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Laminin-1 is a novel carrier glycoprotein for the nonsulfated HNK-1 epitope in mouse kidney

Yasuhiko Kizuka[2], Kyoko Kobayashi[2], Shinako Kakuda[3], Yukari Nakajima[4], Satsuki Itoh[4], Nana Kawasaki[4], and Shogo Oka[1,3]

[2]Department of Biological Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501; [3]Department of Biological Chemistry, Human Health Science, Graduate School of Medicine, Kyoto University, Kyoto 606-8503; and [4]Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, Tokyo 158-8501, Japan

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The HNK-1 epitope has a unique structure comprising the sulfated trisaccharide (HSO_3-3GlcAβ1-3Galβ1-4GlcNAc), and two glucuronyltransferases (GlcAT-P and GlcAT-S) are key enzymes for its biosynthesis. However, the different functional roles of these enzymes in its biosynthesis remain unclear. Recently, we reported that a nonsulfated form of this epitope, which is biosynthesized by GlcAT-S but not by GlcAT-P, is expressed on two metalloproteases in mouse kidney. In this study, we found that a novel glycoprotein carrying the nonsulfated HNK-1 epitope in mouse kidney was enriched in the nuclear fraction. The protein was affinity-purified and identified as laminin-1, and we also confirmed the *N*-linked oligosaccharide structure including nonsulfated HNK-1 epitope derived from laminin-1 by mass spectrometry. Curiously, immunofluorescence staining of kidney sections revealed that laminin-1 appeared not to be colocalized with the nonsulfated HNK-1 epitope. However, proteinase treatment strengthened the signals of both laminin-1 and the nonsulfated HNK-1 epitope, resulting in overlapping of them. These results indicate that the nonsulfated HNK-1 epitope on laminin-1 is usually embedded and masked in the robust basement membrane in tight association with other proteins. To clarify the associated proteins and the functional role of the carbohydrate epitope, we investigated the interaction between laminin-1 and alpha-dystroglycan through their glycans in mouse kidney using the overlay assay technique. We obtained evidence that glucuronic acid as well as sialic acid inhibited this interaction, suggesting that the nonsulfated HNK-1 epitope on laminin-1 may regulate its binding and play a role in maintenance of the proper structure in the kidney basal lamina.

Keywords: Dystroglycan/glucuronyltransferase/HNK-1/laminin

Introduction

It is commonly known that glycosylation is one of the major posttranslational modifications, resulting in structural diversity of proteins, and regulates molecular and cellular recognition (Kleene and Schachner 2004). Among carbohydrates, we have been focusing on the HNK-1 (human natural killer-1) epitope comprising sulfated trisaccharide (HSO_3-3GlcAβ1-3Galβ1-4GlcNAc) (Schwarting et al. 1987; Voshol et al. 1996). The HNK-1 epitope is highly expressed in the nervous system, and is carried on cell adhesion molecules (NCAM, L1, and P0), extracellular matrix proteins (tenascin-R and phosphacan), and glycolipids (SGGL-1 and SGGL-2) (Liedtke et al. 2001; Saghatelyan et al. 2000). We cloned two glucuronyltransferases (GlcAT-P and GlcAT-S) that regulate the biosynthesis of this epitope (Terayama et al. 1997; Seiki et al. 1999). On analysis of GlcAT-P-deficient mice, it was revealed that the HNK-1 epitope synthesized by GlcAT-P plays important roles in high-ordered brain functions such as synaptic plasticity, learning, and memory (Yamamoto et al. 2002). However, the detailed function of GlcAT-S in vivo remained unclear. Recently, we found that GlcAT-S was expressed in kidney rather than brain, and that the nonsulfated type of HNK-1 epitope (GlcAβ1-3Galβ1-4GlcNAc) synthesized by GlcAT-S was expressed on two metalloproteases in mouse kidney and the carbohydrate structure on complex-type *N*-glycans of meprin-alpha had been determined by mass spectrometry (MS) (Tagawa et al. 2005). Although these two glucuronyltransferases exhibit high homology in amino acid sequence and similar transferase activity of glucuronic acid toward glycoprotein in vitro (Kakuda et al. 2004), they seem to differ in physiological function in vivo. To well understand the function of GlcAT-S in vivo, we investigated the expression and function of the nonsulfated HNK-1 epitope synthesized by GlcAT-S in mouse kidney in more detail.

In this study, we demonstrated that a novel carrier protein, laminin-1, was also modified with the nonsulfated HNK-1 epitope in mouse kidney. Laminin-1 (comprising alpha-1, beta-1, and gamma-1 subunits), which is known to be a major component of the basement membrane, consists of many domains that bind various molecules (Sasaki et al. 2004). Moreover, it has been reported that some of these interactions are regulated by carbohydrates such as heparin and the HNK-1 epitope (Hall and Schachner 1998). Here, we focused on the interaction between laminin-1 and alpha-dystroglycan through glycans in mouse kidney, which revealed that the nonsulfated HNK-1 epitope may regulate this interaction. These lines of evidence suggest a novel functional role of the nonsulfated HNK-1 epitope synthesized by GlcAT-S in kidneys.

Results and discussion

Existence of novel carrier glycoproteins bearing the nonsulfated HNK-1 epitope in the nuclear fraction of mouse kidney

Previously, we reported that the nonsulfated HNK-1 epitope synthesized by GlcAT-S is expressed in mouse kidney and

[1]To whom correspondence should be addressed: Tel: +81-75-751-3959; Fax: +81-75-751-3959; e-mail: shogo@hs.med.kyoto-u.ac.jp

