated melanoma differentiation.

α-Dystroglycan (α-DG) is a ubiquitously expressed peripheral membrane glycoprotein, which serves as a receptor for extracellular matrix components, including laminin, agrin, and perlecan (18, 19). α-DG is anchored on the plasma membrane by β-DG, which interacts with cytoskeletal proteins, together comprising the DG complex that provides physical links between the cell and basal lamina (18–20). α-DG undergoes extensive glycosylation in a tissue-specific manner (18, 19, 21), and the attached glycan acts as a critical mediator of the interaction between α-DG and its ligands (22, 23). Although the precise structure of the glycan important for the function of α-DG has not completely been determined, aberrant glycosylation of α-DG has already been identified in the pathogenesis of several types of congenital muscular dystrophy (CMD) accompanied by brain and eye malformations (24, 25). In CMD patients, mutations in six known or putative glycosyltransferase genes involved in the biosynthesis of *O*-mannosyl glycan, including protein *O*-mannosyltransferase 1 (*POMT1*), *POMT2*, protein *O*-mannose β-1,2-*N*-acetylglucosaminyltransferase 1 (*POMGnT1*), *fukutin,* fukutin-related protein (*FKRP*), and like-acetylglucosaminyltransferase (*LARGE*), have been found (26–31). These observations indicate that *O*-mannosyl modification is essential for the functional glycosylation of α-DG. Furthermore, altered glycosylation of α-DG is also implicated in epithelium-derived cancer progression, demonstrating the involvement of α-DG in tumorigenic phenotypes (32, 33).

In this report, we show that HNK-1ST induced in RA-treated S91 melanoma cells suppressed the glycosylation and ligand binding activity of α-DG. The functional loss of α-DG resulted in a reduction in cell migration. Using CHO-K1 cells, we provide direct evidence that HNK-1ST actually has the ability to inhibit the synthesis of glycans on α-DG, suppressing the interaction between α-DG and laminin. Furthermore, the HNK-1ST-dependent incorporation of sulfate groups was detected on α-DG. These results suggest a novel tumor-suppressive role for HNK-1ST, which acts as a functional regulator of α-DG via sulfate transfer.

EXPERIMENTAL PROCEDURES

cDNA Construction—For α-DG-Fc, the coding sequence of human α-DG was amplified by PCR with primers ACGATCGATGCCACCATGAGGATGTCTGT (with ClaI site) and CCGACTAGTACTCACCGCCCCGGGTGATATTCTGCA (with SpeI site), using pcDNA3.1 containing human *DAG1* cDNA as a template, and subcloned into pEF-Fc. Expression plasmids for FLAG-GlcAT-P, pIRES-GlcAT-P/HNK-1ST (pIRES-P/ST), HNK-1ST-EGFP, C4ST1-EGFP, and GalNAc4ST1-EGFP were described previously (13). For LARGE-myc, the full-length cDNA of mouse *LARGE* was amplified from pBC SK^+ containing mouse *LARGE* cDNA, which was provided by Kazusa DNA Research Institute, using primers TCTGAGAGGATGCTGGGAAT and AAAGGGCCCCTGTTGTTCTCAGCTGTGAG (skipping stop codon, with ApaI site). After the resulting fragment had been digested with ApaI, it was ligated to pcDNA3.1/myc-His B (Invitrogen), which had been digested with EcoRV and ApaI. Construction of R189A-EGFP, the plasmid encoding the R189A mutant of HNK-1ST, was described previously (34).

Cell Culture and Transfection—CHO-K1 cells were maintained in α-minimum essential medium with 10% fetal bovine serum (FBS) in 5% CO_2 at 37 °C. S91 murine melanoma cells (a gift from Dr. A. Kurosaka, Kyoto Sangyo University), also known as M3, were maintained in Dulbecco's modified Eagle's medium with 10% FBS in 5% CO_2 at 37 °C. For cDNA transfection, cells were grown overnight and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. To obtain the extracellularly secreted proteins, the culture medium was replaced with serum-free OPTI-MEM I (Invitrogen) after 5 h of incubation.

RA Treatment—RA (all-*trans*-retinoic acid, Sigma) was dissolved in DMSO, kept as a 10 mM stock solution at −20 °C, and then diluted to the final concentration in the growth medium before being added to cells. Cells were harvested after 16 h for the RT-PCR analysis or after 48 h for Western blotting and the migration assay.

siRNA-mediated Knockdown—siRNA oligonucleotides specific for mouse *HNK-1ST* and a negative control siRNA were obtained from Qiagen. Oligonucleotide sequences used were as follows: si-ST1 5′-GGAUGGGUAUAGUGCCAAATT-3′ and 5′-UUUGGCACUAUACCCAUCCGG-3′ and si-ST2 5′-CAGAUUUCUUGCUAAAUUATT-3′ and 5′-UAAUUUAGCAAGAAAUCUGGT-3′. siRNA oligonucleotides were transiently transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. The culture medium was replaced with growth medium after 5 h of incubation.

Purification of Recombinant α-DG-Fc—The culture medium and cells were collected at 48 h post-transfection. The cells were lysed with Tris-buffered saline (pH 7.4) containing 1% Triton X-100 and protease inhibitor mixture (Nakalai Tesque), and then the cell extracts were obtained by centrifugation. The culture medium and the cell extracts were incubated with protein G-Sepharose (GE Healthcare) for 2 h at 4 °C. The beads were washed extensively with phosphate-buffered saline (PBS) containing 0.1% Triton X-100, and the bound proteins were eluted by boiling in Laemmli sample buffer (LSB).

Western Blotting and Laminin Overlay Assay—Proteins solubilized in LSB were separated by SDS-PAGE using 10% polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with 5% nonfat dry milk in PBS containing 0.05% Tween 20, the membranes were incubated with primary antibodies, followed by HRP-conjugated secondary antibodies. Protein bands were detected with Super Signal West Pico chemiluminescence reagent (Thermo Scientific) using an LAS-3000 Luminoimage Analyzer (FUJIFILM). The following primary antibodies were used: HNK-1 mAb (a hybridoma cell line was purchased from American Type Culture Collection); M6749 mAb (against a nonsulfated form of the HNK-1 carbohydrate, a gift from Dr. H. Tanaka, Kumamoto University); GP2 pAb (a rabbit anti-GlcAT-P pAb raised against the catalytic region of the recombinant human GlcAT-P); anti-Fc pAb (Jackson ImmunoResearch); anti-EGFP mAb (Clontech); anti-Myc mAb and IIH6 mAb (Millipore); anti-laminin pAb and anti-

FLAG pAb (Sigma); anti-β-DG mAb (Novocastra); α-DG core pAb (goat polyclonal antibody against the C-terminal domain of the α-DG polypeptide) (35); and anti-GAPDH mAb (Calbiochem). For the laminin overlay assay, nitrocellulose membranes with transferred proteins were blocked with laminin-binding buffer (LBB: 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$) containing 5% nonfat dry milk. After being washed in LBB, the membranes were incubated with 1 μg/ml laminin-1 (Sigma) diluted with LBB containing 3% bovine serum albumin (BSA) for 90 min at room temperature. Bound laminin-1 was detected using anti-laminin antibody by immunoblotting as described above. For the quantification of the intensity of the protein bands, densitometric analyses were performed using image analysis software ImageGauge (FUJIFILM).

