Adhesion of Cultured Bovine Aortic Endothelial Cells to Laminin-1 Mediated by Dystroglycan

(Received for publication, September 28, 1998, and in revised form, January 12, 1999)

Hisao Shimizu‡, Hiroshi Hosokawa‡*†, Haruaki Ninomiya‡, Jeffrey H. Miner‡, and Tomoh Masaki‡†

From the ‡Department of Pharmacology, Faculty of Medicine, Kyoto University, Kyoto 606, Japan and the §Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

Expression of dystroglycan (DG) by cultured bovine aortic endothelial (BAE) cells was confirmed by cDNA cloning from a BAE cDNA library, Northern blotting of mRNA, Western blotting of membrane proteins, and double immunostaining with antibodies against βDG and platelet endothelial cell adhesion molecule-1. Immunocytochemical analysis revealed localization of DG in multiple plaques on the basal side of resting cells. This patchy distribution was obscured in migrating cells, in which the most prominent staining was observed in the trailing edge anchoring the cells to the substratum. Biotin-labeled laminin-1 overlay assay of dissociated BAE membrane proteins indicated the interaction of laminin-1 with αDG. The laminin α5 globular domain fragment expressed in bacteria and labeled with biotin could also bind αDG on the membrane blot, and the unlabeled fragment disrupted the binding of biotin-laminin-1 to αDG. The interaction of biotin-laminin-1 with αDG was inhibited by soluble αDG contained in the conditioned medium from DG cDNA-transfected BAE cells and by a series of glycosaminoglycans (heparin, dextran sulfate, and fucoidan). Soluble αDG in the conditioned medium inhibited the adhesion of BAE cells to laminin-1-coated dishes, whereas it had no effect on their adhesion to fibronectin. All three glycosaminoglycans that disrupted the biotin-laminin-1 binding to αDG inhibited BAE cell adhesion to laminin-1, whereas they failed to inhibit the adhesion to fibronectin. These results indicate a role of DG as a non-integrin laminin receptor involved in vascular endothelial cell adhesion to the extracellular matrix.

Dystroglycan (DG)§ consists of αDG and βDG, the two subunits yielded by proteolytic cleavage of a single precursor protein. αDG is a highly glycosylated extracellular protein with a molecular mass of 120–190 kDa, and βDG is a 43-kDa transmembrane protein (1, 2). On the cell surface, αDG is anchored to βDG by noncovalent bonds (3, 4).

DG is the central component of the dystrophin-associated glycoprotein complex, and its physiological roles have been extensively studied in skeletal muscle, from which it was originally isolated. In the skeletal muscle sarcolemma, DG forms a physical link between the extracellular matrix and intracellular cytoskeleton by the binding of αDG and βDG with laminin-2 in the matrix and dystrophin in the cytoskeleton, respectively (5, 6). The membrane stability of the sarcolemma depends on this link as evidenced by the progressive muscle degeneration in Duchenne’s muscular dystrophy that is caused by an abnormal dystrophin gene (7). A specialized form of the DG complex is found in the neuromuscular junction, where αDG and βDG associate with agrin in the matrix and utrophin in the cytoskeleton, respectively (8–10). This protein complex is critically involved in agrin-induced clustering of acetylcholine receptors (11).

DG is expressed in various tissues and cell lines, and evidence for its functions in non-muscle tissues has been accumulating in recent years. In the peripheral nervous system, DG is expressed by Schwann cells and is involved both in Schwann cell adhesion to the extracellular matrix and in myelination (12–15). In the central nervous system, DG is expressed both by glial cells and by certain groups of neurons and is suggested to be involved in blood brain barrier and synapse formation (16–18). Outside the nervous system, DG has been shown to be involved in epithelial morphogenesis during embryogenesis (19, 20).

Immunohistochemical studies using human brain sections indicated the expression of DG by vascular endothelial cells (21, 22). An immunocytochemical study indicated the expression of DG by cultured human umbilical endothelial cells as well (23). The expression of DG by vascular endothelial cells, however, is still controversial because of the negative immunostaining of anti-DG antibodies found in brain capillary endothelial cells (16). Most recently, Durbeej et al. (24) suggested that the anti-DG immunostaining in some blood vessels emulates not from the endothelial cells, but from the smooth muscle cells, which are a rich source of DG.

