Identification and expression of NEU3, a novel human sialidase associated to the plasma membrane

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Several mammalian sialidases have been described so far, suggesting the existence of numerous polypeptides with different tissue distributions, subcellular localizations and substrate specificities. Among these enzymes, plasma-membrane-associated sialidase(s) have a pivotal role in modulating the ganglioside content of the lipid bilayer, suggesting their involvement in the complex mechanisms governing cell-surface biological functions. Here we describe the identification and expression of a human plasma-membrane-associated sialidase, NEU3, isolated starting from an expressed sequence tag (EST) clone. The cDNA for this sialidase encodes a 428-residue protein containing a putative transmembrane helix, a YRIP (single-letter amino acid codes) motif and three Asp boxes characteristic of sialidases. The polypeptide shows high sequence identity (78%) with the membrane-associated sialidase recently purified and cloned from *Bos taurus*. Northern blot analysis showed a wide pattern of expression of the gene, in both adult and fetal human tissues. Transient expression in COS7 cells permitted the detection of a sialidase activity with high activity towards ganglioside substrates at a pH optimum of 3.8. Immunofluorescence staining of the transfected COS7 cells demonstrated the protein's localization in the plasma membrane.

Key words: expressed sequence tag, gangliosides, gene structure, membrane-bound enzyme.

INTRODUCTION

Sialidases or neuraminidases (EC 3.2.1.18) remove sialic acid residues from various sialo derivatives such as sialo-oligosaccharides, sialoglycoproteins and sialoglycolipids (gangliosides). The biology of viral and bacterial sialidases has been extensively studied [1], whereas the study of their mammalian counterparts has been hampered for a long time by their usually low cellular content and lability during purification procedures. In mammals, several sialidases with different subcellular localizations, tissue distributions and substrate specificities have been described, suggesting great molecular variety in these proteins. In this perspective, the availability of the cDNA species encoding mammalian sialidases is an important step leading to a comprehensive picture of the relationships between the structure and function of these enzymes.

In the past decade several mammalian sialidases have been cloned. The first mammalian protein carrying this enzyme activity to be cloned and characterized at the molecular level was the rat cytosolic sialidase [2], followed by a soluble sialidase secreted into the culture medium by CHO (Chinese hamster ovary) cells [3]. Then the cDNA species encoding the human [4–6] and mouse [7–9] lysosomal sialidases were isolated, permitting the definition of the molecular basis of the human lysosomal disorder sialidosis [10] and the neuraminidase defect in the SM/J mouse [11] respectively. We recently reported the identification of a novel human gene, *NEU2*, encoding a cytosolic sialidase similar to the soluble enzyme from rat and hamster [12,13]. The last gene of the mammalian sialidase family that has so far been cloned encodes a bovine plasma-membrane-associated sialidase specific for gangliosides [14], a protein previously purified from bovine brain [15]. Membrane-associated sialidases have been described in different tissues [16–18] and cell types [19–21]; a form linked to the membrane via a glycosylphosphatidylinositol anchor has also been identified [22,23]. The main role of these forms of sialidases is thought to be the regulation of the sialic acid content of membrane-bound sialyl glycoconjugates. Among these compounds, gangliosides are the most abundant in the plasma membrane of vertebrate cells; they are involved in various biological processes including cell adhesion, cell–cell interactions and cell proliferation, differentiation and oncogenic transformation [24]. Therefore a detailed molecular characterization of membrane-associated sialidases could help in an understanding of the mechanisms underlying these different aspects of ganglioside biology. Here we report the identification of a human plasma-membrane-associated sialidase encoded by a novel human gene, *NEU3*, mapping on chromosome 11q13.

EXPERIMENTAL

General techniques

Standard molecular biological techniques were performed as described by Sambrook et al. [25]. DNA restriction and modifying enzymes were from Boehringer unless indicated otherwise.

Isolation of *NEU3* cDNA and gene

Searches of the database of expressed sequence tags (dbEST) were performed as described previously [26]. The IMAGE cDNA

Abbreviations used: dbEST, database of expressed sequence tags; EST, expressed sequence tag; HA, haemagglutinin; 4-MU, 4-methylumbelliferone; ORF, open reading frame; PAC, P1-derived artificial chromosome.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number Y18563.

clones corresponding to the *NEU3* transcript were obtained from the UK Human Genome Mapping Project Resource Centre.

