Molecular Cloning and Characterization of a Plasma Membrane-associated Sialidase Specific for Gangliosides*

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Gangliosides are plasma membrane components thought to play important roles in cell surface interactions, cell differentiation, and transmembrane signaling. A mammalian sialidase located in plasma membranes is unique in specifically hydrolyzing gangliosides, suggesting crucial roles in regulation of cell surface functions. Here we describe the cloning and expression of a cDNA for the ganglioside sialidase, isolated from a bovine brain cDNA library based on the amino acid sequence of the purified enzyme from bovine brain. This cDNA encodes a 428-amino acid protein containing a putative transmembrane domain and the three Asp boxes characteristic of sialidases and sharing 19–38% sequence identity with other sialidases. Northern blot and polymerase chain reaction analyses revealed a general distribution of the gene in mammalian species, including man, and the mouse. In COS-7 cells transiently expressing the sialidase, the activity was found to be 40-fold that of the control level with ganglioside substrates in the presence of Triton X-100, and the hydrolysis was almost specific to gangliosides other than GM1 and GM2, both α2−3 and α2−8 sialyl linkages being susceptible. The major subcellular localization of the expressed sialidase was assessed to be plasma membrane by Percoll density gradient centrifugation of cell homogenates and by immunofluorescence staining of the transfected COS-7 cells. Analysis of the membrane topology by protease protection assay suggested that this sialidase has a type I membrane orientation with its amino terminus facing to the extracytoplasmic side and lacking a signal sequence.

The sialidase reaction is an initial step of the degradation of glycoproteins and gangliosides. Sialidases of mammalian origin have been implicated not only in lysosomal catabolism but also in modulation of functional molecules involved in many biological processes (1, 2). However, the physiological significance and the regulation mechanisms of desialylation remain obscure because the structure and function of mammalian sialidases are not fully understood. Our previous studies aimed at the biochemical characterization of mammalian sialidases demonstrated four types in rat tissues differing in subcellular location as well as catalytic and immunological properties: intralysosomal (3), cytosolic (4), lysosomal membrane, and plasma membrane (5). The multiple nature of mammalian sialidases suggests that each form may play a unique role depending on its particular subcellular location and catalytic properties. To elucidate the structure and function of these low abundance proteins, cloning of the individual genes is required. We previously cloned a rat cytosolic sialidase gene (6), the first cDNA example of a mammalian species, and established its involvement in differentiation of skeletal muscle cells (7). Recently, human (8–10) and mouse (11, 12) major histocompatibility complex (MHC)-related sialidases were cloned and suggested to be primarily localized in lysosomes (13).

Membrane-associated sialidases hydrolyze gangliosides preferentially (5, 14, 15), and those in the plasma membrane are distinct from lysosomal membrane sialidases in acting specifically on gangliosides (5, 16–18) residing in the same membrane. Gangliosides are thought to play crucial roles in cell surface events, including cell differentiation, cell-cell interactions, and transmembrane signaling (19–21). Plasma membrane sialidases, therefore, have been considered to participate in these phenomena through modulation of gangliosides. In fact, there are observations suggesting important biological roles of ganglioside sialidases, although information as to what types of ganglioside sialidase involved is not available enough. The activity levels fluctuate consistently with cell differentiation, cell growth, and malignant transformation. For example, a sialidase inhibitor, 2,3-dehydro-2-deoxy-N-acetylneuraminic acid, abolishes increase of a differentiation marker enzyme in human neuroblastoma cells (22, 23), and the observations by Usuki et al. (24, 25) led them to propose the participation of ganglioside sialidase in cell growth regulation. In addition, alterations of the levels of ganglioside sialidase expression associated with malignant transformation have been described: loss of cell density-dependent suppression in 3T3-transformed cells (26) and appearance of ganglioside sialidase activity in transformed cell lines of baby hamster kidney fibroblasts (27). We previously reported an increase of plasma membrane sialidase activity associated with induction of anchorage-independent growth in mouse epidermal JB6 cells exposed to phorbol esters (28). However, little is known about the molecular mechanisms underlying such sialidase alterations. To provide tools for their elucidation, we have focused on cloning a cDNA of the sialidase. We recently were able to purify a ganglioside sialidase extensively from bovine brain, which is the

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AB008184.