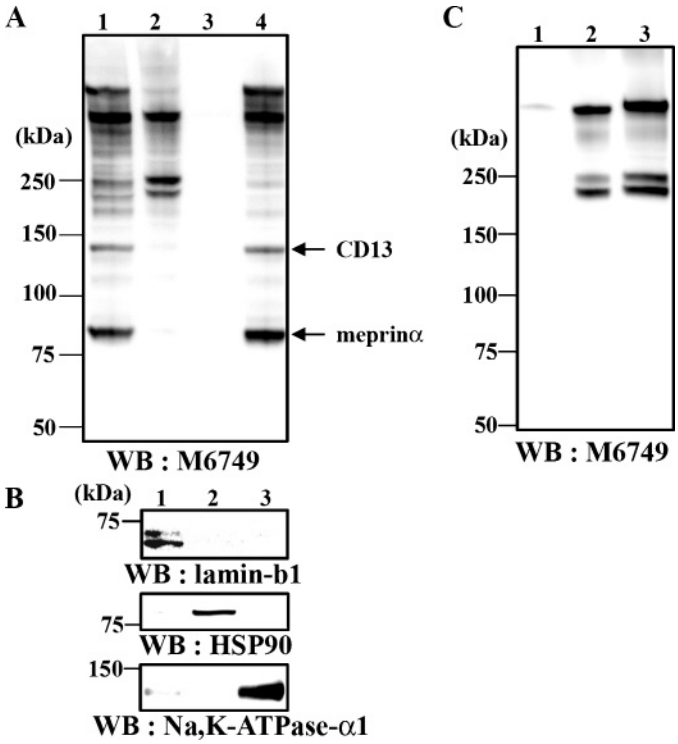

Fig. 1. Western blotting of a fractionated kidney homogenate with M6749 mAb. (**A**) A kidney from a 4-week-old C57BL/6 mouse was homogenized and fractionated as described under *Materials and methods*, and then proteins were Western blotted with M6749 mAb. Lane 1, total homogenate; lane 2, nuclear fraction; lane 3, soluble fraction; lane 4, membrane fraction. (**B**) Fractionated proteins from mouse kidney were Western blotted with anti-lamin-b1 (upper panel), anti-HSP90 (middle panel), and anti-Na,K-ATPase-alpha-1 (lower panel) antibodies. Lane 1, nuclear fraction; lane 2, soluble fraction; lane 3, membrane fraction. (**C**) A kidney nuclear fraction was extracted with three kinds of Tris-buffered saline containing 1% Triton X-100 (lane 1), 1% SDS (lane 2), and 6 M urea (lane 3), respectively. After ultracentrifugation, the supernatant was used as the solubilized proteins, being Western blotted with M6749 mAb.

we identified two carrier glycoproteins, meprin-alpha and CD13/aminopeptidase-*N*, that had been purified from the membrane fraction using an M6749 monoclonal antibody (mAb) column (Tagawa et al. 2005). M6749 mAb, which exhibits similar epitope specificity to HNK-1 mAb, recognizes the HNK-1 epitope with or without the sulfate group at the 3-position of the terminal glucuronic acid (Tagawa et al. 2005), while the HNK-1 antibody only reacts with sulfated glucuronic acid. These two carrier proteins are membrane-anchored metalloproteases; therefore, they are detected in the kidney membrane fraction with M6749 mAb, as shown in Figure 1A, lane 4. During the course of a further study on the function of the epitope, we noticed that other carrier glycoproteins of around 200 kDa and over 250 kDa (approx. 400 kDa) existed in the mouse kidney homogenate (Figure 1A, lane 1). To characterize these molecules, Western blotting was performed using biochemically fractionated kidney proteins with M6749 mAb (Figure 1A, lanes 2–4). To our surprise, three bands were detected for the nuclear fraction (Figure 1A, lane 2). These bands were not detected with HNK-1 mAb (data not shown), suggesting that the nonsulfated HNK-1 carbohydrate is expressed on these glycoproteins as in the case of meprin-alpha and CD13. Though other unidentified carrier proteins other than meprin-alpha and CD13 existed in the membrane fraction (Figure 1A, lane 4), we focused on proteins in the nuclear fraction because many carriers of the HNK-1 epitope so far reported have been membrane proteins or extracellular matrix proteins, suggesting that analysis of the carrier proteins in the nuclear fraction would provide us with new knowledge about the function of the HNK-1 epitope. The nuclear fraction was prepared from a kidney homogenate by low-speed centrifugation (1000 $\times$ *g*), and thus probably included nuclei, cell debris, and unbroken cells; however, bands corresponding to meprin-alpha and CD13 were not detected for the nuclear fraction, suggesting that the protein bands detected with M6749 mAb were not due to simple contamination by membrane glycoproteins. Besides, we also confirmed the validity of our fractionation by Western blotting with the antibodies specific for the resident proteins of each fraction (lamin-b1, HSP90, and Na,K-ATPase-alpha-1) (Figure 1B). To characterize the carrier glycoproteins of the nonsulfated HNK-1 epitope in the nuclear fraction, we examined the solubility of these proteins. As a result, the glycoproteins were found to be hardly solubilized with a neutral detergent like Triton X-100, while urea as well as SDS was able to solubilize them (Figure 1C), indicating that they were tightly associated with the membrane but not membrane proteins. We also examined the expression level of the nonsulfated HNK-1 epitope in the nuclear fraction throughout development and found that higher expression was seen in young mice (from 2- to 4-week-old) than in adult mice (over 10-week-old) (data not shown). Therefore, kidneys from young mice (from 2- to 4-week-old) were used in subsequent experiments.