Vascular endothelial cells undergo drastic morphological and functional changes during angiogenesis, and it is well established that the behavior of the cell is critically influenced by interaction with the extracellular matrix in their milieu. Laminin is the major constituent of the vascular endothelial basement membrane (25), and it is generally accepted that the principal endothelial cell-surface receptor that recognizes laminin is the integrin family of cell adhesion molecules (26–28). Several lines of evidence, however, suggested the existence of non-integrin types of laminin receptors expressed by vascular endothelial cells whose identity remains unclear (29, 30). Given the laminin-binding capacity of DG, the purposes of this study

* This work was supported in part by research grants from the Ministry of Education, Science, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Current address: National Cardiovascular Research Inst., 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. Tel.: 81-6-833-5012 (ext. 2501); Fax: 81-6-833-1421; E-mail: masaki@ri.ncvc.go.jp.
‡ To whom correspondence should be addressed: National Cardiovascular Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan.
§ The abbreviations used are: DG, dystroglycan; BAE, bovine aortic endothelial; DMEM, Dulbecco’s modified Eagle’s medium; PECAM1, platelet endothelial cell adhesion molecule-1; BSA, bovine serum albumin; PCR, polymerase chain reaction; Mes, 4-morpholineethanesulfonic acid.

This paper is available on line at http://www.jbc.org
were to confirm the expression of DG by primary cultured bovine aortic endothelial (BAE) cells and to establish the role of DG as a non-integrin type of laminin receptor involved in BAE cell adhesion to the extracellular matrix.

**Experimental Procedures**

**Materials—Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum, mouse Engelbreth-Holm-Swarm sarcoma laminin-1, and bovine plasma fibronectin were from Gibco BRL (Tokyo, Japan). Glycosaminoglycans (heparin, dextran sulfate, fasciculin, sulfated chondroitin sulfate, and dextran) and oligosaccharides (N-acetylglucosamine and N-acetylgalactosamine) were from Wako (Tokyo). 5′-32P-CTP (2200 TBq/mmol) was from Amersham International (Buckinghamshire, United Kingdom). EZ-link sulfo-NHS-SS-biotin was from Pierce. A mouse monoclonal anti-human βDG antibody was from Novo CASTRA (Claremont Place, United Kingdom). A rabbit polyclonal antibody against platelet endothelial cell adhesion molecule-1 (PECAM) was a kind gift from Dr. K. Fujisawa (National Cardiovascular Research Institute, Saitama, Japan). The cDNA clone for rabbit DG was a kind gift from Dr. K. P. Campbell (Howard Hughes Medical Institute and Department of Physiology and Biophysics, University of Iowa College of Medicine). All other chemicals were of reagent grade and were obtained commercially.

**Cell Culture and Adhesion Assay—** A primary culture of BAE cells was obtained as described (31). The cells were routinely maintained in DMEM and 10% fetal calf serum at 37°C in a humidified atmosphere containing 5% CO2. They were passaged once a week at a ratio of 1:5 to keep an exponentially growing state. All cells used were between passages 5 and 10. For the cell adhesion assay, 96-well plates were incubated with laminin-1 or fibronectin (both at 10 µg/ml in phosphate-buffered saline) for 1 h at 37°C. Both laminin-1 and fibronectin costing of the substratum caused a dose-dependent enhancement of BAE cell adhesion, and a 10 µg/ml concentration of either substance was a supramaximal concentration for the effect (data not shown). Residual protein-binding sites were blocked by incubating the plates in 2% BSA overnight at 4°C. Cells were seeded at 5 × 10³ cells/well in 100 µl of DMEM and 0.2% BSA and then kept in a CO2 incubator. Because a time course analysis showed a linear increment of the attached cell number at least for 2 h (data not shown), the incubation time was set to 2 h in the following experiments. After the incubation, unattached cells were removed by washing the plates with phosphate-buffered saline. The attached cells were lysed in 100 µl of Tris-buffered saline (10 mM Tris-HCl, pH 7.4, and 150 mM NaCl) containing 0.2% Tween 20. The number of attached cells was estimated by the lactate dehydrogenase activity in the lysate measured with a lactate dehydrogenase cytoxic assay kit (Wako) according to the manufacturer’s instructions. This assay gave a linear relationship between the cell number and the measured A₅₅₀, up to the cell number of 16 × 10⁴/well. Under the control condition after 2 h of incubation, (1.8 ± 0.1) × 10⁴ (n = 8; 36% of the cells seeded) attached to laminin-1-coated dishes, and (4.0 ± 0.1) × 10⁴ (n = 8; 96% of the cells seeded) attached to fibronectin-coated dishes.