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The abbreviations used are: MHC, major histocompatibility complex; MOPS, 4-morpholinepropanesulfonic acid; 4MU-NeuAc, 4-methylumbelliferyl-neuraminic acid; PCR, polymerase chain reaction; HSV, Herpes Simplex virus; aa, amino acid(s); PAGE, polyacrylamide gel electrophoresis.

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major ganglioside-hydrolyzing sialidase of the tissue and is located mainly in synaptosomes (18). Using the purified enzyme protein, we have now succeeded in cloning a membrane-associated ganglioside sialidase.

**EXPERIMENTAL PROCEDURES**

cDNA Cloning of the Membrane-associated Sialidase—Membrane-associated sialidase was purified extensively from 5 kg of frozen bovine brain as described previously (18). The concentrated enzyme at the step of thiol-Sepharose column chromatography was electrophoresed on an SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The polyvinylidene difluoride-immobilized enzyme was used to obtain cDNA coding for the enzyme protein. The polyvinylidene difluoride-immobilized enzyme protein was subjected successively to reduction, S-carboxymethylation, and in situ digestion with lysylendopeptidase and endoproteinase Asp-N (29). The digested peptides were fractionated on a reverse-phase high performance liquid chromatography column, and amino acid sequencing was performed. Based on the amino acid sequences of the four peptides derived from the purified sialidase (see Fig. 1a), nine degenerate oligonucleotide primers for both sense and antisense strands were synthesized with deoxynucleosine substitution as shown in Fig. 1. First strand cDNAs were synthesized from the poly(A)-rich RNA of bovine brain using random primers and murine leukemia virus reverse transcriptase (Superscript RNase H−, Life Technologies, Inc.) and used as templates for PCR reactions. The PCR reaction conditions were as follows: denaturation at 94°C for 30 s, annealing at 55°C for 1 min, elongation at 72°C for 2 min; 30–40 cycles. A fragment (482 base pairs) amplified with Ap3S and DN2A primers was judged to be appropriate by sequencing, randomly labeled with [32P]dCTP, and used as a probe to screen a bovine brain cDNA library (CLONTECH). Ten positive clones were isolated by high stringency washing and sequenced by the dideoxy chain termination method in both directions using an AutoRead Sequencing kit (Amersham Pharmacia Biotech). Two overlapping clones (1.5 and 2.8 kilobases) containing full-length open reading frames were isolated by high stringency washing and sequenced by the dideoxy chain termination method in both directions using an AutoRead Sequencing kit (Amersham Pharmacia Biotech). Two overlapping clones (1.5 and 2.8 kilobases) containing full-length open reading frames were subcloned into Bluescript (pBB121 and pBB321). For 5′- and 3′-end amplification, the procedure described by Frohman et al. (30) was employed using specific primers synthesized based on the sequence of the positive clones.

To obtain sialidase cDNA fragments of mouse and human tissues, cDNAs were amplified under the same conditions described above with the primers 5′-GGACCAGGCGGAGATCAACGGCGGCTCCG-3′ (sense) and 5′-CTTGGCCGCCACGGAAGTGGCCA-3′ (antisense) for a region in which the amino acid sequence of the bovine sialidase is identical to that of cytosolic sialidase.

Northern Blotting—Total RNA was extracted from bovine brain by the acid guaniidinium-phenol-chloroform extraction procedure (31), and poly(A)-rich RNA was isolated with oligo(dT)-cellulose column chromatography. Poly(A)-rich RNA from human brain and skeletal muscle were obtained from CLONTECH. Total RNA (15 μg) and Poly(A)-rich RNA (5 μg) were denatured at 65°C in a solution of 50% (v/v) formamide, 6% (v/v) formaldehyde, and 20 mM MOPS (pH 7.0), electrophoresed in a 1% saline-sodium phosphate/EDTA, 5 mM dithiothreitol, 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride, the mixture was centrifuged at 10,000 × g for 20 min, and the resulting pellets were dissolved in 300 μl sodium acetate (pH 4.6) and 0.2 mg of Triton X-100. After incubation at 37°C for 10–30 min, the sialic acid released was determined by the thiobarbituric acid method as described elsewhere (4). Sialidase activity toward 4-methylumbelliferyl-neuraminic acid (4MU-Neu5Ac) was assayed by spectrophotometric measurement of 4MU released (4). One unit of sialidase was defined as the amount of enzyme that catalyzed the release of 1 nmol of sialic acid/h.

