A Close Association of the Ganglioside-specific Sialidase Neu3 with Caveolin in Membrane Microdomains*

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The ganglioside-specific sialidase Neu3 has been suggested to play essential roles in regulation of cell surface functions because of its major localization in the plasma membrane and strict substrate preference for gangliosides involved in signal transduction. Here we show that human Neu3 sialidase is enriched in caveolae microdomains and closely associates with caveolin like other caveolin-binding signaling molecules. Using HeLa cells and Neu3-transfected COS-1 cells, endogenous and exogenous Neu3 was found to co-concentrate caveolin-1 in low density Triton X-100-insoluble membrane fractions on sucrose density gradients of the respective cell extracts, as assessed by enzyme activity assays and immunoblotting with a monoclonal antibody to human Neu3. The presence of a putative caveolin-binding motif within Neu3 prompted us to determine whether Neu3 binds to caveolin-1. In transfectants expressing a polyhistidine-tagged form of Neu3, caveolin-1 co-eluted with Neu3 on affinity column chromatography. A mutation with a single amino acid change in the caveolin-binding motif led to inhibition of recruitment of the sialidase to the microdomain, accompanied by reduction of the enzyme activity. Neu3 also failed to associate with caveolin-enriched microdomains by cholesterol depletion with β-cyclodextrin (with concomitant decrease of the sialidase activity), whereas Neu3 was activated by increased caveolin-1 expression. The tight association of Neu3 with caveolin-1 was supported further by co-immunoprecipitation of Neu3 by anti-caveolin-1 antibody. These results strongly suggest that Neu3 functions as a caveolin-related signaling molecule within caveolin-rich microdomains.

Sialidase catalyzes the removal of sialic acids from glycoproteins and gangliosides, an initial step of the degradation of these molecules. Mammalian forms have been demonstrated to differ from microbial sialidases in various aspects, especially in the presence of multiple types (even in a single cell and in strict substrate specificity), suggesting participation not only in lysosomal degradation but also in many cellular processes (1–3). Three mammalian sialidases that are classified based on subcellular localization have been cloned so far; these are lysosomal, cytosolic, and plasma membrane sialidases, abbreviated to Neu1, 2, and 3, respectively. Although the functional roles of these sialidases are not fully understood, recent progress in sialidase gene cloning has allowed partial clarification. Among the sialidases, plasma membrane sialidase (Neu3) is a key enzyme for ganglioside hydrolysis because of its strict substrate preference to gangliosides. To obtain functional evidence regarding Neu3, we previously cloned and characterized the sialidase cDNAs of mammalian origin (4–7), and we have employed a human Neu3 cDNA (5) in the present study.

Gangliosides, sialic acid-containing glycosphingolipids, are present in surface membranes of cells and are thought to play important functional roles in regulating a wide range of biological processes including cell surface interactions, cell differentiation, and transmembrane signaling (8–10). The ganglioside-specific sialidase Neu3 thus may play important roles in cell surface events through modulation of gangliosides. All of the observations on ganglioside function, however, have been performed using microbial sialidases and exogenous gangliosides to mimic ganglioside expression. To understand better the physiological function of gangliosides, it is necessary to pay attention to the endogenous sialidase, Neu3, responsible for ganglioside hydrolysis inside of the cells.

Accumulating evidence suggests functional importance for plasma membrane-attached vesicular organelles called detergent-insoluble glycosphingolipid-enriched complexes (DIGs) or lipid rafts (11–14). DIGs are closely related to caveolae, 50–100-nm invaginations of the plasma membrane, in lipid composition and resistance to detergent solubilization. A major structural protein of caveolae is caveolin, which is known to act as a scaffold for various signaling molecules (15–17). Recent studies have indicated that caveolin recruits transducer molecules into caveolae and regulates their functions by direct interaction with these proteins including G protein α-subunits, H-Ras, Src family tyrosine kinases, MAP kinase, and endothelial nitric oxide synthase, which recognize a short cytosolic domain derived from the N-terminal region of caveolin-1 (the caveolin scaffolding domain) by caveolin-binding common motifs present within their molecules (14). In addition, interaction of the caveolin scaffolding domain with caveolin-binding motifs of epidermal growth factor receptor (18) and protein kinase C (19) is known to inhibit kinase activity. We now present evidence that Neu3 is closely associated with caveolin-1 within caveolea microdomains and probably acts as a transducer molecule. In this context it is of interest that in the course of the present study, a study (20) was published that described gan-
glioside sialidase activity co-fractionating with rafts from a neuroblastoma cell line.