cDNA Cloning and Northern Blotting—cDNA was synthesized from the total BAE RNA by SuperScript II reverse transcriptase (Gibco BRL) and used as a template for polymerase chain reaction (PCR). The sequences of the PCR primers were 5′-GCCCTGAGCCGACTCTTTAGGCGG-3′ (sense) and 5′-CTGCTCAGCCTGCGAAAAG-3′ (antisense). These sequences correspond to nucleotide sequences 2252−2274 and 2565−2577 of rabbit DG (1), respectively. The PCR product was labeled with [32P]dCTP (110 TBq/mmol; Amersham International) using a random primer DNA labeling system (Gibco BRL) and was used as a probe for hybridization screening of a λgt11 cDNA library from BAE poly(A)+ RNA. A single positive clone was extracted from the plaque, and the EcoRI fragment of the phage was subcloned into pBlueScript to give pBSK/DG. The nucotide sequence of the pBSK/DG insert was determined using a BeA Best dideoxy sequencing kit (Takara, Otsu, Japan). The probe for Northern blotting was prepared by labeling the EcoRI fragment of pBSK/DG with [32P]dCTP as described above. Blotting procedures were as described (32). The blot was visualized for autoradiography with a BAS2000 image analyzer (FujiFilm, Tokyo). Western Blotting—The trichloroacetic acid precipitate of the whole cell lysate was used for Western blotting with the anti-βDG antibody. In brief, the cells were harvested in phosphate-buffered saline by scraping and lysed by sonication, and the cellular protein was precipitated by incubation at 4°C for 1 h in 10% trichloroacetic acid. After centrifugation at 3000 × g for 1 h at 4°C, the pellet was dissolved in Laemmli’s sample buffer and subjected to SDS-10% polyacrylamide gel electrophoresis and Western transfer to polyvinylidene difluoride membranes. Immunostaining of the blots was done as described (33). The blots were developed using an ABC immunodetection kit (Vector Labs, Inc., Burlingame, CA) and Konica immunostain HRP-1000.

**Immunocytochemistry—** Cells cultured on poly-l-lysine-coated glass-bottom dishes were used for immunostaining. All procedures were done at room temperature. The cells were rinsed with a buffer (10 mM Mes, pH 6.1, 150 mM NaCl, 5 mM EGTA, 5 mM MgCl₂, and 5 mM glucose), fixed with 4% paraformaldehyde in the same buffer for 10 min, and then permeabilized with 0.1% Triton X-100 for 1 min. After blocking with 1% BSA and 2% goat serum, they were incubated with the anti-human DG antibodies were detected with Cy3-conjugated anti-rabbit IgG, respectively. Fluorescent images of the cells were obtained using an Axiosvert 25 fluorescent microscope (Carl Zeiss, Inc.) or an MRC1024 confocal microscope (Bio-Rad).

**Statistical Analysis—** Where necessary, statistical analysis was done by analysis of variance.

**Results**

**Expression of DG by Cultured BAE Cells—** To examine whether BAE cells expressed DG, we conducted Northern and Western blotting. The bovine DG cDNA probe for Northern blotting was prepared by reverse transcription-PCR followed by hybridization screening of a BAE cDNA library in λgt11 using the PCR product as a probe. The primers for the PCR were designed based on the rabbit DG cDNA sequence (1). In this process, we eventually isolated a full-length cDNA encoding bovine DG (GenBank™/EBI accession number AB009079). Northern blotting of BAE mRNA using the whole coding sequence of the DG cDNA as a probe detected a major band at 5.4 kilobase pairs and a minor band at 5.0 kilobase pairs (Fig. 1). On Northern blotting of bovine multiple-tissue mRNAs, we found a ubiquitous distribution of the 5.4-kilobase pair band (data not shown). For Western blotting, we used a monoclonal anti-human βDG antibody. This antibody recognizes an epitope in the carboxyl-terminal 10-amino acid sequence of human βDG that is identical to that of bovine βDG. The antibody detected a single band at 43 kDa in the BAE cell lysate (Fig.
Immunofluorescent staining of the confluent cell layer revealed that almost all of the cells expressed both βDG and the endothelial cell-specific cell-cell adhesion molecule PECAM1 (36) (Fig. 1c). These results indicated that the DG mRNA and protein detected in our primary culture derived from endothelial cells, but not from contaminating smooth muscle cells.