Phage library plating and screening conditions were performed with standard procedures [25]. Human cDNA clones were isolated from a human skeletal muscle cDNA library (Clontech; in λ GT11 vector) by using a probe derived from IMAGE clone 1041654 with primers HS1F (5′-AGGCCTCATTCTCTGATG-3′) and HS1R (5′-CTTACCACACTCCCTTGG-3′). Inserts of recombinant phages were isolated by PCR with vector oligonucleotide primers.

DNA sequencing

Automated sequencing (with an Applied Biosystems ABI 377 fluorescence sequencer) was performed with vector- and gene-specific oligonucleotide primers. The sequences of the exon–intron boundaries were determined by sequencing P1-derived artificial chromosome (PAC) clones with cDNA-specific primers.

Computer sequence analysis

Sequence assembly and editing was performed with both Auto-Assembler version 1.4 (Perkin Elmer–Applied Biosystems) and DNA Strider version 1.2 [27] programs. Multiple sequence alignment was performed with the ClustalW algorithm [28]. Nucleotide and amino acid sequences were compared with the non-redundant sequence databases present at the NCBI (National Center for Biotechnology Information) with BLAST version 2.0 [29].

Chromosomal localization and PAC library screening

For radiation-hybrid mapping we used the Genebridge 4 panel (Research Genetics, Huntsville, AL, U.S.A.), which includes 93 human and hamster clones. DNA (25 ng) for each of the hybrid clones was used for PCR amplification in 96-well microtitre plates with the HS1F and HS1R primers, at 55 °C as the annealing temperature. Results were sent to the Radiation Hybrid Mapper server at Whitehead Institute/MIT Center for Genome Research (http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl).

For the RPCI-5 PAC library screening, the same set of primers was used.

Northern blot analysis

Commercial human RNA Master Blot (Clontech) containing dotted human poly(A)$^+$ RNA species from 50 different tissues was hybridized with human sialidase (HS) probe. This probe was PCR-amplified from the cDNA HSM5 clone by using the primer set HSNt (5′-ATGGAAGAAGTGACAACATG-3′) and HSCt (5′-TTAATTGCTTTTGAATTGG-3′) and spanned the entire *NEU3* open reading frame (ORF). The experiment was performed under the conditions recommended by the manufacturer.

Expression in COS7 cells

The cDNA region containing the entire *NEU3* ORF was amplified by PCR with cloned *Pfu* polymerase (Stratagene), a sense primer with a *Bgl*II site (HSNtB, 5′-GAAGATCTATG-GAAGAAGTGACAACATG-3′), an anti-sense primer with an *Eco*RI site (HSCtE, 5′-CATGAATTCTTAATTGCTTTTGA-ATTGG-3′) and HSM5 cDNA clone as template. pCDNAI-NEU3 was constructed by cloning the amplified insert 5′ in frame with the haemagglutinin (HA) epitope into plasmid pCDNAI (Invitrogen). The amplified insert was also cloned into a Rous-sarcoma-virus-promoted vector [30], yielding the pCDL-NEU3 construct. COS7 cells were grown in Petri dishes (100 mm diameter) by using Dulbecco's modified Eagle's medium with 10 % (v/v) fetal calf serum. Transfections were performed overnight with 6 μg of plasmid DNA and LipofectAMINE reagent, in accordance with the manufacturer's guidelines (Life Technologies). Cells were harvested by scraping, washed in PBS and resuspended in the same buffer containing 1 mM EDTA, 1 μg/ml pepstatin A, 10 μg/ml apoprotinin and 10 μg/ml leupeptin. Total cell extracts were prepared at various times up to 72 h after transfection by sonication. The supernatant obtained after centrifugation at 800 ***g*** for 10 min was designated the crude cell extract and was subsequently centrifuged at 200000 ***g*** for 15 min on an Optima TL 100 ultracentrifuge (Beckman). Aliquots of the original crude cell extract, 200000 ***g*** supernatant and pellet were used for assays for protein (Coomassie Protein Assay Reagent; Pierce) and sialidase activity, and for Western blot analysis.