Identification of the carrier protein for the nonsulfated HNK-1 epitope as laminin-1

To identify the carrier proteins for the nonsulfated HNK-1 epitope, proteins solubilized with SDS from the kidney nuclear fraction were immunoprecipitated with M6749 mAb. The precipitate was subjected to SDS–PAGE, and then we performed Western blotting and protein staining (Figure 2A, left and right panel, respectively). Then, protein bands corresponding to ones immunoreactive with M6749 mAb (200 and 400 kDa) were excised, and analyzed by LC/MS/MS (data not shown). As a result, the proteins were identified as laminin-alpha-1 (400 kDa), -beta-1 (200 kDa), and -gamma-1 (200 kDa) (Figure 2A), which are well-known subunits of laminin-1. To confirm this, we performed an immunoprecipitation experiment on an SDS lysate of the kidney nuclear fraction using an anti-laminin-1 polyclonal antibody (pAb), which recognizes all subunits comprising laminin-1. As shown in Figure 2B, the immunoprecipitates with anti-laminin-1 pAb were reactive with M6749 mAb, surely indicating that laminin-1 is a carrier glycoprotein of the nonsulfated HNK-1 epitope. However, as shown in Figure 2B, left panel, it seemed that the beta-1 and gamma-1 subunits were sometimes not separated under similar conditions, so it was difficult to determine whether or not both subunits were actually carrier proteins of the nonsulfated HNK-1 epitope. To clarify this, we performed immunoprecipitation experiments with three antibodies specific for the respective laminin-1 subunits (Figure 2C). Because they were denatured on the addition of SDS and beta-mercaptoethanol, it was considered that the three subunits (alpha-1, beta-1, and gamma-1) became dissociated and

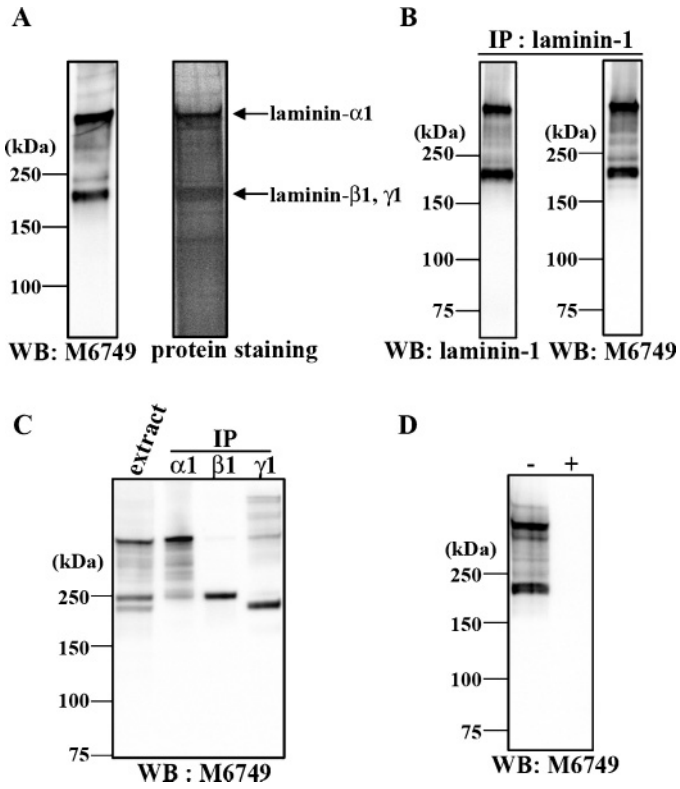

Fig. 2. Identification of laminin-1 as a novel carrier protein for the nonsulfated HNK-1 epitope in mouse kidney. (**A**) Proteins in the nuclear fraction were solubilized with SDS and then immunoprecipitated with M6749 mAb. The immunoprecipitate was subjected to SDS–PAGE and Western blotting with M6749 mAb (left panel) or protein staining (right panel). (**B**) Solubilized proteins from the nuclear fraction were immunoprecipitated with anti-laminin-1 pAb, and then subjected to Western blotting with anti-laminin-1 pAb (left panel) or M6749 mAb (right panel). (**C**) Solubilized proteins from the nuclear fraction (extract) were immunoprecipitated with anti-laminin-α1 pAb (α1), anti-laminin-β1 pAb (β1), or anti-laminin-γ1 pAb (γ1), and then Western blotted with M6749 mAb. (**D**) Proteins in the nuclear fraction were solubilized and then incubated in the absence (−) or presence (+) of *N*-glycosidase F. After that, the proteins were subjected to Western blotting with M6749 mAb.

thus could be precipitated separately. As shown in Figure 2C, we confirmed that all three subunits were modified by the nonsulfated HNK-1 epitope. Next, the glycoproteins in the nuclear fraction were treated with *N*-glycosidase F and then subjected to Western blotting with M6749 mAb to determine whether the nonsulfated HNK-1 epitope of laminin-1 was on *N*-glycan or not (Figure 2D). Expectedly, after treatment with *N*-glycosidase F, bands immunoreactive with M6749 mAb disappeared, suggesting that the nonsulfated HNK-1 epitope was expressed on the *N*-glycan of laminin-1. To confirm the specificities of anti-laminin-1 pAb and M6749 mAb, we also performed similar experiments using immunoprecipitated laminin-1 of the kidney nuclear fraction (Supplementary Figure 1). The immunoreactivity with M6749 mAb disappeared after *N*-glycosidase F treatment, while that with anti-laminin-1 pAb still remained even after the glycosidase treatment.

Laminin-1 does not seem to localize in nucleus, because laminin-1 is well known as a glycoprotein comprising basement membrane. Furthermore, immunohistochemical staining of mouse kidney with anti-laminin-1 antibodies indicated that laminin-1 was detected at the basolateral side of cells but not at the nucleus (see Figure 4). These lines of evidence suggest that laminin-1 detected in the nuclear fraction was not localized in the nucleus but was tightly associated with the plasma membrane and recovered in the nuclear fraction for unknown reasons.

LC/MS/MS of the nonsulfated HNK-1 carbohydrate of laminin-1

Next, we performed the structural study by MS about *N*-glycans of laminin-1 derived from mouse kidney to confirm that the nonsulfated HNK-1 carbohydrate was actually expressed on laminin-1. For this purpose, we extracted and purified laminin-1 from mouse kidney nuclear fraction using immunoprecipitation with anti-laminin-1 pAb. The purified laminin-1 was subjected to SDS–PAGE. The gel containing alpha-1 subunit of laminin-1 was excised and treated with *N*-glycosidase F. The released *N*-Linked oligosaccharides were subjected to LC/MS/MS analysis. Figure 3 shows the MS/MS spectrum of one of the nonsulfated HNK-1 carbohydrates ($dHex_2Hex_5HexA_1HexNAc_5$). The presence of the nonsulfated HNK-1 motif-distinctive B ion, $GlcAGalGlcNAc^+$ (*m/z* 542), and the Y ions, including $[M\,(GlcAGalGlcNAc) + H]^+$ (*m/z* 1,773), clearly indicated that the nonsulfated HNK-1 carbohydrate is expressed on *N*-linked oligosaccharides of laminin-1.