Biotinylation of Cell Surface Proteins—Cells cultured in 60-mm dishes were washed twice with ice-cold PBS and incubated with 1 mg/ml EZ-link Sulfo-NHS-SS-biotin (Thermo Scientific) in PBS for 30 min at 4 °C. Cells were washed twice with PBS and lysed with Tris-buffered saline (pH 7.4) containing 1% Triton X-100 and protease inhibitor mixture, and then the cell extracts were obtained by centrifugation. The biotinylated proteins were precipitated with immobilized streptavidin (Thermo Scientific) and analyzed by Western blotting as described above.

Radioactive Metabolic Labeling—CHO cells were grown overnight in 25-cm^2 cell culture flasks under normal conditions. At 5 h post-transfection, the culture medium was replaced with sulfate-free M8028 minimum Eagle's medium (Sigma), and incubation was continued for 1 h. The cells were then labeled with 30 μCi/ml [^{35}S]sodium sulfate (ARC Inc.). The culture medium was collected after two nights of labeling and incubated with protein G-Sepharose for 2 h. Bound proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and then subjected to autoradiography or Western blotting using anti-Fc pAb.

RNA Isolation and RT-PCR—S91 cells were harvested and homogenized in TRIzol (Invitrogen) and extracted with chloroform and isopropyl alcohol. The RNA was dissolved in RNase-free water. One microgram of total RNA was digested with DNaseI and then converted to cDNA using SuperScriptII (Invitrogen). The following primer pairs were used for detecting the mRNA expression: HNK-1ST, GACCCGGGGGATCCAGTTTGAAGAT and GTCTCGTTTGCTGATGCCCAGGAAG; α-DG, GCCAGATTCGCCCAACACTGACAAT and CCACCCAGGCATCTACCCTGTCAAT; LARGE, GTCAGATGCAGAAGCCCAGCAGTTC and TGGGGAAAGAGAGTCTGTAGCGCAG; LARGE2, CGAGAGCTGCTCACTCTGAT and GGCATCCAAAGAGCTCTCTT; POMGnT1, TCGTGGGACGAAAAGGAGGTCC and TGGGCCGGTTCCCTGCAATG; POMT1, TTGCCCGCATCACCCAAGGC and GGCTGCGACATCGTGCGTGTT; POMT2, TTGCTGGCTACCTGAGCGGG and AGGGGGCAGAGAAAGGCCTGTT; and GAPDH, GGAAGGGCTCATGACCACAGTCCAT and CATACTTGGCAGGTTTCTCCAGGCG. Quantitative PCR was performed with a Chromo 4 Real Time System (Bio-Rad) using SYBR Green I. Each sample was run in triplicate, and GAPDH mRNA was amplified from the same sample to normalize the expression level.

Migration Assays—Cell migration was assayed using 24-well transwell plates (8 μm pore size) (BD Biosciences) according to the manufacturer's manual. Prior to the assay, S91 cells were treated with 1 μM RA or 0.1% DMSO for 48 h, and the insert membranes were coated with laminin-1 (10 μg/ml) (BD Biosciences). The 1×10^5 S91 cells suspended in serum-free DMEM were seeded into the upper chamber, and DMEM supplemented with 10% FCS was placed in the lower chamber. 1 μM RA or 0.1% DMSO was added to both chambers during the migration period. After 24 h, the inserts were fixed in ice-cold methanol, and nonmigrating cells were removed from the upper surface of the membrane. The inserts were stained in 0.1% Toluidine Blue O solution for 15 min and washed two times with PBS. The membranes were cut and mounted on glass slides. Five randomly selected fields per membrane were counted, and the average was shown as the migrated cell number.

Immunofluorescence—Cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature, and then incubated with primary antibodies, followed by Alexa Fluor-conjugated secondary antibodies. For permeabilization of the plasma membrane, cells were incubated with PBS containing 3% BSA and 0.1% Triton X-100 for 15 min after the fixation. Immunofluorescent images were acquired by a Fluoview laser confocal microscope system (Olympus). The following antibodies were used: IIH6 mAb (Millipore); anti-HIS pAb (Santa Cruz Biotechnology); GM130 mAb (BD Biosciences); and anti-β-DG mAb (Novocastra).

Statistics—Statistical significance was determined by a two-tailed Student's *t* test for comparisons between two groups and by an analysis of variance with Ryan's test for comparisons among multiple groups.

RESULTS

Specific Induction of HNK-1ST Expression and Alternate Glycosylation of α-DG in RA-treated Melanoma Cells—To investigate whether HNK-1ST governs tumor-related phenotypes of melanoma cells via production of the HNK-1 carbohydrate or not, we evaluated the mRNA expression of the enzymes synthesizing HNK-1 in S91 murine melanoma cells, which undergo RA-mediated differentiation (6, 36). Following treatment with 1 μM RA, a marked increase of HNK-1ST mRNA was detected using RT-PCR (Fig. 1, *A* and *B*), consistent with a previous report (6). However, neither of the glucuronyltransferases (GlcAT-P and GlcAT-S) responsible for producing HNK-1 was observed (Fig. 1*A*), suggesting the absence of the HNK-1 carbohydrate in S91 cells. To confirm this notion, we examined the expression of the HNK-1 carbohydrate in S91 cells treated with DMSO or RA using an HNK-1 monoclonal antibody (mAb). As expected, HNK-1 carbohydrate was not detected even in RA-treated cells (Fig. 1*C*). As we reported previously that the HNK-1 mAb specifically recognizes sulfated form of HNK-1 epitope but not nonsulfated one (17), we tried to examine whether the sulfated form of HNK-1 epitope was expressed in S91 cells by the transfection of GlcAT-P. As expected, HNK-1 immunoreactivity was detected by GlcAT-P transfection, sug-