EXPERIMENTAL PROCEDURES

Cells and DNA Transfection—HeLa cells and COS-1 cells were obtained from The Cell Bank of Tohoku University and RIKEN Cell Bank, respectively, and were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in 5% CO₂. To observe the effects of cholesterol depletion on caveolae, cells were incubated with 10–20 mM methyl-β-cyclodextrin (Sigma) for 50 min before harvesting. Sialidase expression vectors were constructed by subcloning a cDNA fragment containing the open reading frame (ORF) of human membrane-associated ganglioside sialidase (human Neu3) or the mutated DNA into the EcoRI site of pME18S, a eukaryote expression vector under control of the SRα promoter, a generous gift from Dr. T. Maruyama (Tokyo Medical and Dental University). Introduction of mutations on human Neu3 cDNA was performed by recombination PCR (21) with the following mutation primers: for the Y181A mutation, 5'-GCGTATACCGCGTACATCCCTGG-3' and 5'-GGGATGTAC-GCGGT ATACGCAGGGATG-3'; for the F187R mutation, 5'-CCTTCC-TGGCGCTTTT GCTTCCAGCTAC-3' and 5'-AAGCA AAAAGCG CCAG-GAAGGGATGTAG-3'. PCR reactions were carried out using primers, a plasmid containing the wild type human Neu3 ORF (pME-hmSD) as a template, and LA-Taq polymerase (Takara Shuzo, Otsu, Japan) at 95 °C for 5 min followed by 25 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 5 min. Mutated DNAs, with sequences confirmed by the di-
deoxy chain termination method using an AutoRead sequencing kit (Amersham Biosciences), finally were subcloned into the EcoRI site of the pME18S vector. As a control, a cytosolic sialidase expression plasmid was constructed by subcloning a cDNA fragment covering the ORF into pME18S and was used for transfection. A caveolin-1 expression vector was constructed by subcloning the ORF of human caveolin-1 cDNA (22) into pCEP4 (Invitrogen).

COS-1 cells were transfected by electroporation using the procedures described previously (4). For transfection of caveolin-1 to HeLa cells, Effectene transfection reagents (Qiagen) were used as recommended by the supplier. After 48 h of transfection, cells were collected and stored at −80 °C until use. To correct for transfection efficiency, a luciferase expression plasmid was co-transfected with the sialidase plasmid, and the sialidase activity was then normalized to the luciferase activity as detailed earlier (23).

Sucrose Density Gradient Analysis—Low density Triton X-100-insoluble membrane domains were isolated from cultured cells using the method described (24) with slight modifications. Briefly, cultured cells (∼3 × 108) were collected in 1 ml of ice-cold lysis buffer containing 25 mM MES (pH 6.5), 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% Triton X-100. After homogenization, the cell lysate was adjusted to 40% sucrose by addition of 1 ml of the above lysis buffer without Triton X-100 containing 80% sucrose. A linear sucrose gradient was layered over the lysate and centrifuged at 190,000 × g for 16–20 h at 4 °C. The dialyzed fraction was applied to 1 ml of the above lysis buffer described above, assayed for sialidase activity, and dialyzed against the start buffer (20 mM phosphate buffer, pH 7.5, 10 mM imidazole, 0.5 M NaCl, and 0.1% Triton X-100) for 3 h at 4 °C. The dialyzed fraction was applied to 1 ml of the start buffer (20 mM phosphate buffer, pH 7.5, 500 mM imidazole, 0.5 M NaCl, and 1% Triton X-100). Washes and eluates then were subjected to immunoblot analysis.

Sialidase Assays—The cell lysate and the fractions from the sucrose density gradient were used routinely for sialidase assays with gangliosides as the substrate. The reaction mixture contained 50 nmol of substrate as bound sialic acid, 0.2 mg of bovine serum albumin, 10 μmol of sodium acetate (pH 4.6), and 0.2 mg of Triton X-100 in 200 μl. After incubation at 37 °C for 10–60 min, released sialic acid was determined by the thiobarbituric acid method after passing through an AG1X-2 mini-column as described elsewhere (7). Protein was determined by dye-binding assay (Bio-Rad). One unit of sialidase was defined as the amount of enzyme that catalyzed the release of 1 nmol of sialic acid/h.