Percoll density gradient centrifugation of the sialidase in transfected cells was conducted as follows: 1 ml of cell homogenate (1,000 × g supernatant) was applied on top of 16 ml of 40% Percoll (in 0.25 M sucrose) and 1 ml of 60% Percoll (in 1.5 M sucrose) and centrifuged at 48,000 × g for 45 min. Fractions of 300 μl were collected and assayed for sialidase, N-acetyl-β-hexosaminidase and 5′-nucleotidase activities (5).

For immunofluorescence staining, transfected cells expressing HSV epitope-tagged sialidase were fixed with 4% (w/v) paraformaldehyde for 10 min, permeabilized with 0.2% (w/v) Triton X-100 in phosphate-buffered saline for 2 min, and immunostained with HSV tag monoclonal antibody (Novagen). As a control, cells transfected with pMEcSD-HSVTag, in which the open reading frame of membrane sialidase was replaced by that of cytosolic sialidase (6), were stained in the same manner. To test antibody binding to the cell surface, non-permeabilized cells transfected with pMEemSD-HSVTag or pMEemSD were stained with HSV tag monoclonal or anti-sialidase peptide antibodies, respectively. The anti-peptide antibody was prepared by immunizing rabbits with keyhole limpet hemocyanin-coupled oligopeptides corresponding to amino acid residues 109–128, according to standard procedures. The specific antibody was affinity purified from the sera of immunized animals using immobilized peptides. Fluorescein isothiocyanate-conjugated anti-mouse (Tago) and anti-rabbit (Bio-Rad) IgG Fab fragments were used as secondary antibodies. To determine the membrane topology of the sialidase, a protein P protection assay was performed. Transfected cells (5 × 106) harboring pMEemSD-HSVTag or pMEemSD were suspended in 9 volumes of 0.25 M sucrose containing 10 mM Tris-HCl, 1 mM dithiothreitol, 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride and disrupted by 10 passages through a 22-gauge needle. The vesicles obtained were subjected to proteolysis with proteinase K (0.5 μg/ml) for 5 h at 0°C in the presence or absence of 1% Triton X-100. After stopping the reaction with 5 mM phenylmethylsulfonyl fluoride, the mixture was centrifuged at 10,000 × g for 20 min, and the resulting pellets were dissolved in 300 μl of SDS-PAGE sample buffer. Expressed sialidase proteins were then analyzed by Western blotting using HSV tag monoclonal or anti-sialidase peptide antibodies followed by exposure to alkaline phosphatase-conju-
FIG. 2. Nucleotide and deduced amino acid sequences of the bovine ganglioside sialidase. a, The predicted amino acid sequence is shown by the single letter amino acid code under the nucleotide sequence. The positions of the four peptide sequences obtained from the purified...
envelope antibody or anti-rabbit IgG Fab fragments, respectively. The presence of N-linked glycosylation in the expressed sialidase was assessed by tunicamycin and N-glycosidase treatments. Tunicamycin was added into the culture medium at 2.5 μg/ml at 28 h prior to harvesting. For N-glycosidase sensitivity test, homogenates (30 μg of protein) of the transfected cells were incubated with 1 milliunit of N-glycosidase F (Flavobacterium meningosepticum, Takara, Japan) at 37 °C overnight under denaturation conditions as recommended by the supplier. Fetuin (25 μg) was treated simultaneously as a control glycoprotein.