Subcellular Localization of DG in Migrating BAE Cells—
Immunofluorescent staining of βDG in subconfluent cells revealed a distinct difference in the staining patterns between the resting and migrating cells (Fig. 2). In the resting cells, the antibody stained multiple plaques located on the basal side of the cells as revealed by confocal microscopy. The plaques appeared to be diffusely distributed in the central portion of the cell floor (Fig. 2a). In the migrating cells, the patchy staining was obscured, and instead, strong immunostaining was observed in the trailing edge (Fig. 2b). The trailing edge was retracted when the cells were detached and actually moved (Fig. 2c).

Biotin-Laminin-1 Binding to αDG—The interaction of αDG with laminin-1 was characterized by biotin-laminin-1 overlay on the Western blot of BAE membrane proteins. Biotin-laminin-1 detected a major band at 130 kDa (Fig. 3b) and a minor band at 110 kDa (Figs. 3b and 5b, lane 4) on the blot. The same assay of the soluble proteins contained in the conditioned medium from pRE9P/DG- or vector-transfected cells verified the presence of the soluble form of αDG. The binding of biotin-laminin-1 to the 130-kDa BAE membrane protein (Fig. 3b), indicating the identity of this protein as αDG. The minor band at 110 kDa was also abolished by the conditioned medium from pRE9P/DG-transfected cells, suggesting that the 110-kDa protein also derived from αDG. The appearance of the 110-kDa minor band was not as consistent as that of the 130-kDa band on the blot of membrane proteins, and it was not detected on the blot of soluble proteins. Therefore, it is most likely that the 110-kDa minor band represents immature αDG with less glycosylation.

The binding to the 130-kDa protein was totally abolished in the presence of EDTA (Fig. 4) as expected from the Ca²⁺ dependence of the αDG-laminin interaction (1). αDG is heavily glycosylated, and its mucin-like carbohydrate moiety is required for binding with laminin (37, 38). Therefore, we tested the ability of a series of glycosaminoglycans to inhibit the biotin-laminin-1 binding to BAE cell αDG and found that the binding was inhibited by heparin, dextran sulfate, and fucoidan, but not by sulfatide, chondroitin sulfate, and dextran (Fig. 4). We also tested two oligosaccharides, N-acetylgalactosamine and N-acetylmuramic acid and found that neither of them could displace the binding (Fig. 4).

Binding of Recombinant Laminin α5 to αDG—Because of the recent identification of laminin α5 as a major subtype of laminin α chains present in the basement membrane of endothelial cells (35, 39, 40), we tested the binding capacity of a recombinant laminin α5 fragment for αDG. The ~110-kDa fragment that includes the globular domains of laminin α5 was expressed in E. coli, purified (Fig. 5a), and labeled with biotin. The biotin-labeled laminin α5 protein detected both the 130-
Dystroglycan in Endothelial Cells

and 110-kDa bands on the Western blot of BAE membrane proteins (Fig. 5b, lane 1). These bindings were abolished in the presence of a high concentration of unlabeled laminin-1 (lane 2) and also in the presence of the conditioned medium from pREP9/DG-transfected cells (lane 3). In another set of experiments, unlabeled laminin α5 protein disrupted the binding of dystroglycan to the 130/110-kDa proteins (lanes 4 and 5).

**Adhesion of BAE Cells to Laminin-1 Mediated by DG**—The immunocytochemical localization of DG on the basal side of BAE cells and the interaction of DG with laminin in the ligand overlay assay suggested the involvement of DG in BAE cell adhesion to the extracellular matrix. To obtain direct evidence for it, we examined whether 1) the soluble αDG in the conditioned medium from pREP9/DG-transfected cells and/or 2) the glycosaminoglycans that inhibited the biotin-laminin-1 binding to αDG in the overlay assay could inhibit the BAE cell adhesion to laminin-1-coated dishes. To test the substratum specificity, we also conducted the same assay using fibronectin-coated dishes.

The conditioned medium from pREP9/DG-transfected cells, but not that from vector-transfected cells, caused significant inhibition of the cell adhesion to laminin-1-coated dishes (Fig. 6). It caused no further decrement of the adhesion in the presence of heparin. In contrast to its effects on laminin-1-coated dishes, the conditioned medium caused no effect on BAE cell adhesion to fibronectin-coated dishes (Fig. 6). Of the six glycosaminoglycans, heparin, dextran sulfate, fucoidan, and sulfatide caused significant inhibition of the cell adhesion to laminin-1 (Fig. 7). Chondroitin sulfate and dextran failed to inhibit the adhesion and so did the two oligosaccharides tested, N-acetylgalactosamine and N-acetylneuraminic acid. Of the four effective glycosaminoglycans, sulfatide was by far the most effective compared with the modest inhibition by the other three. None of the glycosaminoglycans/oligosaccharides caused any effects on BAE cell adhesion to fibronectin-coated dishes, except for sulfatide. Sulfatide again caused a drastic inhibition of the adhesion to fibronectin-coated dishes (Fig. 7).