Localization of laminin-1 and the M6749 epitope in mouse kidney

Laminin-1 is well known as one of the components of the basement membrane at the basolateral side of cells in many epithelial tissues (Sasaki et al. 2004), while we have reported that the nonsulfated HNK-1 epitope is mainly localized at the apical side of proximal tubules in mouse kidney (Tagawa et al. 2005, and see Figure 4B and E). Furthermore, as shown in Figure 1A, it seemed that laminin-1 is one of the major constituents of the nonsulfated HNK-1 epitope in mouse kidney. These results raised the question of whether or not nonsulfated HNK-1 epitope is colocalized with laminin-1 in mouse kidney. To investigate this, kidney sections were immunostained with both anti-laminin-1 pAb and M6749 mAb (Figure 4). As expected, the apical side of proximal tubules in the renal cortex was stained with M6749 mAb (Figure 4B), while the immunoreactivity of laminin-1 was detected on the opposite side of the tubules (Figure 4A). That is to say, laminin-1 appeared not to be colocalized with the M6749 epitope (Figure 4C). Regarding this discrepancy, it has been reported that several epitopes on laminin-1 were difficult to detect because the basement membrane is structurally so strong that certain epitopes are masked (Sasaki et al. 2002). For the exposure of the laminin-1 epitope usually embedded, kidney sections were treated with proteinase K and then immunostained (Figure 4G, H, and I). The level of staining of laminin-1 was enhanced by this treatment (compare Figure 4D and G). Moreover, the signal with M6749 mAb was also increased by the proteinase treatment and it seemed that the signal at the lateral side of tubules with M6749 mAb emerged (compare Figure 4E and H), resulting in overlapping of laminin-1 and the nonsulfated HNK-1 epitope at the lateral side of tubules in the kidney cortex (Figure 4I). These results indicated that most of the nonsulfated HNK-1 epitope on laminin-1 was usually masked at the lateral side of renal tubules in mouse kidney.

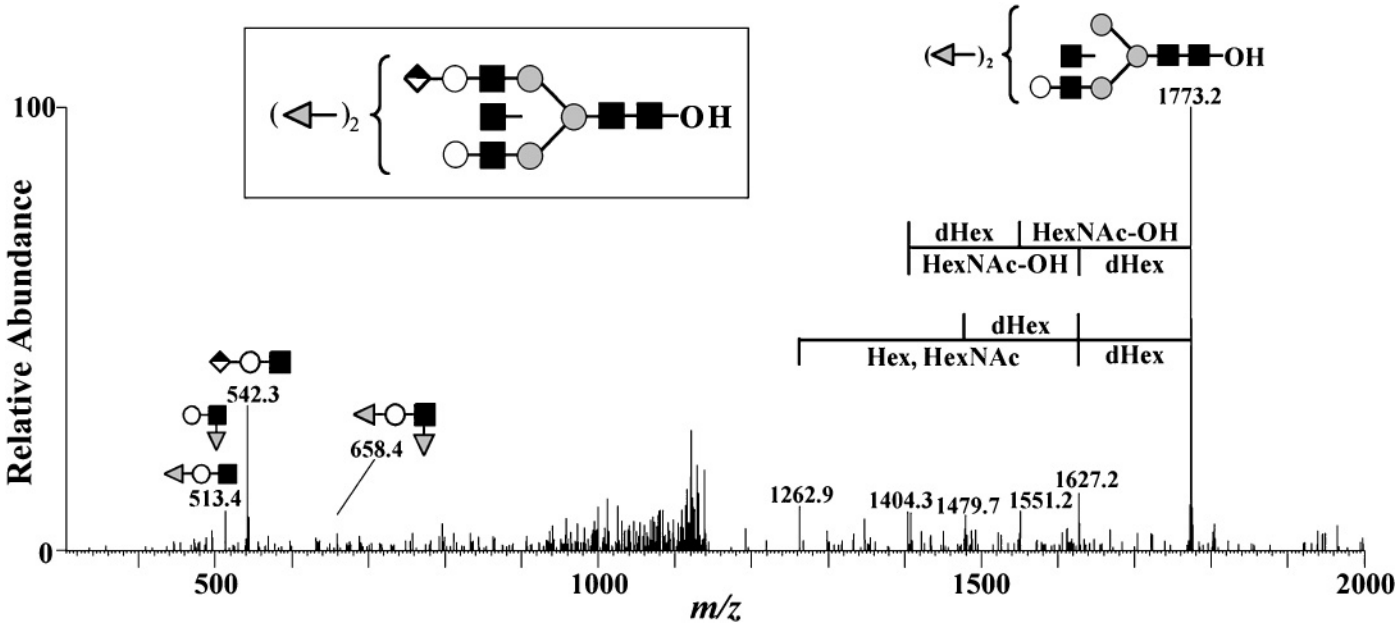

Fig. 3. LC/MS/MS of *N*-linked oligosaccharides from alpha-1 subunit of laminin-1. The MS/MS spectrum of *N*-linked oligosaccharide containing nonsulfated HNK-1 carbohydrate released from the alpha-1 subunit of laminin-1 (precursor ion: *m/z* 1159.0). Circle, Hex; square, HexNAc; triangle, dHex; diamond, HexA. The deduced structure of the nonsulfated HNK-1 carbohydrate is shown in the inset.