RESULTS

The PVDF-immobilized enzyme protein was digested with lysylendopeptidase followed by endoproteinase and microsequence. Based on the four peptides thus obtained (Fig. 1, top), degenerate oligonucleotides for sense and antisense primers were prepared (Fig. 1, bottom) and used in the PCR reaction with bovine brain random-primed cDNA as template. An amplified cDNA fragment (482 base pairs) produced with Ap3S and DN2A primers was identified as a candidate because the deduced amino acid sequence contained two Asp boxes (32) and demonstrated 30% identity with cytosolic sialidase unexpectedly. With this cDNA as a probe, we screened a bovine brain λgt10 library (2 × 10^6 plaques) and obtained ten positive clones. Two overlapping clones were found to contain the entire open reading frame, and the 5′-end amplification by PCR resulted in further extension at the 5′-end of 83 nucleotides. The longest positive clone (pBB321) and the amplified cDNA with an extended 5′-end were used to generate a cDNA (Fig. 2a) of 2898 nucleotides having an extended 5′-end of 258 nucleotides with a putative initiating methionine in perfect agreement with the consensus for eukaryotic genes. We predict that the methionine codon at nucleotides 259–261 is the initiation site rather than the residue at nucleotides 229–231 because of the existence of a clone different in the 5′-portion upstream of nucleotide 249. None of the positive clones were found to possess a polyadenylation signal but ATTTA motifs were present in the 3′-untranslated region, thought to be involved in destabilization of mRNAs coding for proto-oncogenes and cytokines and therefore in the regulation of gene expression during cell growth and differentiation (33). Despite repeated attempts, amplification of the 3′-end portion of the gene by PCR was unsuccessful. The deduced protein has 428 amino acids, with a molecular mass of 47,916 Da, that includes the four sequenced peptides, three Asp boxes, and one potential glycosylation site. Comparison of the primary sequence with those of other mammalian and bacterial sialidases revealed 19–38% sequence identities: the highest degree of homology (38%) was found with rat (6) and hamster (34) cytosolic sialidases, and 24, 21, and 19% with human MHC-related (8–10), Clostridium perfringens (35), and Salmonella typhimurium (36) sialidases, respectively. Searches for primary sequence homology in protein data bases showed no significant similarities to other proteins, but interestingly, one region of the sialidase (59–103 aa) contained 35.6% identity to a region (291–335 aa) of the mouse embryonic growth factor (GDF1) (37), a member of the TGF-β superfamily. A hydropathy plot generated by the method of Hopp and Wood (38) suggested that the sialidase has a hydrophobic segment of 21 amino acids (174–194 aa) and no signal sequence as shown in Fig. 2b. The hydrophobic stretch divides the sialidase sequence into two parts and can be assumed to be a transmembrane segment flanked by a positively charged residue on the carboxyl-terminal side. Like viral and bacterial sialidases, this enzyme has a high content of cysteine residues (21 cysteines) and β-sheet structures. S. typhimurium sialidase, whose three-dimensional structure has been determined by x-ray crystallography (39), was used to investigate active site residues of our bovine sialidase. Alignment of the two sequences using the mode described by Milner et al. (9) revealed a strikingly similar spatial arrangement of the catalytic residues: 8 of the 13 active site residues are conserved in the bovine sialidase. Two of the three active residues involved in binding the acetyl group of sialic acid (Asp-62 and Asp-100) and
three of the four residues forming the hydrophobic pocket (Trp-121, Trp-128, and Leu-175 in the bacterial sialidase) demonstrated replacement by Glu-51, Asn-88, Val-107, Arg-114, and Gly-162, respectively, in the bovine enzyme. The three active site residues in the hydrophobic pocket are identical to the corresponding residues in the rat cytosolic sialidase, and all four residues differ from those of human MHC-sialidase, probably reflecting differences in substrate specificity.

Northern blot analysis of bovine brain revealed an approximate 7.5-kb length of the mRNA using a cDNA covering the entire coding sequence as a probe (Fig. 3a), indicating a long stretch (5.8 kilobases) of the gene at the 3′-untranslated region. The same size transcripts were found to be present in human skeletal muscle and brain. Analysis of the partial sequences obtained by PCR reaction for the human and mouse sialidase genes, as shown in Fig. 3b, demonstrated extensive sequence identity to the bovine gene. In this region, the four active site residues forming the hydrophobic pocket, as aligned with S. typhimurium sialidase, were found, and their sequences were all identical. In fact, the primary sequence of the corresponding human gene displayed an 83% overall identity (data not shown).