Preparation of Monoclonal Antibody to Human Neu3—The foot pads of 6-week-old female BALB/c mice were injected every week with 2 mg of protein of the particulate fraction of human Neu3-transfected COS cells emulsified with incomplete adjuvant. After five successive injections followed by a booster injection, spleens and bilateral inguinal and parietal abdominal lymph nodes of immunized mice were harvested, and B cells were fused with the mouse myeloma X63 Ag8.653 (The Cancer Cell Repository, Tohoku University, Sendai, Japan) in 50% polyethylene glycol glycol 1000 (Wako). Screening of hybridomas was carried out by examining the specific immunoreactivity of the supernatant of their culture medium in enzyme-linked immunosorbent assay system (Zymed Laboratories) and by fluorescence-activated cell sorter analysis. For this, the polystyrene microtiter plates precoated with homogenates

Fig. 2. Localization of human Neu3 expressed in COS-1 cells in caveolin-rich fractions. The fractions on a sucrose density gradient were assayed for ganglioside sialidase activity and immunoblotted for Neu3 and caveolin proteins with mAb anti-human Neu3 and anti-caveolin-1, respectively (a). As a control, cytosolic sialidase expressed in COS-1 cells was assayed for activity using 4MU NeuAc as a substrate (b).
of the Neu3 transfectants were incubated with the supernatants of hybridomas at 37 °C for 1–2 h and with alkaline phosphatase-conjugated goat anti-IgG and then were detected by color development. To avoid the problems related to extraction procedures for antigen, including partial disruption of antigenic determinants, the hybridomas also were tested by incubation with the Neu3 transfectants in the presence of 0.5% saponin followed by anti-mouse F(ab')2 fragments of IgG conjugated with fluorescein for fluorescence-activated cell sorter analysis. The homogenates from vehicle-transfected COS cells were used to exclude false positives caused by reactions with parent COS cell proteins. The positive clones were screened by dilution to obtain single specific clones. The subclass of monoclonal antibody (mAb) was determined to be IgG1 with a mouse isotyping kit.

Immunoblotting and Immunoprecipitation—Cell lysates, sucrose density gradient fractions, or fractions from HiTrap affinity columns were separated on SDS-polyacrylamide electrophoresis gels (10%) under reducing conditions. Proteins were transferred to polyvinylidene difluoride membranes and immunoblotted with antibodies specific to respective proteins. The individual bands were detected with peroxidase-conjugated goat anti-mouse or -rabbit IgG (Promega), and development was achieved with an enhanced chemiluminescence system (ECL Plus Western blotting reagent, Amersham Biosciences), according to the manufacturer’s recommendations. In immunoprecipitation experiments, cell lysates were incubated overnight at 4 °C with protein A-Sepharose (Amersham Biosciences) and polyclonal anti-caveolin-1 (Santa Cruz Biotechnology) or monoclonal anti-human Neu3 antibody, and the precipitates after washing were applied to SDS-PAGE and immunoblotted as described above. Quantification of the protein bands was determined by NIH image analysis. Antibodies and their sources were as follows: anti-caveolin-1 (mAb 2297, BD Transduction Laboratories) and anti-caveolin-1 (Santa Cruz Biotechnology); anti-c-Src (Santa Cruz Biotechnology); anti-MAP kinase (New England Biolabs); anti-14–3-3 (Santa Cruz Biotechnology).

RESULTS

Co-fractionation of Human Neu3 with Caveolin-1—To examine whether endogenous ganglioside-specific sialidase is associated with caveolin-enriched microdomains, we used HeLa cells having relatively high sialidase activity. Cell lysates were fractionated on a sucrose density gradient, and the fractions were analyzed for protein concentration, caveolin-1 immunoreactivity, and sialidase activity with gangliosides as substrates. Fig. 1a shows ganglioside sialidase activity co-fractionating in low density fractions 4–6 with caveolin-1 and cSrc (a signal transducer) as assessed by immunoblot analysis, whereas greater than 90% of the total protein loaded on the gradient was recovered in high density fractions 9–10. To obtain evidence that Neu3 protein is present in the caveolin-rich fraction, we prepared mAb specific to human Neu3 and observed the protein distribution by immunoblotting. The antibody was evaluated to be reactive only with Neu3 (48-kDa protein) from HeLa cells (in the experiments with homogenates in Fig. 1b) as well as from Neu3-transfected COS-1 cells. Upon sucrose gra-
dient Neu3 protein from HeLa cells was detected mainly in fractions 5–6, in parallel with sialidase activity together with caveolin-1. We then examined COS-1 cells transiently transfected with human Neu3 cDNA. More than 70% of the ganglioside-specific sialidase activity was found in fractions 4–6, rich in caveolin-1, and ~30% of the activity remained in fractions 9–10 (Fig. 2a). The activity detected in the high density fractions was not caused by overloading because reloading on the gradient resulted in recovery of the activity in the same fractions. Neu3 proteins were detected in the fractions having high activity in a manner similar to the case of HeLa cells. On the other hand, when we tested cytosolic sialidase (Neu2)-transfected COS-1 cells by measuring sialidase activity at pH 6.0 with 4MU-NeuAc (4-methylumbelliferyl-neuraminic acid), most of the sialidase was found in the high density fractions (Fig. 2b). In addition, endogenous lysosomal sialidase (Neu1) in COS-1 cells, assayed with 4MU-NeuAc as substrate at pH 4.6, showed a pattern nearly identical to that of the exogenously expressed cytosolic sialidase (data not shown). These results indicate human Neu3 indeed to be localized in the caveolin-rich microdomains, in contrast to Neu2 and Neu1 sialidases.