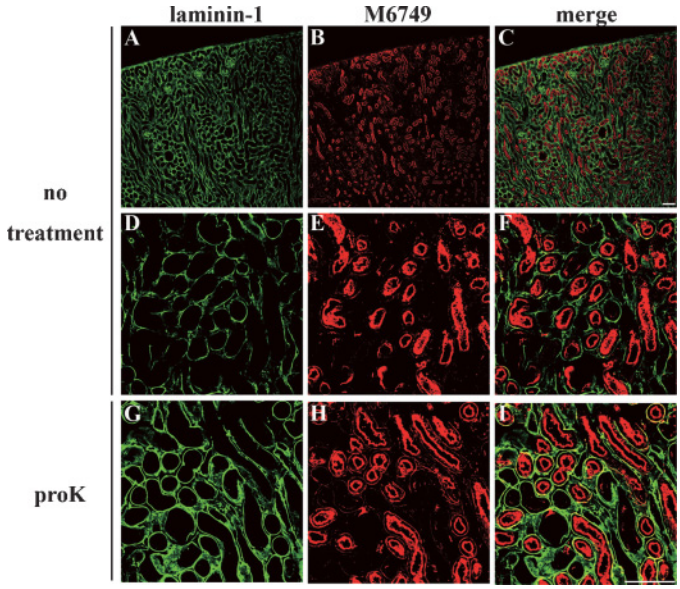

Fig. 4. Double immunofluorescence staining of kidney sections with anti-laminin-1 pAb and M6749 mAb. Kidney sections (40 μm thick) were immunostained with both anti-laminin-1 pAb (**A**, **D,** and **G**) and M6749 mAb (**B**, **E,** and **H**), overlay images being shown in C, F, and I. A–C: low magnification of kidney sections of the renal cortex. D–I: high-magnification images. To partially disrupt the robust basement membrane structures, sections were treated with proteinase K before immunostaining (**G–I**) as described under *Materials and methods*. Bars: 100 μm.

Influence of the nonsulfated HNK-1 epitope on the binding of laminin-1 and alpha-dystroglycan

As described above, laminin-1 was recovered in the nuclear fraction due to its tight association with membrane and the nonsulfated HNK-1 epitope expressed on laminin-1 was masked probably due to the formation of a protein complex. To search for an associated protein that conceals the nonsulfated HNK-1 epitope on laminin-1, we carried out an overlay assay analysis. Thus, a mouse kidney homogenate was biochemically fractionated and then subjected to laminin-1 overlay assaying (Figure 5A). For this method, anti-laminin-1 pAb was used to detect the overlaid laminin-1. Laminin-1 endogenously expressed in mouse kidney was also detected, as shown in Figure 5A, left panel, asterisks. However, overlaying of laminin-1 protein allowed new bands (around 120 kDa) to be seen, as shown in Figure 5A, right panel. Considering the high intensity of this signal and the molecular weight of the material in this band, it was suspected that this laminin-1-binding protein was alpha-dystroglycan, which is well known to interact with laminin (Ervasti and Campbell 1993). Moreover, in fact, a previous study demonstrated that alpha-dystroglycan in kidney interacted with laminin-1 (Durbeej and Campbell 1999). To determine whether or not these bands in Figure 5A, right panel, were derived from alpha-dystroglycan, the same samples were subjected to Western blotting with IIH6 mAb (Figure 5B), which was thought to recognize the glycosylated form of alpha-dystroglycan (Ervasti and Campbell 1993). As a result, the expression pattern of the IIH6 epitope was found to be very similar to that of the proteins detected with laminin-1, indicating that the protein associated with laminin-1 in mouse kidney was alpha-dystroglycan.

To determine whether the glycans on both laminin-1 and alpha-dystroglycan were involved in this interaction or not, we performed the overlay assay in the presence of various inhibitors (Figure 5C). As expected, IIH6 mAb inhibited this interaction (Figure 5C, middle panel), which indicated that the glycan (s) on alpha-dystroglycan was involved in it. Next, the laminin-1 overlay assay was carried out in the presence of EDTA. As described previously (Ervasti and Campbell 1993), the interaction of