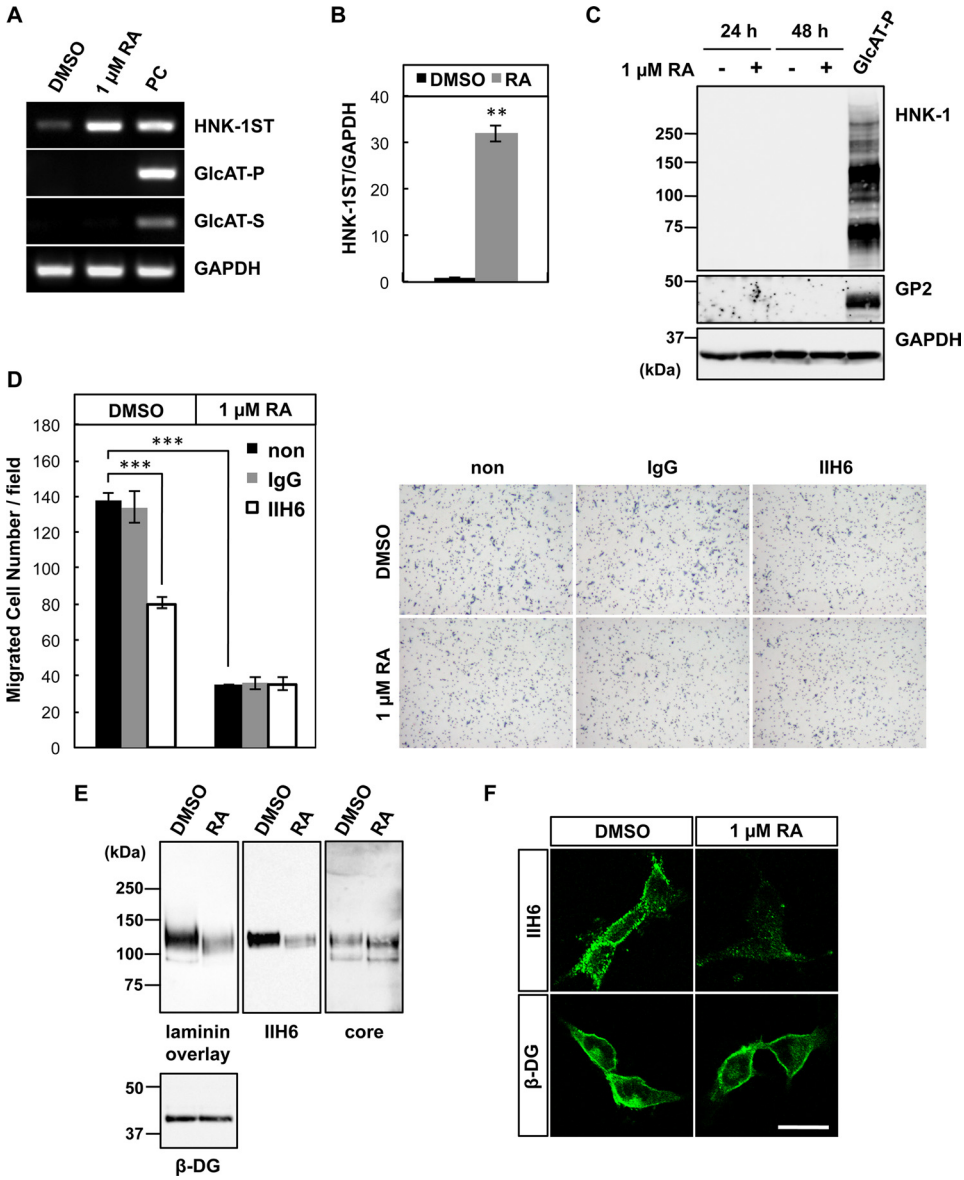

FIGURE 1. **Altered glycosylation of α-DG associated with HNK-1ST induction in RA-treated melanoma cells.** *A,* RT-PCR was performed using mRNA extracted from S91 cells treated with DMSO or 1 μM RA for 16 h. mRNA prepared from 2-week-old mouse brain was used as a positive control (*PC*). *B,* amount of HNK-1ST mRNA was quantified by quantitative RT-PCR, normalized to that of GAPDH mRNA, and shown as HNK-1ST/GAPDH. The value for DMSO-treated cells was set at 1. The *graphs* indicate the mean ± S.E. for three independent experiments. **, $p < 0.01$. *C,* S91 cells were treated with DMSO or 1 μM RA for the periods indicated. The cells were lysed, and an equal amount of protein (50 μg) from each sample was analyzed by Western blotting with HNK-1 mAb, GP2 (anti-GlcAT-P) pAb, and anti-GAPDH mAb. As a positive control for the HNK-1 mAb, GlcAT-P cDNA was transiently transfected into S91 cells (GlcAT-P). *D,* migration assay was carried out using transwell chambers with insert membranes coated with 10 μg/ml laminin-1. The migration of S91 cells pretreated with DMSO or 1 μM RA for 48 h was assessed (*non*). For antibody treatment assays, IIH6 mAb or normal mouse IgG was added in the upper chamber (IIH6 and IgG, respectively). The *graphs* indicate the mean ± S.E. for three independent experiments (*left panel*). ***, $p < 0.001$. The representative toluidine blue staining images of the insert membranes are shown (*right panel*). *E* and *F,* effect of RA on α-DG was investigated using S91 cells treated with DMSO or 1 μM RA for 48 h. *E,* cell surface proteins were biotinylated, pulled down by streptavidin-agarose beads, and analyzed by laminin overlay assay or Western blotting with IIH6 mAb, anti-β-DG mAb, and α-DG core pAb. *F,* cells were immunostained by IIH6 or anti-β-DG mAb. *Scale bar,* 20 μm.

gesting that a functional HNK-1ST was expressed in the cells (Fig. 1*C*). These results indicate that HNK-1ST acts solely as a tumor suppressor and its function is independent of HNK-1 biosynthesis.