**DISCUSSION**

In this study, expression of DG by cultured BAE cells was confirmed by cDNA cloning from a BAE cDNA library, Northern blotting of mRNA, and Western blotting of membrane proteins (Fig. 1, a and b). Coexpression of βD and PECAM1 by the cells confirmed the endothelial cell origin of the DG mRNA and protein detected (Fig. 1c). The length of the deduced amino acid sequence of bovine DG (885 amino acids) was exactly the same as those of the rabbit, mouse, and human versions, and 91–93% of the amino acid sequence was identical to the three reported sequences (6). Together with the similar size of the mRNA and its ubiquitous distribution on the Northern blot, these results indicate high conservation of the DG precursor gene among mammalian species.

Immunocytochemical analysis revealed localization of DG on the basal side of BAE cells and a drastic change in the localization associated with cell migration (Fig. 2). In the resting

**Fig. 3. Detection of αDG by biotin-laminin-1 overlay assay.** Soluble proteins in the conditioned medium (a) or membrane proteins (b) were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. In both cases, 10 μg of protein was loaded on a lane. The membranes were probed with biotin-laminin-1 as described under “Experimental Procedures.” a, the conditioned medium was from cells transfected with vector plasmid (lane 1) or pREP9/DG (lane 2), b, the membrane strip was incubated with biotin-laminin-1 in the absence (lane 1) or presence of the concentrated conditioned medium (10%, v/v) from cells transfected with vector plasmid (lane 2) or pREP9/DG (lane 3). Molecular sizes are given in kilodaltons.

**Fig. 4. Inhibition of biotin-laminin-1 binding to αDG by glycosaminoglycans.** BAE membrane proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membrane strips were incubated with biotin-laminin-1 in the absence (None) or presence of EDTA (5 mM), glycosaminoglycan, or oligosaccharide as indicated. ChS, chondroitin sulfate; Dex, dextran; DexS, dextran sulfate; Fuc, fucoidan; Hep, sodium heparin; Sul, sulfatide; NAX, N-acetylgalactosamine; NAG, N-acetylglucosamine. The glycosaminoglycans/oligosaccharides were used at 2 mg/ml.

**Fig. 5. Binding of recombinant laminin α5 fragment to αDG.** a, Coomassie Blue staining of the 110-kDa recombinant α5 fragment (arrow) expressed in E. coli. The protein was expressed with (lane 1) or without (lane 2) isopropyl-β-D-thiogalactopyranoside induction. b, biotin-laminin overlay assay. Membrane strips were cut from a Western blot of BAE membrane proteins. They were incubated with the biotin-labeled laminin α5 fragment (1 μg/ml) in the absence (lane 1) or presence of unlabeled laminin-1 (1 mg/ml) (lane 2) or the concentrated conditioned medium from pREP9/DG-transfected cells (10%, v/v) (lane 3). In another set of experiments, the strips were incubated with biotin-labeled laminin-1 (1 μg/ml) in the absence (lane 4) or presence (lane 5) of the unlabeled laminin α5 fragment (1 mg/ml). Molecular masses (in kilodaltons) are indicated.
FIG. 6. Effects of soluble αDG on BAE cell adhesion. The same number of cells in 100 μl of cell suspension was seeded on plastic plates coated with laminin-1 or fibronectin. The wells contained 100 μl of DMEM and 0.2% BSA with or without heparin (2 mg/ml) as indicated and 10 μl of the concentrated conditioned medium from vector-transfected cells (open bars) or from pREP9/DG-transfected cells (closed bars). After 2 h of incubation, the number of attached cells was estimated by the lactate dehydrogenase activity in the cell lysate as described under “Experimental Procedures.” The results are expressed as a percentage of the values in the absence of heparin and the conditioned medium (100%). Shown are the mean ± S.E. of three determinations, each done in triplicate. *, p < 0.01 (significantly different from the values in the presence of the concentrated conditioned medium from vector-transfected cells).