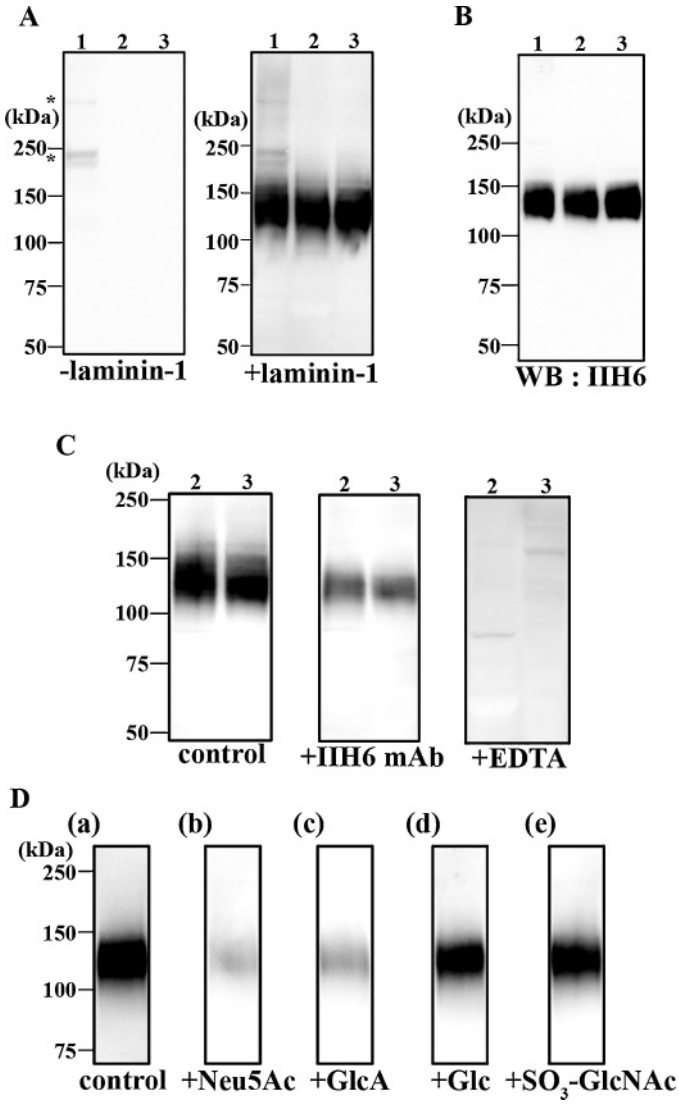

Fig. 5. Laminin-1 overlay assaying of fractionated kidney proteins. (**A**) Fractionated kidney proteins were subjected to SDS–PAGE and then transferred to a nitrocellulose membrane. Then, after the membrane had been incubated with (right panel) or without (left panel) purified laminin-1, Western blotting was performed with anti-laminin-1 pAb to detect bound laminin-1. Lane 1, nuclear fraction; lane 2, soluble fraction; lane 3, membrane fraction. The asterisks indicate the endogenously expressed laminin-1 that reacted with anti-laminin-1 pAb. (**B**) The kidney nuclear fraction (lane 1), soluble fraction (lane 2), and membrane fraction (lane 3) were subjected to SDS–PAGE and Western blotting with anti-alpha-dystroglycan mAb, clone IIH6. (**C**) Lane 2, soluble fraction; lane 3, membrane fraction. The laminin-1 overlay assay was carried out with or without an inhibitor. During incubation with laminin-1, IIH6 mAb (middle panel), or 1 mM EDTA (right panel), or no inhibitor (left panel) was added. (**D**) Soluble fraction from mouse kidney was subjected to the laminin-1 overlay assay with various monosaccharides. During incubation with laminin-1, 20 mM Neu5Ac (b), GlcA (c), Glc (d), 6-sulfo-GlcNAc (e), or the same volume of water (a) was added.

laminin-1 and alpha-dystroglycan depends on Ca^{2+}, and EDTA blocks this interaction. Consistent with this, the addition of EDTA abolished the binding of laminin-1 to alpha-dystroglycan in mouse kidney (Figure 5C, right panel). It was previously reported that the *O*-mannosylated glycan terminating sialic acid of alpha-dystroglycan was important for the binding with laminin (Chiba et al. 1997), and that the loss of *O*-mannosylated glycan due to glycosylation defects causes certain myopathy (Barresi et al. 2004). The importance of the sialic acid residue on the *O*-mannosylated glycan of alpha-dystroglycan had previously been shown by the finding that sialidase treatment weakened the interaction with laminin (Yamada et al. 1996). In addition to this, we had expected that the nonsulfated HNK-1 epitope was relevant to this interaction. In other words, the terminal glucuronic acid has an effect on this interaction, so we performed an inhibition experiment using glucuronic acid or sialic acid (Figure 5D). As a result, this interaction was found to be significantly inhibited in the presence of 20 mM sialic acid (Neu5Ac) monosaccharide (Figure 5D, panel (b)). Besides, glucuronic acid monosaccharide (20 mM) also inhibited the interaction of laminin-1 with alpha-dystroglycan, even at a slightly weaker level than that of sialic acid (Figure 5D, panel (c)), while glucose monosaccharide had no effect on this interaction (Figure 5D, panel (d)). We also checked the dose dependence of Neu5Ac and GlcA in inhibition toward the interaction (Supplementary Figure 2). It was revealed that these two acidic monosaccharides inhibited the binding in a dose-dependent manner and that the concentration of 20 mM was sufficient for the effective inhibition under our experimental conditions. In addition, we confirmed that 6-sulfated *N*-acetylglucosamine monosaccharide (20 mM) hardly inhibited this interaction (Figure 5D, panel (e)), excluding a possibility that whatever kind of negatively charged monosaccharide has an ability to inhibit the laminin-1-alpha-dystroglycan binding. These results also suggested that carboxy group in these two monosaccharides may be important for the interaction. These results constituted evidence that in addition to the sialic acid residue, the nonsulfated HNK-1 epitope was relevant to this binding, and this is the first finding that GlcA may be involved in this interaction.

As to the significance of the nonsulfated HNK-1 epitope of laminin-1 in the interaction with alpha-dystroglycan, it should be noted that alpha-dystroglycan in mouse kidney was not thought to be modified by the nonsulfated HNK-1 epitope because of the lack of a major and broad band with M6749 mAb at around 120 kDa (Figure 1A). Therefore, the GlcA on laminin-1 but not on alpha-dystroglycan was important for the interaction. As described above (see *Introduction*), laminin-1 is known to interact with various molecules including alpha-dystroglycan, and it has been reported that carbohydrate molecules are involved in some of these interactions. These reports indicated that laminin-1 recognizes and binds with the carbohydrate moieties of counterpart molecules. In this study, however, we show that the unique carbohydrate epitope is expressed on laminin-1 itself, and a significant inhibition of GlcA monosaccharide was observed. Unfortunately, EHS-laminin used in the overlay assay was not modified by the nonsulfated HNK-1 epitope as shown in Supplementary Figure 3. In other words, it indicated that EHS-laminin could interact with alpha-dystroglycan without GlcA. These lines of evidence suggest that GlcA regulates the binding properties of laminin-1 rather than acting as a component of the binding site between laminin-1 and alpha-dystroglycan. However, further studies are necessary to clarify these issues.

Interaction between laminin-1 and alpha-dystroglycan in mouse kidney

As described above, laminin-1 interacts with alpha-dystroglycan in vitro. However, there has been no report showing that

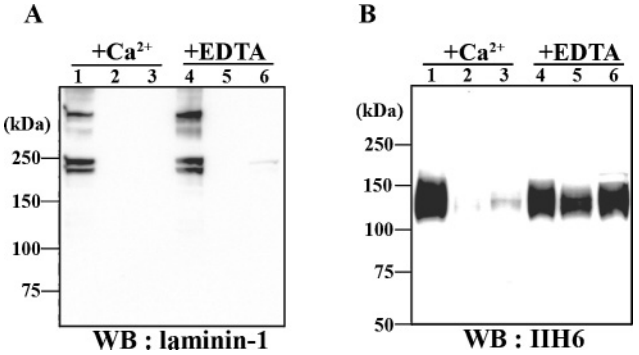

Fig. 6. Effect of Ca^{2+} on the distributions of laminin-1 and alpha-dystroglycan. A kidney was homogenized in the presence of 1 mM $CaCl_2$ (lanes 1–3) or 1 mM EDTA (lanes 4–6), and then fractionated to a nuclear fraction (lanes 1 and 4), a soluble fraction (lanes 2 and 5), and a membrane fraction (lanes 3 and 6), respectively. Proteins were subjected to SDS–PAGE and Western blotted with anti-laminin-1 pAb (**A**) or IIH6 mAb (**B**).