It has been demonstrated that α-DG, especially the glycan attached to it, is involved in tumor invasiveness in various cancer types such as breast, prostate, and lung carcinomas (32, 33). Because HNK-1ST associates with the modification of glycans, up-regulation of HNK-1ST expression might affect the glycosylation in cells. Hence, we speculated that RA treatment causes a compositional change in the glycan on α-DG, leading to a suppression of melanoma invasiveness. To test this possibility, we explored the involvement of α-DG in the migratory behavior of S91 cells using a transwell migration assay with laminin-coated membranes. The RA-treated cells showed significantly decreased migration (Fig. 1*D*), which confirmed the anti-tumor effect of RA. Intriguingly, on the addition of an IIH6 mAb, which recognizes the laminin-binding glycan on α-DG and can disturb the α-DG-ligand interaction (23), S91 cells showed substantially reduced migration (Fig. 1*D*). Moreover, RA treatment

eliminated the susceptibility to the IIH6 mAb (Fig. 1*D*). These findings suggest that the glycan recognized by IIH6 on α-DG positively regulates the motility of control S91 cells, and the reduced motility in RA-treated cells is due to the glycosylation state of α-DG. Then, we employed biochemical analyses to clarify the functional alteration of α-DG caused by RA. α-DG was enriched from RA-treated cells using cell surface biotinylation and subjected to a laminin overlay assay and immunoblotting with the IIH6 mAb. RA-treated S91 cells exhibited considerably decreased laminin binding activity of α-DG (to 43.8%) and drastically reduced IIH6 immunoreactivity (to 14.5%) (Fig. 1*E* and supplemental Fig. S1*A*). The expression of α-DG core protein and β-DG was unaltered by RA (Fig. 1*E*), indicating that the treatment resulted in a change in the glycosylation of α-DG but not in the cell surface abundance of α-DG itself. Immunofluorescence analyses also demonstrated RA-dependent disappearance of the IIH6 epitope, which intrinsically localized on the plasma membrane of S91 cells (Fig. 1*F*). Although the precise glycan structure recognized by the IIH6 mAb is still unknown, LARGE, a putative glycosyltransferase, is one of the most potent inducers of the IIH6-positive laminin-binding glycan on α-DG (37, 38). The IIH6-positive laminin-binding glycan on α-DG was induced by the transfection of LARGE in S91 cells (supplemental Fig. S2, *A* and *B*). However, RA treatment eliminated the generation of the IIH6 epitope even in cells overexpressing LARGE (supplemental Fig. S2, *A* and *B*). Taken together, these results revealed that RA had a strong effect inducing a functional change of α-DG by altering its glycosylation, which contributed at least in part to the RA-mediated suppression of cell motility.

Involvement of HNK-1ST in Functional Glycan Synthesis on α-DG and Cell Migration—In DMSO- and RA-treated S91 cells, the expression patterns of α-DG and various glycosyltransferases involved in the synthesis of laminin-binding glycan and IIH6 epitope were unchanged (Fig. 2*A*). Therefore, we sought the role of HNK-1ST, which showed dynamic induction by RA, as a key determinant controlling α-DG glycosylation. To examine whether the RA-dependent regulation of α-DG was mediated by HNK-1ST, we performed knockdown analyses of HNK-1ST using siRNA. Two different siRNAs against *HNK-1ST* (si-ST1 and -2) were used. Western blot analysis showed that both si-ST1 and -2 substantially restored the laminin binding activity (from 46.4 to 77 and 60.3%) and IIH6 epitope of α-DG (from 19.1 to 60 and 42.7%) in RA-treated S91 cells, compared with the control siRNA (si-Cont) (Fig. 2*B* and supplemental Fig. S1*B*). Then we assessed the knockdown efficacy in siRNA-transfected cells by quantitative RT-PCR. Compared with the RA-treated control, the amount of HNK-1ST mRNA was reduced to 38.9 and 47.1% in si-ST1- and -2-transfected cells, respectively (Fig. 2*C*). In contrast, forced expression of EGFP-tagged HNK-1ST effectively reduced the laminin binding activity of α-DG and IIH6 epitope production regardless of LARGE overexpression (Fig. 2*D*). Collectively, these analyses provide direct evidence that HNK-1ST negatively regulates the glycosylation of α-DG, which is a novel role for HNK-1ST as a functional regulator of α-DG. Furthermore, we analyzed the effect of down-regulation of HNK-1ST on the migration of S91 cells. Using the transwell assay, both si-ST1 and -2 were found to partially ameliorate the migration of RA-treated S91 cells (Fig. 2*E*). si-ST1 induced a much more effective recovery of migration than si-ST2, which was well correlated with the amount of IIH6 epitope shown in Fig. 2*B*, indicating significant involvement of this glyco-epitope in the migration of S91 cells.

Expression of HNK-1ST Abrogates LARGE-dependent Glycosylation on α-DG—HNK-1ST was found to have the potential to suppress the glycosylation by LARGE, prompting us to further investigate the functional interaction between HNK-1ST and LARGE in the glycosylation of α-DG. We generated an expression plasmid encoding α-DG fused to a human IgG Fc fragment (α-DG-Fc), which would be secreted into the culture medium. In addition to α-DG-Fc, LARGE-myc and HNK-1ST-EGFP were simultaneously transfected into CHO-K1 cells. α-DG-Fc was pulled down from the culture medium and analyzed by Western blotting. The extensive glycosylation induced by LARGE was detected by laminin overlay assay and immunoblotting with IIH6 mAb, as a broad and high molecular band (Fig. 3*A*). However, when α-DG-Fc was co-transfected with HNK-1ST-EGFP, there was a remarkable decrease in the laminin binding activity and almost complete loss of IIH6 immunoreactivity, despite the comparable expression of LARGE-myc (Fig. 3, *A* and *B*). The results obtained from this simple expression system clearly demonstrated that HNK-1ST actually inhibits the formation of the glycan on α-DG. Furthermore, to explore whether a similar effect could be found with other homologous sulfotransferases, we co-transfected LARGE-myc and C4ST1-EGFP or GalNAc4ST1-EGFP, both of which belong to the HNK-1ST family (39, 40). LARGE-dependent glycosylation of α-DG was not suppressed by either C4ST1 or GalNAc4ST1 (Fig. 4, *A* and *B*), indicating that the α-DG-modulating function is specific to HNK-1ST.

Interaction between α-DG and LARGE Is Unaltered in the Presence of HNK-1ST—To investigate the molecular basis underlying the inhibitory effect of HNK-1ST on the glycosylation of α-DG, we tested the following two possibilities: that HNK-1ST, causing steric hindrance, prevents glycosyltransferases from approaching α-DG, and that HNK-1ST acts as a sulfotransferase to suppress the glycosylation of α-DG. First, we analyzed whether the interaction between α-DG and LARGE is attenuated in the presence of HNK-1ST because the interaction is a crucial step in the LARGE-dependent glycosylation of α-DG (41). We observed no significant change in the interaction between α-DG-Fc and LARGE-myc, regardless of HNK-1ST-EGFP expression (supplemental Fig. S3*A*), indicating that HNK-1ST does not cause steric hindrance. In addition, we confirmed that the subcellular localization of LARGE-myc in the Golgi apparatus (42) was unaltered by co-expression with HNK-1ST-EGFP (supplemental Fig. S3*B*).