FIG. 7. Effects of glycosaminoglycans and oligosaccharides on BAE cell adhesion. The same number of cells in 100 μl of cell suspension was seeded on plastic plates coated with laminin-1 or fibronectin. The wells contained 100 μl of DMEM and 0.2% BSA with or without the glycosaminoglycan or oligosaccharide as indicated. The glycosaminoglycans/oligosaccharides were used at 2 mg/ml. Shown are the mean ± S.E. of three determinations, each done in triplicate. *, p < 0.01 (significantly different from the control values in the absence of the glycosaminoglycan/oligosaccharide). ChS, chondroitin sulfate; Dex, dextran; DeaS, dextran sulfate; Fuc, fucoidan; Hep, sodium heparin; Sul, sulfatide; NAcN, N-acetylneuraminic acid; NAG, N-acetylgalactosamine.

cells, DG was confined in multiple filopodia, the morphology of which is apparently close to that of the laminin/agrin-induced DG plaques in skeletal muscle cells (41). The plaques were obscured in migrating cells, in which the trailing edge was most intensely stained. The trailing edge was then retracted when the cells actually moved in space. A straightforward explanation for these observations is that DG, with its tight association with the extracellular matrix, is left in the last part of the cell that detaches from the substratum when it moves. Although it is necessary to monitor the location of DG in live cells to verify this scenario, it is at least clear that the subcellular localization of DG in migrating BAE cells is quite different from that of integrins that have been shown to be recruited to the frontal portion of migrating leukocytes (42).

Two lines of biochemical evidence were presented in this study that indicated the role of DG as a non-integrin laminin receptor involved in BAE cell adhesion to the extracellular matrix. The first is the inhibition of BAE cell adhesion to laminin-1 by soluble αDG contained in the conditioned medium from DG cDNA-transfected cells (Fig. 6). Soluble αDG could also inhibit the biotin-laminin-1 binding to αDG in the dissociated membrane proteins (Fig. 3). Secretion of αDG to the medium was first indicated in RT4 schwannoma cells (15), and we have confirmed the finding in cultured BAE cells. The molecular mechanism that generates the soluble form and its physiological role must be the subjects in a future study.

The second line of biochemical evidence is the inhibition of BAE cell adhesion to laminin-1 by a set of glycosaminoglycans (heparin, dextran sulfate, fucoidan, and sulfatide) (Fig. 7). Of the four glycosaminoglycans, sulfatide was by far the most effective; however, it did not inhibit the αDG-laminin-1 interaction in the ligand overlay assay (Fig. 4), and it also inhibited the BAE cell adhesion to fibronectin (Fig. 7). Therefore, it is plausible to conclude that the other three, but not sulfatide, inhibited BAE cell adhesion to laminin-1 by specifically disrupting the αDG-laminin-1 interaction. The mechanism for sulfatide inhibition is still unknown. The three glycosaminoglycans (heparin, dextran sulfate, and fucoidan) have been shown to inhibit the adhesion of RT4 schwannoma cells to laminin-1 (15) as well as agrin-induced acetylcholine receptor clustering in myotubes (43), suggesting that the similar carbohydrate moiety on αDG was involved in the binding to laminin-1/agrin in these cell lines. Together with the failure of oligosaccharides to inhibit the adhesion of RT4 (15) and BAE (Fig. 7) cells, these results indicated the importance of the high anionic charge and polymeric structure of the glycosaminoglycans in interrupting the αDG-laminin-1 interaction. The soluble αDG in the conditioned medium caused no further decrement of the cell adhesion in the presence heparin (Fig. 6), providing supportive evidence for the interruption of the αDG-laminin-1 interaction by heparin.

Of the three glycosaminoglycans, heparin has been known for its activities to stimulate both endothelial cell proliferation (44) and migration (45). Polyanions such as heparin bind to a wide variety of glycoproteins, including extracellular matrix proteins, growth factors, and protease inhibitors, and the mechanism of action of heparin to modulate endothelial cell behavior is still unknown. Studies are underway in our laboratory to test the hypothesis that the cell behavior modulation by heparin is due to the inhibition of the αDG-laminin interaction.

Both in the cases of the soluble αDG and the three glycosaminoglycans, inhibition was not observed in fibronectin-coated dishes, suggesting the laminin specificity of the DG-mediated adhesion. Laminin is a group of heterogeneous proteins, and care must be taken to interpret the results obtained with a purified protein. The mouse Engelbreth-Holm-Swarm laminin-1 used in this study is composed of the three subunits, α1, β1, and γ1. BAE cells have been shown to express β1 and γ1, but not α1 (40). Recent histochemical analysis indicated the presence of α5, but not α1, in the vascular basal laminae of murine heart (23). The laminin-5 in the vascular basal laminae may be produced by the underlying smooth muscle cells because neither BAE nor mouse aortic endothelial cells produce laminin-5, but only laminin-4 (46). Therefore, it is at least clear that laminin-1 is unlikely to be an in vivo ligand for DG in vascular endothelial cells. We have, however, shown in the ligand overlay assay that the α5 protein expressed in E. coli bound αDG and that it could disrupt the binding of laminin-1 to αDG (Fig. 5). The recombinant α5 protein immobilized on the dishes promoted the BAE cell adhesion (data not shown). These results support the idea that endothelial DG works as a laminin receptor in vivo. Further studies with laminin-α5 purified from tissues or gene knockout experiments will be required to test the idea directly.
Dystroglycan in Endothelial Cells