alpha-dystroglycan was really in the same protein complex with laminin-1 in vivo, while Durbeej and Campbell performed a laminin-1 overlay assay of a kidney homogenate before us (Durbeej and Campbell 1999). Unfortunately, however, we were not able to confirm this interaction under natural conditions by coimmunoprecipitation analysis because in our investigation, laminin-1 was not solubilized unless denaturing agents such as SDS and urea were present (Figure 1C). Then, we utilized the property that EDTA inhibited the binding between alpha-dystroglycan and laminin-1 by chelating Ca^{2+} to demonstrate the physical association through Ca^{2+} in mouse kidney under natural conditions. For this purpose, a kidney was homogenized and fractionated with Ca^{2+} or EDTA, and then Western blotting was performed with anti-laminin-1 pAb or IIH6 mAb (Figure 6). As a result, when 1 mM Ca^{2+} was present, both laminin-1 and alpha-dystroglycan were detected in the same fraction (Figure 6A, lane 1 and B, lane 1), that is, in the nuclear fraction. On the other hand, alpha-dystroglycan was detected not only in the nuclear fraction but also in the soluble and membrane fractions under the Ca^{2+} depletion conditions (i.e., the presence of 1 mM EDTA during homogenization and fractionation) (Figure 6B, lanes 4, 5, and 6), while the laminin-1 distribution remained unchanged regardless of Ca^{2+} (Figure 6A, lane 4), indicating that alpha-dystroglycan was dissociated from the protein complex in the kidney basement membrane by EDTA. These results suggest that laminin-1 and alpha-dystroglycan endogenously bind in mouse kidney.

In this study, we demonstrated that the nonsulfated HNK-1 epitope was expressed on laminin-1 in mouse kidney and obtained evidence that the epitope on laminin-1 may be involved in the interaction with alpha-dystroglycan. We are now trying to generate GlcAT-S-gene-deficient mice and if we are successful, more details of the role of the nonsulfated HNK-1 epitope on laminin-1 in mouse kidney will probably be revealed.

Materials and methods

Materials

M6749 mAb was a generous gift from Dr H. Tanaka (Kumamoto University, Kumamoto, Japan). Rabbit anti-laminin-1 pAb was purchased from Sigma. Goat anti-laminin-alpha-1 pAb, rabbit anti-laminin-beta-1 pAb, and rabbit anti-laminin-gamma-1 pAb were purchased from Santa Cruz. Anti-alpha-dystroglycan mAb (clone IIH6C4) and anti-Na,K-ATPase-alpha-1 mAb were from Upstate. Anti-lamin-b1 mAb, horseradish peroxidase (HRP)-conjugated anti-mouse IgM, and HRP-conjugated anti-rabbit IgG were from Zymed Laboratories Inc. Anti-HSP90 pAb was obtained from Lab Vision. Alexa Fluor 546-conjugated anti-mouse IgM and Alexa Fluor 488-conjugated anti-rabbit IgG were obtained from Molecular Probes. D-Glucuronic acid (GlcA) and proteinase K were purchased from Wako Chemicals (Osaka, Japan), *N*-acetylneuraminic acid (Neu5Ac) from bovine milk was from Seikagaku Corporation (Tokyo, Japan), D-glucose (Glc) was from Nacalai Tesque (Kyoto, Japan), and *N*-acetylglucosamine 6-sulfate (6-sulfated GlcNAc) sodium salt was from Sigma. Before use, acidic monosaccharides (GlcA and Neu5Ac) were solubilized in water and neutralized with 1 M NaOH.

Fractionation of mouse kidney homogenate

A whole kidney from a C57BL/6 (from 2- to 4-week-old) mouse was homogenized with a Polytron homogenizer in nine volumes of 20 mM Tris–HCl (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, and protease inhibitors (Nacalai Tesque). The homogenate was centrifuged at 1000 × *g* for 10 min at 4°C and the resulting pellet was used as the nuclear fraction. The supernatant was centrifuged at 105,000 × *g* for 1 h at 4°C, and the resulting pellet and supernatant were used as the membrane and soluble fractions, respectively.

SDS–PAGE and Western blotting

SDS–PAGE and Western blotting were carried out as described previously (Tagawa et al. 2005). Protein bands were detected with SuperSignal West Pico (Pierce) using a Luminoimage Analyzer LAS-3000 (Fuji, Tokyo, Japan).

Extraction of glycoproteins bearing the nonsulfated HNK-1 carbohydrate from the nuclear fraction

A nuclear fraction prepared from mouse kidney as described above was treated with three buffers, Tris-buffered saline (TBS, 20 mM Tris–HCl, pH 7.4, containing 150 mM NaCl) containing 1% Triton X-100, TBS containing 1% SDS, or TBS containing 6 M urea. After ultracentrifugation (105,000 × *g*, 1 h), the supernatant was used for Western blotting.

Immunoprecipitation

The nuclear fraction was lysed with a lysis buffer consisting of 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% SDS, and 1% β-mercaptoethanol, and then boiled. After centrifugation, the clarified lysate was diluted with TBS containing Nonidet P-40 (final 0.5%) to reduce the concentration of SDS (final 0.1%). The solution was incubated with anti-laminin-1 antibodies (final 4 μg/mL) for 30 min at 4°C followed by incubation with protein G-Sepharose TM4 Fast Flow (Amersham Bioscience) for 2 h with gentle shaking. The beads were precipitated by centrifugation and washed three times with an excess volume of TBS containing 0.1% Triton X-100. Proteins bound to the beads were eluted by boiling in a Laemmli sample buffer.