Sulfotransferase Activity Is Prerequisite for HNK-1ST to Modulate α-DG Glycosylation—Next, we generated R189A-EGFP, a plasmid encoding a form of HNK-1ST that harbors a mutation of Arg^{189} to Ala, exhibiting almost no enzymatic activity due to impaired binding to the donor substrate, 3′-phosphoadenosine 5′-phosphosulfate (PAPS) (34, 43). R189A-EGFP did not synthesize the HNK-1 carbohydrate when co-transfected with GlcAT-P, which confirmed the disappearance of its sulfotrans-

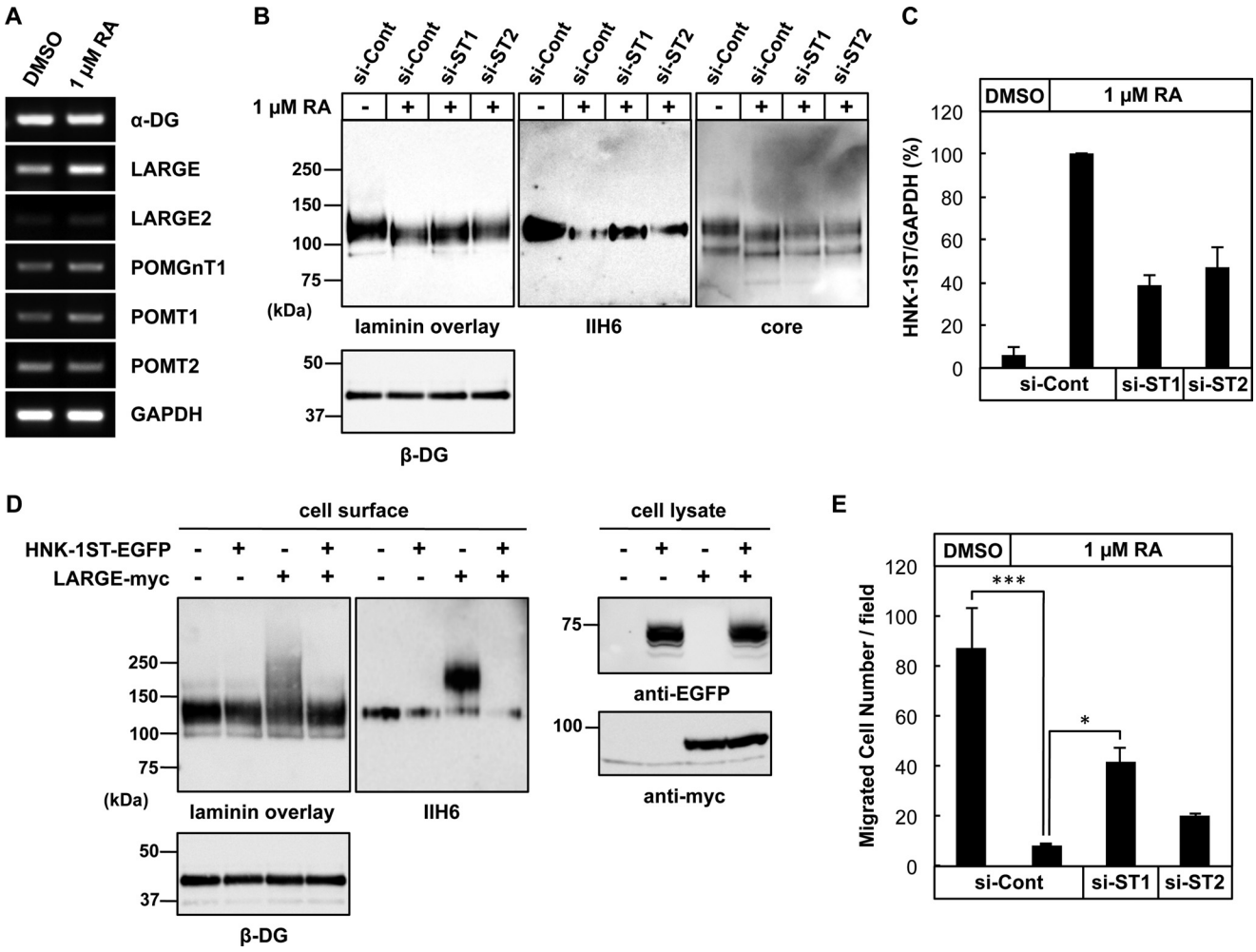

FIGURE 2. **Effect of HNK-1ST knockdown on the glycosylation of α-DG and cell motility.** *A,* S91 cells were treated with DMSO or 1 μM RA for 16 h. mRNA was extracted and subjected to RT-PCR analyses using primer sets as indicated. *B,* S91 cells were transfected with siRNA and treated with DMSO (−) or 1 μM RA (+) for 48 h. Then cell surface proteins were biotinylated, pulled down by streptavidin-agarose beads, and analyzed by laminin overlay assay or Western blotting with IIH6 mAb, anti-β-DG mAb, and α-DG core pAb. *C,* S91 cells were transfected with siRNA and treated with DMSO (−) or 1 μM RA (+) for 16 h. The amount of HNK-1ST mRNA was evaluated by quantitative RT-PCR, normalized to that of GAPDH mRNA, and shown as HNK-1ST/GAPDH. The value for RA-treated and si-Cont-transfected cells was set at 100. The graphs indicate the mean ± S.E. for three independent experiments. *D,* HNK-1ST-EGFP and LARGE-myc were transiently co-expressed in S91 cells as indicated. The cell surface proteins were biotinylated, pulled down, and analyzed by laminin overlay assay or Western blotting with IIH6 and anti-β-DG mAb (cell surface). Cell lysates were analyzed by Western blotting using anti-Myc and anti-EGFP mAbs to assess the expression of LARGE-myc and HNK-1ST-EGFP (cell lysate). *E,* S91 cells were transfected with siRNA, treated with DMSO or 1 μM RA for 48 h, and then subjected to the transwell migration assay. The insert membranes were coated with 10 μg/ml laminin-1. The graphs indicate the mean ± S.E. for three independent experiments. *, $p < 0.05$; ***, $p < 0.001$.

ferase activity (supplemental Fig. S4*A*). While showing no enzymatic activity, R189A-EGFP properly localized in the Golgi apparatus (supplemental Fig. S4*B*). Then we utilized the mutant to determine the requirement of the sulfotransferase activity of HNK-1ST in the modulation of α-DG glycosylation. Judging from the laminin overlay assay and immunoblotting with IIH6 mAb, R189A-EGFP did not suppress the LARGE-dependent modification (Fig. 5, *A* and *B*), indicating that sulfotransferase activity is essential for HNK-1ST to regulate the glycosylation of α-DG. To further confirm this evidence, we carried out an inhibition assay for PAPS production using sodium chlorate ($NaClO_3$). $NaClO_3$ is a specific inhibitor of ATP sulfurylase, an enzyme responsible for the production of PAPS in cells, resulting in depression of the intracellular sulfation (44). Treatment with 50 mM $NaClO_3$ obviously suppressed HNK-1 carbohydrate synthesis, showing that sulfate transfer is effectively abrogated in CHO-K1 cells (supplemental Fig. S5). As expected, $NaClO_3$ treatment considerably restored the LARGE-dependent glycosylation in HNK-1ST-EGFP-expressing cells (Fig. 5, *C* and *D*). Taken together, these results provide strong evidence that the sulfate transfer induced by HNK-1ST plays a regulatory role in the formation of functional glycans on α-DG.