In conclusion, we have confirmed the expression of DG by cultured BAE cells and presented evidence for the role of DG as a non-integrin laminin receptor involved in BAE cell adhesion to the extracellular matrix. These findings expand our knowledge on the physiological roles of DG in non-muscle tissues. The distinct subcellular localization of DG in BAE cells and the essential role of the carbohydrate moiety in the DG-laminin interaction suggest the influence of DG-mediated cell adhesion that is quite different from that of integrin-mediated cell adhesion. The control of cell behavior by the DG-laminin interaction must be the subject for future studies.

REFERENCES
1. Ibraghimov-Beskrovnaya, O., Ervasti, J. M., Leveille, C. J., Slaughter, C. A., Serratt, S. W., and Campbell, K. P. (1992) Nature 355, 696–702
2. Ibraghimov-Beskrovnaya, O., Milatovich, A., Ozcelik, T., Yang, B., Koepnick, K., Francke, U., and Campbell, K. P. (1993) Hum. Mol. Genet. 2, 1651–1657
3. Yoshida, M., Suzuki, A., Yamamoto, H., Noguchi, S., Mizuno, Y., and Ozawa, E. (1994) Eur. J. Biochem. 222, 1055–1061
4. Jung, D., Yang, B., Meyer, J., Chamberlain, J. S., and Campbell, K. P. (1995) J. Biol. Chem. 270, 27305–27310
5. Suzuki, A., Yoshida, M., Hayashi, K., Mizuno, Y., Hagiwara, Y., and Ozawa, E. (1994) Eur. J. Biochem. 220, 283–292
6. Henry, M. D., and Campbell, K. P. (1996) Curr. Opin. Cell Biol. 8, 625–631
7. Campbell, K. P. (1995) Cell 86, 675–679
8. Bowe, M. A., Deyst, K. A., Leszyk, J. D., and Fallon, J. F. (1994) Neuron 10, 1173–1180
9. Gee, S. H., Montanaro, F., Lindenbaum, M. H., and Carbonetto, S. (1994) Cell 77, 675–686
10. Sugiyama, J., Bowen, D. C., and Hall, Z. W. (1994) Cell Adhes. Commun. 1173–1180
11. Campanelli, J. T., Roberds, S. L., Campbell, K. P., and Scheller, R. H. (1994) Neuron 10, 285–300
12. Ibraghimov-Beskrovnaya, O., Milatovich, A., Ozcelik, T., Yang, B., Koepnick, K., Francke, U., and Campbell, K. P. (1994) FEBS Lett. 352, 49–53
13. Yamada, H., Shimizu, T., Tanaka, T., Campbell, K. P., and Matsumura, K. (1994) FEBS Lett. 352, 49–53
14. Yamada, H., Chiba, A., Endo, T., Kobata, A., Anderson, L. V. B., Hori, H., Fukuta-Ohi, H., Kanazawa, I., Campbell, K. P., Shimizu, T., and Matsumura, K. (1996) J. Neurochem. 66, 1518–1524
15. Yamada, H., Denzer, A. J., Hori, H., Tanaka, T., Anderson, L. V. B., Fujita, S., Fukuta-Ohi, H., Shimizu, T., Ruegg, M. A., and Matsumura, K. (1996) J. Biol. Chem. 271, 23418–23423
16. Matsumura, K., Chiba, A., Yamada, H., Fukuta-Ohi, H., Fujita, S., Endo, T., Kobata, A., Anderson, L. V. B., Kanazawa, I., Campbell, K. P., and Shimizu, T. (1997) J. Biol. Chem. 272, 13904–13910
17. Tan, M., Jacobson, C., Gee, S. H., Campbell, K. P., Carbonetto, S., and Jucker, M. (1996) Eur. J. Neurosci. 8, 2729–2747
18. Drenckhahn, D., Holbach, M., Nesp, W., Schmitz, F., and Anderson, L. V. (1996) Neuroscience 73, 605–612
19. Powell, S. K., and Kleinman, H. K. (1997) Int. J. Biochem. Cell Biol. 29, 401–414