**Association of Human Neu3 with Caveolin-1**

To determine whether Neu3 is specifically associated with caveolin in the microdomain, we searched for a functional binding site for caveolin-1 in the Neu3 molecule. Caveolin-binding motifs were recently deduced (ΦXΦXXXΦ and ΦXXΦΦXXΦ, in which Φ is aromatic residue W, F, or Y) (17). These motifs are present in most caveole-associated proteins including protein kinase Cα, MAP kinase, and epidermal growth factor receptor. In the human Neu3, we identified a possible region (YTYYIPSW,
179–186 residues) within the hydrophobic stretch of the putative transmembrane domain sequence (4). To determine whether the binding site in Neu3 is functional, the following strategies were employed: introduction of mutations into Neu3, affinity purification of His-tagged Neu3 forms, and immunoprecipitation with antibody to either caveolin-1 or Neu3. Two mutants were expressed, and the cell lysates were analyzed by fractionation on a sucrose density gradient (Fig. 3). Mutant F187R with one amino acid change next to the motif sequence demonstrated reduction of ganglioside sialidase activity in the cell lysate to only 45% of the wild type level, and mutant Y181A with a single amino acid change within the motif almost lost the sialidase activity, although Neu3 protein level was similar to the wild type (Fig. 3a). Upon sucrose gradient fractionation, mutant F187R exhibited only a low amount of Neu3 protein in fraction 5 with low sialidase activity, much more protein, and activity being evident in fractions 9–10. In mutant Y181A, Neu3 protein was hardly recovered in the fractions 4–6 where caveolin was still concentrated. In contradiction to the high density fractions of F187R retaining some sialidase activity, those of Y181A hardly showed sialidase activity. This partly may concern the conformational changes of the latter protein. These results suggest that the predicted caveolin-binding motif in Neu3 may be involved in binding to caveolin-1 and loss of the binding ability possibly leading to failure of recruitment to caveolae and subsequently to reduction of the enzyme activity.

To obtain further evidence for specific binding to caveolin-1, we next used His-tagged forms of Neu3 constructed by introducing the His tag epitope into the C terminus (Fig. 4, a and b). Upon transient expression in COS-1 cells, the His-tagged form could be purified by Ni²⁺ ion-charged HiTrap chelating column chromatography, the bound proteins being specifically eluted by affinity elution with imidazole (25). As a control for nonspecific binding, human Neu3 without the tag epitope was also expressed. Both Neu3 forms showed sialidase activity at the same level. After affinity purification on HiTrap column, fractions were analyzed by immunoblotting with antibodies to human Neu3 and caveolin-1 (Fig. 4a). His-tagged Neu3 was specifically eluted by affinity elution with imidazole, whereas no binding to the column was observed with Neu3 lacking the His tag. Under these conditions endogenous caveolin-1 and MAP kinase co-eluted with His-tagged Neu3, but co-eluted with 14–3–3 protein in the washing effluents. When the cell lysates from

![Figure 5](http://www.jbc.org/)