α-DG Undergoes Sulfate Transfer by HNK-1ST—Considering that HNK-1ST also suppressed the laminin binding activity of α-DG in the absence of LARGE (Fig. 6*A*), we assumed that α-DG is the target of sulfation by HNK-1ST, rather than LARGE. Hence, to verify the incorporation of the sulfate moiety into α-DG, we labeled CHO-K1 cells with radioactive [^{35}S]-sodium sulfate. The HNK-1ST-dependent incorporation of sulfate into α-DG-Fc was detected by autoradiography (Fig. 6*B*), suggesting that a sulfated glycan is generated by HNK-1ST on α-DG, which might have a crucial effect on the formation of functional glycans on α-DG.

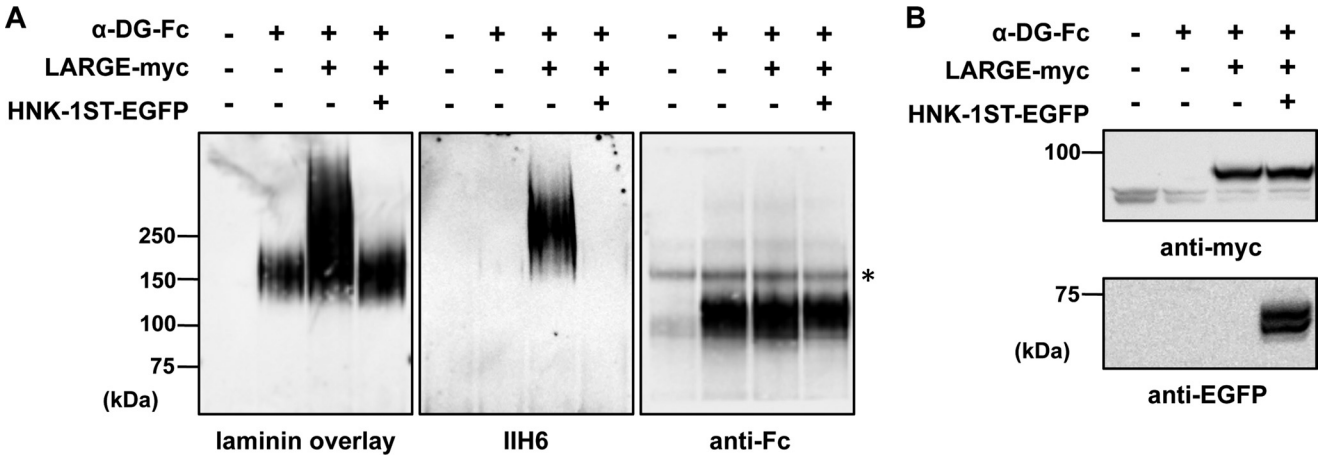

FIGURE 3. **Influence of HNK-1ST on the glycosylation and function of α-DG.** *A,* α-DG-Fc, LARGE-myc, and HNK-1ST-EGFP were transiently co-expressed in CHO-K1 cells as shown. α-DG-Fc was pulled down from the culture medium and assayed for laminin binding activity by the ligand overlay assay and for glycosylation by Western blotting with IIH6 mAb. Anti-Fc pAb was used to confirm equal protein loading. * indicates nonspecific bands. *B,* CHO-K1 cell lysates were analyzed by Western blotting using anti-Myc and anti-EGFP mAbs to assess the expression of LARGE-myc and HNK-1ST-EGFP.

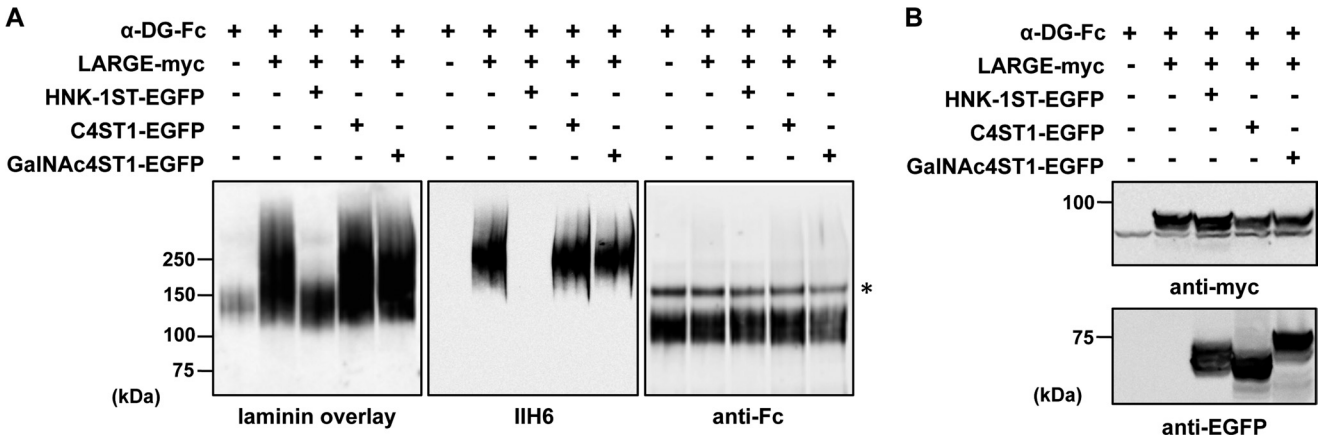

FIGURE 4. **Effect of other sulfotransferases on the glycosylation of α-DG.** *A,* CHO-K1 cells were co-transfected with α-DG-Fc, LARGE-myc, and various EGFP-fused sulfotransferases belonging to the HNK-1ST family as indicated. α-DG-Fc was pulled down from the culture medium and analyzed by laminin overlay assay and Western blotting with IIH6 mAb and anti-Fc pAb. * indicates nonspecific bands. *B,* expression of LARGE-myc and sulfotransferases was confirmed by Western blotting of cell lysates using anti-Myc mAb and anti-EGFP mAb.