To confirm that the isolated cDNA clone encodes the ganglioside-hydrolyzing sialidase, plasmids (pMEmSD) were constructed by introduction of the cDNA into a eukaryotic expression vector, pME18S, containing the SRα promotor. COS-7 cells transiently transfected with pMEmSD showed an over 40-fold increase in sialidase activity toward gangliosides in the presence of 0.1% Triton X-100 using cell homogenates and particulates as enzyme sources in comparison with untransfected cells or with vector-transfected cells, while the activity level toward 4MU-NeuAc, a synthetic substrate, was not changed (Fig. 4a). The sialidase acted preferentially on gangliosides other than GM1 and GM2 and thus on both sialyl linkages (Fig. 4b). The sialidase activity toward gangliosides in the presence of Triton X-100 using cell homogenates and particulates as enzyme sources in comparison with untransfected cells or with vector-transfected cells, while the activity level toward 4MU-NeuAc, a synthetic substrate, was not changed.

FIG. 4. Expression of the ganglioside sialidase in COS-7 cells. a, sialidase activity in cells transfected with pMEmSD was assayed using gangliosides or 4-MUNeuAc as substrates. The values are means ± S.D. of five independent experiments. b, substrate specificity of the sialidase expressed in COS-7 cells. Sialidase activities toward various sialoconjugates were examined in the particulate fractions and expressed as the percentage of sialic acid released relative to the desialylation of GD3.

When the transfected cell homogenates were subjected to Percoll density gradient centrifugation, most sialidase activity toward gangliosides in the presence of Triton X-100 co-migrated with 5′-nucleotidase, a plasma membrane marker enzyme, but little was detected in the fractions corresponding to lysosomes (Fig. 5a). To confirm the association with the plasma membrane, transfected cells with HSV epitope-tagged sialidase genes were fixed, permeabilized, and immunostained with anti-HSV antibody (Novagen) and analyzed by confocal microscopy (Fig. 5b). Strong surface staining was detected in cells expressing the sialidase, whereas transfection of the tagged cytosolic sialidase gene resulted in cytosolic and nuclear expression, as demonstrated previously in skeletal muscle cells by electron microscopy (40), indicating no interference of the HSV tag with the sialidase expression.

We then investigated whether the sialidase is a transmembrane protein and how it is oriented using an antibody to the HSV peptide fused at the carboxyl terminus and an anti-peptide antibody to the oligopeptide corresponding to amino acid residues 109–129 upstream of the putative transmembrane domain. Cells expressing pMEmSD-HSV exhibited the same degree of sialidase activity as pMEmSD-transfected cells. As shown in Fig. 5c, the overexpressed sialidase was recognized specifically as a diffuse band of 48–54 kDa on Western blotting with anti-HSV (Fig. 5c, lanes 1 and 4) and anti-peptide (lane 6) antibodies. The presence of tunicamycin in cell medium gave a little change in mobility of the recombinant protein on Western blots (lane 2), but incubation of the homogenates with N-glycosidase resulted in no change under the condition where a control glycoprotein enhanced sufficiently its mobility (lane 3), indicating little possibility of the presence of N-glycosylation. Prepared membrane vesicles were digested with proteinase K, and the products were analyzed by Western blotting. An approximately 22-kDa fragment (lower arrow) with its degraded forms and an unprocessed form (upper arrow) was detected with the anti-peptide antibody (lanes 7 and 8), which recognizes the peptide on the amino-terminal side of the sialidase, but no protection was apparent with the HSV epitope-tagged sialidase (lane 5). The fragment unaffected by proteinase K treatment corresponds to the amino terminus and a part of the membrane-spanning domain region, being completely degraded with proteinase K in the presence of Triton X-100 (lane 9). This expressed sialidase, therefore, appears to be a transmembrane protein with an extracytoplasmic amino ter-
minus. Support for this conclusion was provided by immuno-staining of non-permeabilized transfected cells. The anti-sialidase peptide antibody gave rise to a cell surface staining, even if low staining intensity (data not shown), whereas staining was hardly observed without permeablization using anti-HSV antibody. The prediction for transmembrane proteins by the Phdtopology (41) and Tmpred (42) programs is consistent with these results. Fusion protein expression plasmids at the amino terminus demonstrated decreased sialidase activity, and introduction of the constructs with prokaryote expression vectors into E. coli gave no sialidase activity. Taken together, these results strongly indicate that the ganglioside sialidase is associated with plasma membrane and suggest that it is an atypical type I membrane protein lacking a signal sequence and exposing the amino terminus to the extracytoplasmic side.