**FIG. 5.** Alterations of Neu3 sialidase activity by β-cyclodextrin treatment and by caveolin transfection. Cells were treated with 10 mM β-cyclodextrin for 50 min at 37°C, and the lysates of collected cells were assayed for sialidase activity (a) and fractionated on a sucrose density gradient for detection of Neu3 activity and the protein (b). Caveolin-1 expression plasmid was transiently transfected to HeLa cells, and the endogenous sialidase activity and caveolin-1 protein were determined (c); left panel, sense DNA; right panel, antisense DNA.
mutant Y181A with the His tag epitope were applied on a Hitrap column, most of caveolin-1 protein was detected in the washings, even though all of the Neu3 protein was detected in the affinity eluates (Fig. 4b). We further evaluated the association of Neu3 with caveolin-1 by immunoprecipitation with antibody to either caveolin-1 or Neu3 (Fig. 4c). Under the conditions in which an almost equal amount of endogenous caveolin-1 was applied for immunoprecipitation (lower panel), wild type Neu3 was co-immunoprecipitated with caveolin-1 by anti-caveolin-1 antibody, whereas much less mutant Neu3 protein was recovered compared with wild type level (upper panel), the protein level relative to wild type in the immunoprecipitates being 67 and 15% in mutants of F187R and Y181A, respectively. Caveolin-1 was not detected in anti-Neu3 immunoprecipitates, possibly because of the epitope of the antibody near caveolin-binding motif. To understand the physiological significance of the tight association between Neu3 and caveolin, we first tested whether Neu3 sialidase activity is altered by treatment with 10 mM β-cyclodextrin, known to disrupt caveolae by cholesterol depletion (26). The treatment of Neu3-transfected COS cells caused dissociation of Neu3 from caveola fractions and significant decrease in the sialidase activity (Fig. 5a), although caveolin-1 still remained in low density fractions at the concentration of β-cyclodextrin. Secondly, we examined the effects of caveolin-1 expression on Neu3 activity by transient transfection of sense and antisense caveolin-1 expression plasmid. HeLa cells transfected with the sense cDNA showed higher endogenous Neu3 activity in proportion to the caveolin level, and in contrast the cells with the antisense cDNA had lower sialidase activity than the vehicle-transfected cells, without a change of Neu3 protein level (Fig. 5b), when endogenous Neu1 sialidase activity was not altered (data not shown). These data together indicate that Neu3 is associated with caveolin-1, probably through the predicted caveolin-binding motif sequence, and that Neu3 activity is down-regulated by dissociation from caveolae and also by decreased expression of caveolin-1.

**DISCUSSION**

Ganglioside-specific sialidase Neu3 is a unique glycosidase in its localization within the plasma membrane and the strict substrate specificity. Unlike lysosomes, the membranes do not contain a set of glycosidases to degrade glycoproteins and glycolipids (27), suggesting that Neu3 is involved in cell surface events other than catabolism of glycoconjugates. In fact, there are several observations suggesting important functional roles of sialidase in processes like cell differentiation (6, 28), apoptosis (29), and malignant transformation (30–33). However, no clear evidence for the physiological functions of Neu3 has been presented hitherto in molecular basis. Here we show for the first time that this sialidase is closely associated with caveolin-1 and that its activity is likely to be regulated by caveolin-mediated recruitment to caveolae microdomains. Neu3 has been expected to participate in cell signaling at cell surface through modulation of gangliosides. In addition to this ganglioside-mediated action, present observations suggest an another mode of Neu3 action through binding to signal-related molecules including caveolin-1. Although clear evidence for direct interaction of Neu3 with caveolin has not been provided, direct involvement of Neu3 in caveolin-mediated signaling appears plausible.

Many caveolin-associated proteins are now known to contain caveolin-binding motifs, and this is considered to be a general mechanism for caveolin-mediated sequestration and subsequent inactivation within caveolae. In the case of Neu3, mutation of the putative caveolin-binding site led to blocking of the recruitment to caveolae. The possibility cannot be excluded that suppression of the sialidase activity in the mutants is primarily caused by the conformational changes but that separation of Neu3 from caveolin in microdomains by cholesterol depletion causes the similar activity change. Reduced expression of caveolin with the antisense DNA also caused down-regulation of the sialidase activity. This suggests that Neu3 activity is regulated rather than inactivated by caveolin-mediated sequestration, similar to the case of the insulin receptor promoting IRS-1 phosphorylation (34). Although the exact mechanisms underlying caveolin-mediated regulation of Neu3 activity have not been elucidated, one possible explanation for caveolin-mediated sequestration may be the following. To activate Neu3 in vitro, detergents such as Triton X-100 are required (4). There is no presently known endogenous activator located in the plasma membrane that can be replaced by the detergent. Because we have observed that phospholipids can stimulate the Neu3 sialidase activity, it is most feasible that phospholipids constituting caveolae may activate Neu3 after recruitment.

Our present observations indicate that Neu3 and caveolin-1 are closely associated in microdomains and that Neu3 activity probably is regulated by complex formation with caveolin within caveolae, suggesting that Neu3 may play important roles as a signal molecule through cross-talk with other signal transducers.

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