DISCUSSION

Melanoma is one of the most malignant tumors, showing high metastatic ability and a rapid progression, which leads to a poor prognosis. Expression of the HNK-1 epitope is found in both primary and metastatic lesions in cases of melanoma (45, 46) and correlates with metastatic behavior (46). In addition, the HNK-1 carbohydrate positively affects the invasive and adhesive functions of melanoma cells, demonstrating the relationship between HNK-1 expression and the aggressiveness of melanomas (47). Meanwhile, HNK-1ST, one of the enzymes producing the HNK-1 carbohydrate, was identified as a candidate suppressor for melanoma invasiveness by Zhao *et al.* (6). Apparent confounding issues are that HNK-1ST functions as a tumor suppressor, although the resulting product, the HNK-1 epitope, promotes metastasis. However, it should be noted that whereas Zhao *et al.* (6) reported that HNK-1ST functions as a tumor suppressor, they failed to detect the HNK-1 epitope in 56 primary and 20 metastatic melanomas. This means that HNK-1ST might regulate invasiveness through an HNK-1 epitope-independent pathway. As a possible solution to this problem, our findings revealed that α-DG-dependent migration is another mechanism of metastasis independent of the HNK-1 epitope. Furthermore, we disclosed here a novel role of HNK-1ST, the functional regulation of α-DG via post-translational modification. This distinct function of HNK-1ST does not require GlcAT-P and GlcAT-S (Fig. 3), which accounts for the absence of the HNK-1 epitope despite the expression of HNK-1ST. Moreover, HNK-1 is not constantly expressed in melanoma lesions or cell lines (45–47), indicating that there are at least two subpopulations of melanomas, *i.e.* HNK-1-positive and -negative. Hence, α-DG-dependent migration controlled by HNK-1ST might predominate in HNK-1-negative melanomas. The unique glycan structure expressed on α-DG has been shown to have a close relationship to tumor-related phenotypes such as invasiveness (32, 33). Previous studies reported that the IIH6 mAb-reactive glycan of α-DG had a suppressive effect on tumor invasion in cases of breast, prostate, and lung carcinoma (32, 33), although we obtained the opposite results using melanoma cells (Figs. 1 and 2), suggesting that the role of α-DG varies depending on the type of cancer. Therefore, we found that

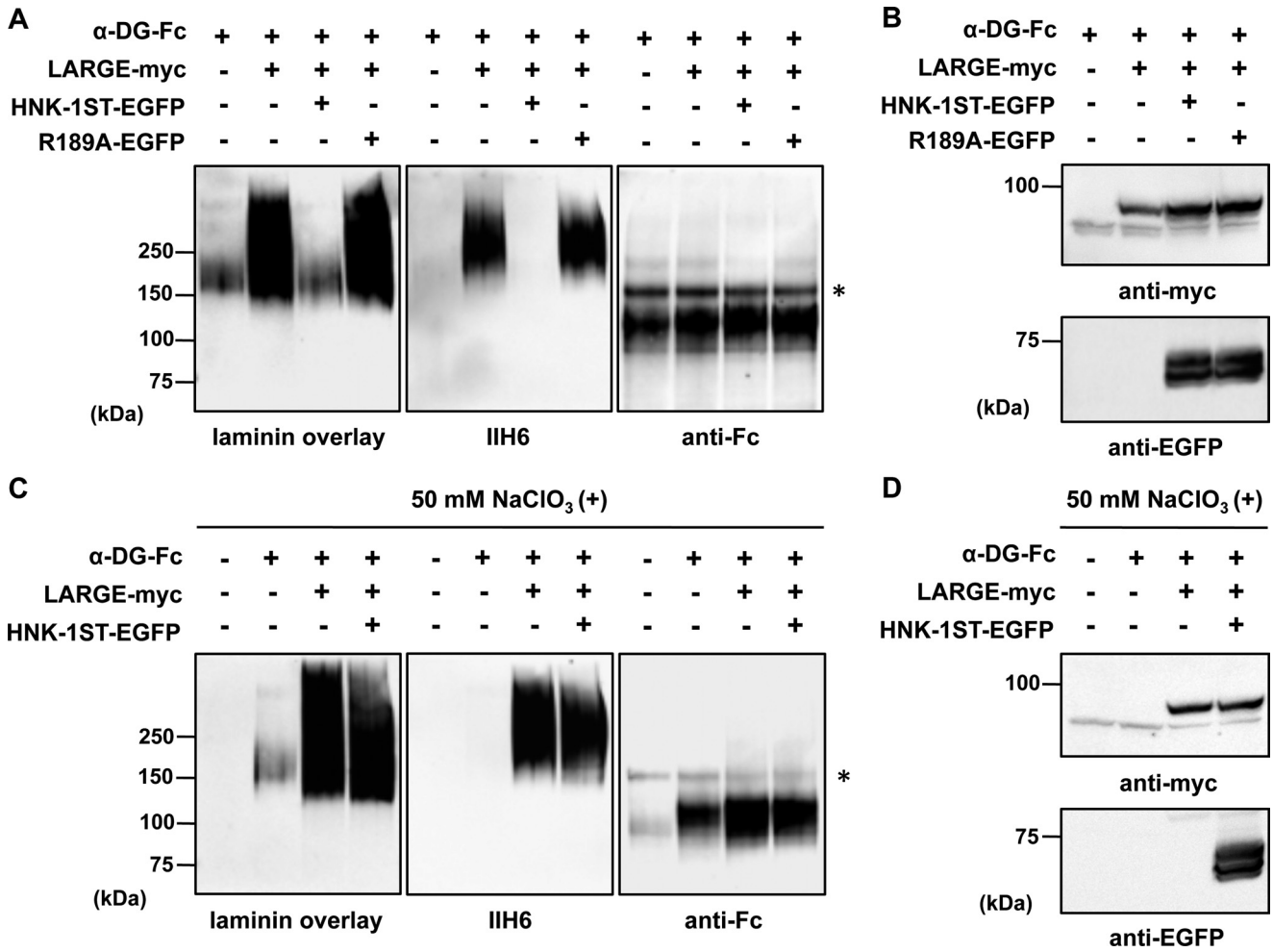

FIGURE 5. **Importance of sulfotransferase activity of HNK-1ST to the α-DG-modulating function.** *A* and *B,* requirement of the sulfotransferase activity was investigated using an activity-abolished mutant of HNK-1ST (R189A-EGFP). *A,* α-DG-Fc, LARGE-myc, and wild-type or R189A HNK-1ST-EGFP were transiently co-expressed in CHO-K1 cells as shown. α-DG-Fc was precipitated from the culture medium and analyzed by laminin overlay assay and Western blotting with IIH6 mAb and anti-Fc pAb. * indicates nonspecific bands. *B,* cell lysates were subjected to Western blotting using anti-Myc and anti-EGFP mAbs to assess the expression of LARGE-myc and wild-type or R189A HNK-1ST-EGFP. *C* and *D,* importance of the sulfotransferase activity was examined by PAPS inhibition experiments using sodium chlorate ($NaClO_3$). *C,* CHO-K1 cells were transiently transfected with α-DG-Fc, LARGE-myc, and HNK-1ST-EGFP in combination as indicated and then treated with $NaClO_3$ for 48 h. α-DG-Fc was precipitated from the culture medium and analyzed by laminin overlay assay and Western blotting with IIH6 mAb and anti-Fc pAb. * indicates nonspecific bands. *D,* expression of LARGE-myc and HNK-1ST-EGFP was confirmed by Western blotting of CHO-K1 cell lysates using anti-Myc and anti-EGFP mAbs.