DISCUSSION

Two types of mammalian sialidase, whose major locations are the cytosol (6, 33) and lysosomes (8–12), have been cloned to date. Their primary sequences are not very similar but contain several conserved sequences including ASP boxes characteristic of sialidases. The plasma membrane-associated sialidase has been found to be clearly distinct from cytosolic and lysosomal sialidases in enzymatic properties, especially in its strict substrate specificity with hydrolysis of gangliosides but not glycoproteins or oligosaccharides to any great extent (5, 17, 18). In the present study, we have cloned a bovine cDNA encoding a ganglioside sialidase associated mainly with the plasma membrane, which is considered to be the major sialidase for ganglioside hydrolysis in bovine brain. Like other sialidases, the primary sequence contained several consensus amino acid sequences, including some putative active site amino acid residues and ASP boxes that are conserved in bacterial sialidases, suggesting a structure related to microbial counterparts and supporting the idea that mammalian and microbial sialidases have a common phylogenic origin (43). The primary structure of the sialidase gene showed the highest homology (38%) to rat and hamster cytosolic sialidases among sialidases so far cloned and less similarity (18%) to human and...
mouse lysosomal sialidases. This is in line with the differences in substrate specificity of these three types because the former two can efficiently hydrolyze gangliosides to be poor substrates for MHC-related lysosomal sialidase (9). Although the similarities in partial sequence and structure indicate that the three mammalian sialidases share a common ancestor with bacterial sialidases, there must be some sequences responsible for the substrate specificities, active site residues in the hydrophobic pocket presumably being involved because of their difference from those of MHC-related sialidase as well as bacterial sialidases. Interestingly, the region containing these residues was predicted to be on the extracellular side of the plasma membrane by protease protection assays. Sequences for subcellular localization are characteristic for each sialidase: the ganglioside sialidase possesses a putative transmembrane domain, whereas no such sequences exist for cytosolic and lysosomal sialidases, which, respectively, have a nuclear translocation signal and a lysosomal targeting sequence.

COS-7 cells transiently transfected with the cDNA showed increased sialidase activity toward ganglioside substrate in the presence of Triton X-100 in the assays. This is consistent with observations using cell homogenates as the enzyme source by Lieser et al. (44) that plasma membrane sialidase is stimulated by non-ionic detergents, suggesting that the increased activity was because of plasma membrane sialidase. The fact that the substrate specificity of the expressed sialidase toward gangliosides was similar to that of the purified enzyme from bovine brain provides further support that the cDNA encodes the sialidase protein.

The subcellular localization of this sialidase was determined to be the plasma membrane by sucrose density gradient centrifugation of cell homogenates and by immunofluorescence staining of the transfected cells. Immunostaining was able to demonstrate differences in subcellular localization between the ganglioside sialidase and rat cytosolic sialidase used as a control, precluding an effect of the HSV peptide on expression of these genes because of the consistency with our previous data for anti-peptide antibody specific to the cytosolic sialidase (40). Examination of the transmembrane topology of the sialidase by protease protection assays provided evidence for an extracytoplasmic side at the amino terminus, implying that a single putative N-glycosylation site resides unusually in the cytoplasm. This raised a question whether the N-glycosylation site is functional on the sialidase protein expressed in COS-7 cells because our previous data, that RCA120-lectin binds the sialidase fraction at a purification step from bovine brain particulates, have suggested that the sialidase may be an N-glycosylated protein (18). N-glycosidase treatment, however, resulted in no change in the size of the protein under appropriate conditions, indicating that this protein does not undergo N-glycosylation although it seems to be inconsistent with the tunicamycin results. Tunicamycin treatment in this case may result in changes in other post-translational modification. If it can be concluded that the sialidase is not N-glycosylated, it raises a question about the RCA-lectin binding ability of the brain enzyme. One explanation for this may be that the enzyme is bound to the lectin indirectly through the mediation of some protein(s) having the ability, which COS-7 cells do not express. As the alignment of the sequence suggested that possible active site residues are distributed all over the molecule, it is not clear whether this sialidase is catalytic in the membrane-anchored state. Rather, it is likely that the catalysis would occur in the cytoplasmic side of the membrane. There are two possibilities. First, like lectins, the sialidase could bind ganglioside substrates at the cell surface by its extracytoplasmic region covering 7 of 13 active site residues, become inter-
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