20. Durbeej, M., and Eriksson, P. (1997) Exp. Lung Res. 23, 109–118
21. Durbeej, M., Larsson, E., Ibraghimov-Beskrovnaya, O., Roberds, S. L., Campbell, K. P., and Eriksson, P. (1995) J. Cell Biol. 130, 79–91
22. Uchino, M., Harra, A., Mizuno, Y., Fujiki, M., Nakamura, T., Tokunaga, M., Hiran, T., Yamashita, T., Uyama, E., Ando, Y., Mita, S., and Ando, M. (1996) Intern. Med. 35, 189–194
23. Belkin, A. M., and Smalheiser, N. R. (1996) Cell Adhes. Commun. 4, 281–296
24. Durbeej, M., Henry, M. D., Ferletta, M., Campbell, K. P., and Ekblom, P. (1998) J. Histochem. Cytochem. 46, 449–457
25. Sanes, J., Engvall, E., Butkowski, R., and Hunter, D. D. (1990) J. Cell Biol. 111, 1685–1699
26. Languino, L. R., Gehlsen, K. R., Wayner, E., Carter, W. G., Engvall, E., and Ruoslahti, E. (1989) J. Cell Biol. 109, 2455–2462
27. Cheng, Y. F., and Kramer, R. H. (1989) J. Cell. Physiol. 139, 275–286
28. Albelda, S. M., Daize, M., Levine, E. M., and Buck, C. A. (1989) Clin. Invest. 83, 1992–2002
29. Tressler, R. J., Belloni, P. N., and Nicolson, G. L. (1989) Cancer Commun. 1, 55–63
30. Basson, C. T., Knowles, W. J., Bell, L., Albeda, S. M., Castronovo, V., Liotta, L. A., and Madri, J. A. (1990) J. Cell Biol. 110, 789–801
31. Kamata, H., Hori, H., Kiyono, H., Shimada, K., Tsuchida, K., Koyama, K., Miwa, S., and Matsumura, T. (1994) Biochem. Biophys. Res. Commun. 203, 1417–1422
32. Sugawara, F., Ninomiya, H., Okamoto, Y., Miwa, S., Mazda, O., Katsura, Y., and Masaki, T. (1995) Mol. Pharmacol. 49, 447–457
33. Miner, J. H. (1995) J. Biol. Chem. 270, 28525–28526
34. Miner, J. H., Patton, B. L., Lentz, S. I., Gilbert, D. J., Snider, W. D., Jenkins, N. A., Copeland, N. G., and Sanes, J. R. (1997) J. Cell Biol. 137, 685–701
35. Osawa, M., Masuda, M., Harada, N., Lopes, R. B., and Fujikawa, K. (1997) Eur. J. Cell Biol. 72, 229–237
36. Brancaccio, A., Schultheiss, T., Gesemann, M., and Engel, J. (1995) FEBS Lett. 368, 139–142
37. Chiba, A., Matsumura, K., Yamada, H., Inazu, T., Shimizu, T., Kusunoki, S., Kanazawa, I., Kobata, A., and Endo, T. (1997) J. Biol. Chem. 272, 2156–2162
38. Sorokin, L. M., Pausch, F., Frieser, M., Kroger, S., Ohage, E., and Deutzmann, R. (1997) Dev. Biol. 189, 285–300
39. Sorokin, L., Girg, W., Gepfert, T., Hallmann, R., and Deutzmann, R. (1994) Eur. J. Biochem. 223, 603–610
40. Cohen, M. W., Jacobson, C., Yurchenco, P. D., Morris, G. E., and Carbonetto, S. (1997) J. Cell Biol. 136, 1047–1058
41. Lawson, M. A., and Maxfield, F. R. (1995) Nature 377, 75–79
42. Azizkhan, R. G., Azizkhan, J. C., Zetter, B. R., and Folkman, J. (1980) Science 203, 623–631
43. Thornton, S. C., Mueller, S. N., and Levine, E. M. (1983) Science 222, 623–625
44. Azizkhan, R. G., Azizkhan, J. C., Zetter, B. R., and Folkmann, J. (1980) J. Exp. Med. 152, 931–934
45. Frieser, M., Noekel, H., Pausch, F., Roder, C., Hahn, A., Deutzmann, R., and Sorokin, L. M. (1997) Eur. J. Biochem. 246, 727–735