HNK-1ST has a potential role modulating invasiveness by controlling the glycosylation of α-DG, leading to tumor suppression in melanoma cases.

Of particular interest was that overexpression or RA-mediated up-regulation of HNK-1ST did not completely abolish the laminin binding activity of α-DG in S91 and CHO-K1 cells, although IIH6 immunoreactivity disappeared in the same samples (Figs. 1–5). This suggests that sulfation by HNK-1ST evokes inhibitory effects predominantly on the IIH6 mAb-reactive glycan among the heterogeneous carbohydrate structures of α-DG, resulting in substantial laminin binding activity of α-DG remaining. The IIH6-reactive epitope and laminin-binding glycan are known to somewhat overlap (23). However, whether these two moieties are identical or not is still unclear despite a number of structural analyses on the glycosylation of α-DG (48–50). Chiba *et al.* (51) reported that a unique *O*-mannosyltetrasaccharide on α-DG has the ability to bind to laminin. More recently, a novel phosphate-containing glycan was identified on α-DG (52). The phosphate is attached to the 6-*O*-position of *O*-linked mannose, and post-phosphoryl glycosylation mediated by LARGE is essential for α-DG-ligand interaction and IIH6 epitope production (52). We demonstrated that LARGE could not generate the IIH6 epitope on α-DG in the presence of HNK-1ST (Figs. 3–5) and HNK-1ST indeed transferred a sulfate group onto α-DG (Fig. 6*B*), suggesting that HNK-1ST inhibits the LARGE-dependent post-phosphoryl modification of α-DG by sulfate transfer. Therefore, identification of the specific site of α-DG sulfated by HNK-1ST and the structure of the resulting sulfated glycan might be important for elucidating LARGE-dependent glycosylation.

During the preparation of this manuscript, Campbell and co-workers (53) reported that LARGE could act as a bifunctional glycosyltransferase with both xylosyl- and glucuronyltransferase activities and could generate a linear polysaccharide structure composed of repeating disaccharide units (-3-xylose-α1,3-GlcAβ1-) on α-DG. HNK-1ST has the ability to transfer a

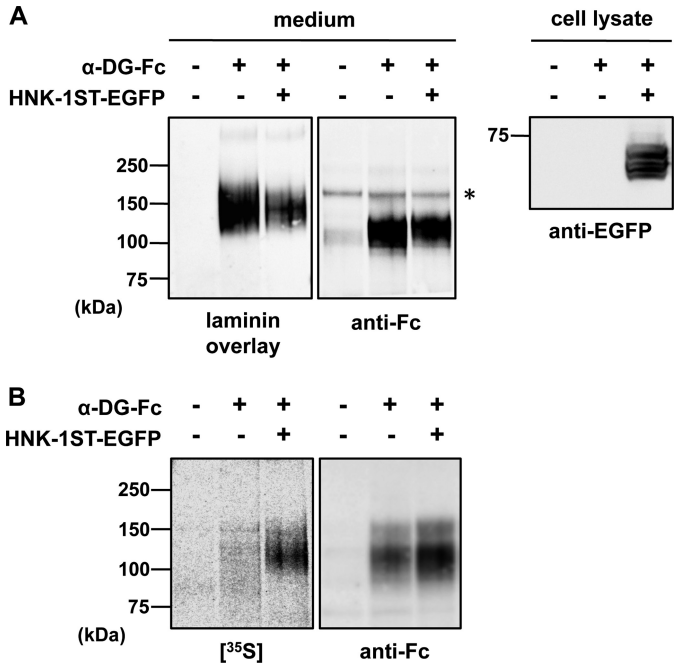

FIGURE 6. **HNK-1ST-mediated incorporation of sulfate into α-DG.** *A,* α-DG-Fc and HNK-1ST-EGFP were transiently expressed in CHO-K1 cells as shown. α-DG-Fc was pulled down from the cultured medium and analyzed by laminin overlay assay and Western blotting with anti-Fc pAb (*medium*). The cell lysates were subjected to Western blotting using anti-EGFP mAb to assess the expression of HNK-1ST-EGFP (*cell lysate*). * indicates nonspecific bands. *B,* CHO-K1 cells transiently expressing α-DG-Fc with (+) or without (−) HNK-1ST-EGFP were labeled with radioactive [^{35}S]sodium sulfate. α-DG-Fc was pulled down from the culture medium, separated by SDS-PAGE, and subjected to autoradiography and Western blotting with anti-Fc.

sulfate group to the C-3 position of terminal GlcA, where xylose is transferred; therefore, it makes sense that HNK-1ST inhibits IIH6-reactive glycan produced by LARGE. Our data presented in this study are highly important for understanding dystroglycan function via its glycosylation.

In mammals, an apparent molecular mass of α-DG varies from highly limited (about 120 kDa, *e.g.* brain) to rather broad (120–200 kDa, *e.g.* muscle) due to its glycosylation in a tissue-dependent manner (18, 19, 21). In contrast, in experiments using cell lines, forced expression of LARGE always yields an extensively glycosylated α-DG that appears as a band of ≥200 kDa on SDS-PAGE (Figs. 2–5) (37, 38, 41), implying the presence of an unidentified machinery that negatively regulates the glycosylation of α-DG *in vivo*. Hence, we propose HNK-1ST to be one such suppressive factor for α-DG function, acting as a "molecular brake" to generate properly glycosylated α-DG. In this regard, future studies might identify a pathogenic mutation of *HNK-1ST* in CMD patients, which causes hyperactivation of HNK-1ST resulting in hypoglycosylation of α-DG. Investigating the α-DG-modulating function of HNK-1ST could be a powerful means of uncovering the regulatory system of α-DG glycosylation, contributing to the development of therapeutic strategies for glycosylation-defective CMDs.

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