Purification and identification of glycoproteins bearing the nonsulfated HNK-1 carbohydrate

The nuclear fraction was lysed, and immunoprecipitation was performed with M6749 mAb and rat anti-mouse IgM Sepharose4B (Zymed). The precipitated glycoproteins bound to M6749 mAb were subjected to SDS–PAGE and then stained with a VisPro 5 Minutes Protein Stain Kit Avegene (Taipei, Taiwan) according to the manufacturer's protocol. A piece of polyacrylamide gel containing around 200 kDa and over 250 kDa (approx. 400 kDa) glycoproteins was excised from the polyacrylamide gel. After carboxymethylation, the glycoproteins in the gel were digested with trypsin. The peptides extracted from the gel were separated with a capillary HPLC (Paradigm; Michrom BioResources, Auburn, CA) coupled online with a linear ion-trap mass spectrometer (Finnigan LTQ; Thermo Fisher Scientific, Waltham, MA). The LC was equipped with a C18 column (Magic C18 0.2 × 50 mm, 3 μ; Michrom BioResources, Auburn, CA). The eluents consisted of H_2O containing 2% CH_3CN and 0.1% formic acid (pump A), and 90% CH_3CN and 0.1% formic acid (pump B). The peptides were eluted with a linear gradient of 5–65% from pump B in 20 min at a flow rate of 3 μL/min. The data were acquired in the mass range of *m*/*z* 450–2000 in the positive-ion mode, and the most intense ion in each scan was subjected to a data-dependent MS/MS as the precursor. Proteins were identified by searching Swiss Prot (mouse) using the Mascot search engine (Matrixscience, London, UK) and TurboSEQUEST search engine (Thermo Fisher Scientific).

N-Glycosidase F digestion

Treatment of kidney nuclear fraction proteins with *N*-glycosidase F was performed as described previously (Tagawa et al. 2005).

Release of N-linked oligosaccharides from the gel-separated glycoprotein

The gel containing alpha-1 subunit of laminin-1 was excised and cut into pieces. The gel pieces were dehydrated with 50% CH_3CN after destaining, then equilibrated with a 100 mM sodium phosphate buffer (pH 7.2) and incubated with five units of *N*-glycosidase F at 37°C for 18 h. *N*-Linked oligosaccharides were extracted from the gel pieces by intermittent sonication for 30 min in water three times. All extracts were combined and lyophilized. Released *N*-linked oligosaccharides were reduced with $NaBH_4$.

LC/MS/MS (multistage tandem mass spectrometry) of N-linked oligosaccharides

The borohydride-reduced oligosaccharides were separated in a graphitized carbon column (Hypercarb, 5 μ, 0.075 × 150 mm, Thermo Fisher Scientific) at a flow rate of 200 nL/min in a HPLC system (nanoFrontier nLC, Hitachi, Tokyo). The eluents consisted of 5 mM ammonium acetate (pH 9.6) containing 2% CH_3CN (pump A), and 5 mM ammonium acetate (pH 9.6) containing 80% CH_3CN (pump B). The oligosaccharides were eluted with a linear gradient of 5–50% of pump B over 110 min, and analyzed using a LTQ with a full mass scan (*m*/*z* 450–2000) followed by a data-dependent MS/MS for the top three abundant ions in positive-ion mode. LC/MS/MS was performed using a capillary voltage of 2.0 kV, a tube lens offset of 110 V, capillary temperature of 300°C, and collision energy of 35%.

Immunofluorescence staining

C57BL/6 mice (from 2- to 4-week-old) were deeply anesthetized by diethyl ether inhalation, and then perfused with phosphate-buffered saline (PBS) containing 0.1% heparin and then with 4% paraformaldehyde in PBS. Their kidneys were postfixed overnight, followed by dipping in 30% sucrose in PBS. For immunofluorescence staining, sections (40 μm thick) were prepared, incubated with the primary antibodies (M6749 mAb and anti-laminin-1 pAb), and then incubated with the fluorescent labeling secondary antibodies (for M6749; Alexa Fluor 546-conjugated anti-mouse IgM, for anti-laminin-1 pAb; Alexa Fluor 488-conjugated anti-rabbit IgG). For proteinase treatment, sections were incubated with 30 μg/mL proteinase K in PBS for 30 min at 37°C. After washing with PBS three times, sections were stained as described above. These sections were visualized with a Fluoview laser confocal microscope system (Olympus, Japan).

Laminin-1 overlay assay

After SDS–PAGE, nitrocellulose membrane-transferred proteins were incubated for 1 h with a binding buffer (20 mM Tris–HCl, pH 7.4; 150 mM NaCl; 1 mM $CaCl_2$; and 1 mM $MgCl_2$) containing 5% nonfat dry milk. The membrane was washed with the binding buffer and then incubated with 1 μg/mL EHS-laminin (referred to as laminin-1, purchased from Sigma) in the presence or absence of an inhibitor (IIH6 mAb, or monosaccharide) in the binding buffer containing 3% BSA (bovine serum albumin) for 2 h at room temperature. After washing with the binding buffer, bound laminin-1 was detected with anti-laminin-1 pAb using the binding buffer containing 3% BSA. In this overlay assay, anti-laminin-1 pAb was used at a lower concentration (0.3 μg/mL) than usual (1.0 μg/mL) to reduce the signal of endogenously expressed laminin-1 in mouse kidney. For inhibition, IIH6 mAb was used at 100-fold dilution of mouse ascites (Upstate). When 1mM EDTA was present, TBS was used instead of the binding buffer during laminin-1 overlay.

Supplementary Data

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

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Conflict of interest statement

None declared.

Abbreviations

BSA, bovine serum albumin; Glc, glucose; GlcA, glucuronic acid; GlcAT, glucuronyltransferase; HNK-1, human natural killer-1; HRP, horseradish peroxidase; MS, mass spectrometry; mAb, monoclonal antibodies; Neu5Ac, *N*-acetylneuraminic

acid; pAb, polyclonal antibodies; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

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