Western blot analysis

Protein samples corresponding to 10 μg of each cell fraction were subjected to SDS/PAGE [10 % (w/v) gel] and subsequently transferred to nitrocellulose extra blotting membrane (Sartorius) by electroblotting. The membranes were incubated for 30 min in TBS/0.1 % (v/v) Tween 20 (TTBS) containing 10 % (w/v) dried milk (blocking buffer; BB). The primary antibody, anti-HA monoclonal antibody (Boehringer), was added at an appropriate dilution in BB and blots were incubated for 1 h. After being washed in TTBS, the membranes were incubated for 1 h with the appropriate horseradish-peroxidase-conjugated IgG (Amersham) diluted in BB. After a final wash in TTBS, detection of antibody binding was performed with enhanced chemiluminescence (Amersham) in accordance with the manufacturer's instructions. All incubations were performed at room temperature, with constant shaking.

Preparation of unlabelled and ^{3}H-labelled gangliosides and neutral glycolipids

Gangliosides GM1, GD1a, GD1b, GT1b and lactosylceramide from calf brain and GM3 (NeuAc) from human spleen were prepared and analysed structurally as described previously [31]. GM1, GM3 and GD1a were ^{3}H-labelled at C-3 of the sphingosine moiety; the separated and purified *erythro* forms were used [32]. In addition, GM3 was ^{3}H-labelled at the level of the *N*-acetyl group of sialic acid ([*NeuAc*-^{3}H]GM3) by the method of Chigorno et al. [33]. The radiochemical purity of [^{3}H]GM1, [^{3}H]GD1 and [*NeuAc*-^{3}H]GM3 was greater than 99 % and the specific radioactivities were 1.25, 0.96 and 0.30 Ci/mmol respectively. Gangliosides were stored at -20 °C in propan-1-ol/water (4:1, v/v).

Sialidase assay

The enzymic activity of NEU2 in total cell lysates and in cellular subfractions was determined by various methods. The sialidase activity towards ganglioside substrates, in the presence or absence of Triton X-100 (0.1 %, v/v), was determined as described by Venerando et al. [34]. All reaction mixtures were set up at least in duplicate, with 25 μg of total protein, in a final volume of 100 μl and in the presence of 12.5 mM sodium citrate/phosphate buffer, pH 3.8. In all cases 1 unit of sialidase activity is defined as the liberation of 1 μmol of NeuAc/min at 37 °C.

Fluorimetric method

Reactions were set in the presence of 400 μg of BSA, with 0.2 mM 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid

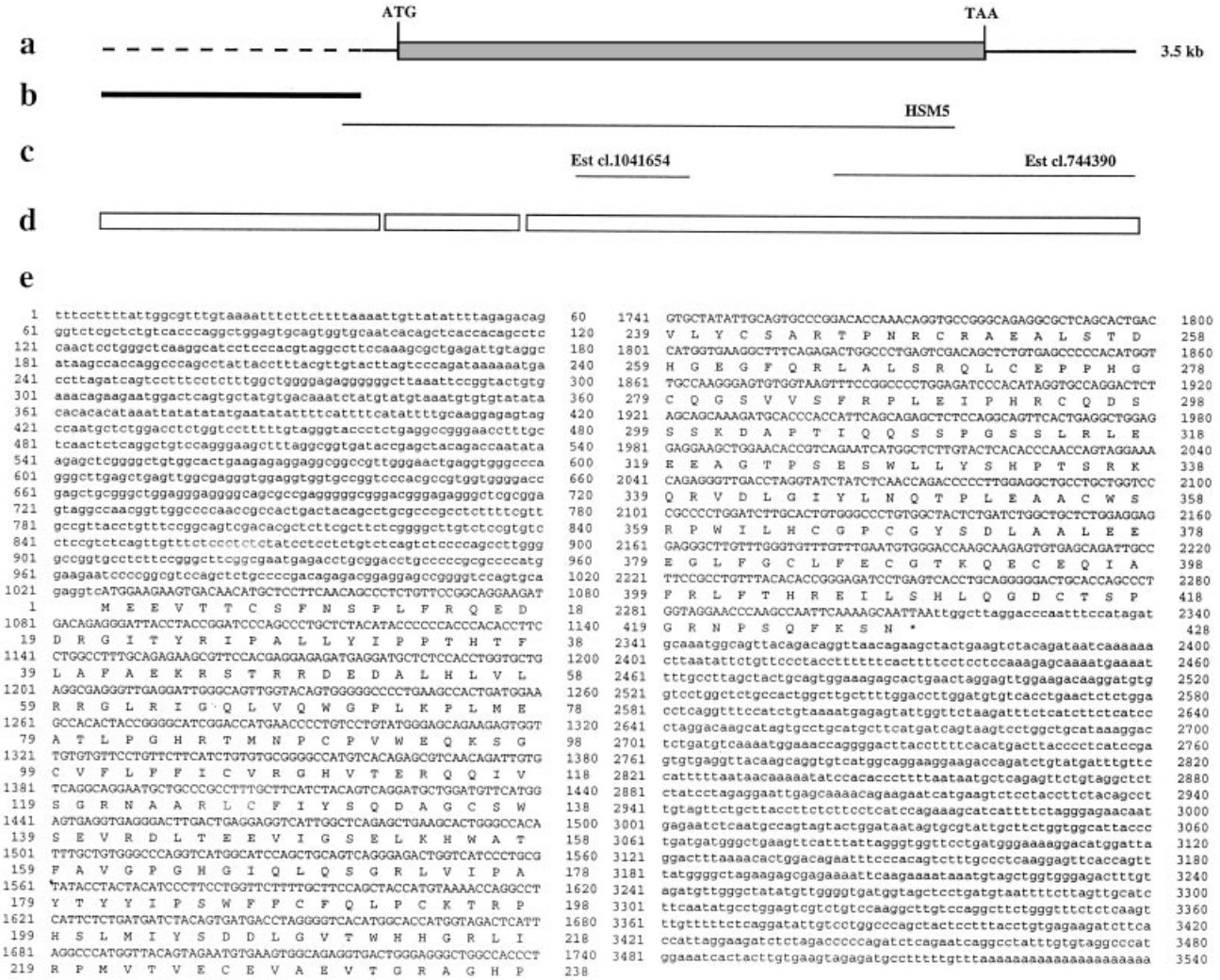


Figure 1 NEU3 cDNA contig, gene structure and translation

(**a**) Schematic representation of the *NEU3* consensus sequence; the grey box indicates the coding region. The broken line in (**a**) and the heavy bar in (**b**) represent the portion of the consensus sequence obtained from genomic DNA. (**c**) The contig of cDNA and EST clones (cl.). (**d**) The genomic structure of *NEU3*. (**e**) The *NEU3* consensus sequence and the corresponding protein translation (one-letter amino acid codes).

(4-MU-NeuAc; Sigma), and incubated at 37 °C for 25 min. Reactions were stopped by the addition of 1.5 ml of 0.2 M glycine/NaOH, pH 10.2. Fluorescence emission was measured on a Jasco FP-770 fluorimeter with excitation at 365 nm and emission at 445 nm, with the use of 4-methylumbelliferone (4-MU) to obtain a calibration curve. Determination of the kinetic parameters was performed with this artificial substrate in the concentration range 0.02–0.6 mM.

Colorimetric method

Reactions were set up with various concentrations of GD1b and GT1b, from 0.03 to 0.6 mM. After incubation at 37 °C for up to 25 min, the release of sialic acid was determined by the thiobarbituric acid method [35].

Radiochemical method

Incubation mixtures containing 0.025–0.6 mM of ^{3}H-labelled GM1, GM3 and GD1a gangliosides, carrying 100000 d.p.m. of ^{3}H label, were incubated at 37 °C for up to 25 min. The incubations were terminated by cooling the tubes in an ice bath and adding 400 μl of tetrahydrofuran. The mixtures were centrifuged at 10000 ***g*** for 5 min; 10 μl of the resulting supernatant (in triplicate) was subjected to high-performance TLC on silica-gel plates with chloroform/methanol/0.2% aqueous $CaCl_2$ (50:42:11, by vol.) as solvent system, to separate the reaction products from the substrates [36]. The separated glycolipids were quantified by radiochromatoscanning (Beta Imager 2000; Biospace Mesures, Paris, France, with the software interface Beta Vision from Dell Optiplex Systems), and the enzyme activity was calculated as described [36].

Immunofluorescence localization

For immunofluorescence studies, COS7 cells were grown in eight-well chamber slide culture chambers (Nunc). Transfections were performed as described previously, with 0.2 μg of DNA in each well. Indirect immunostaining of HA-NEU3 was performed

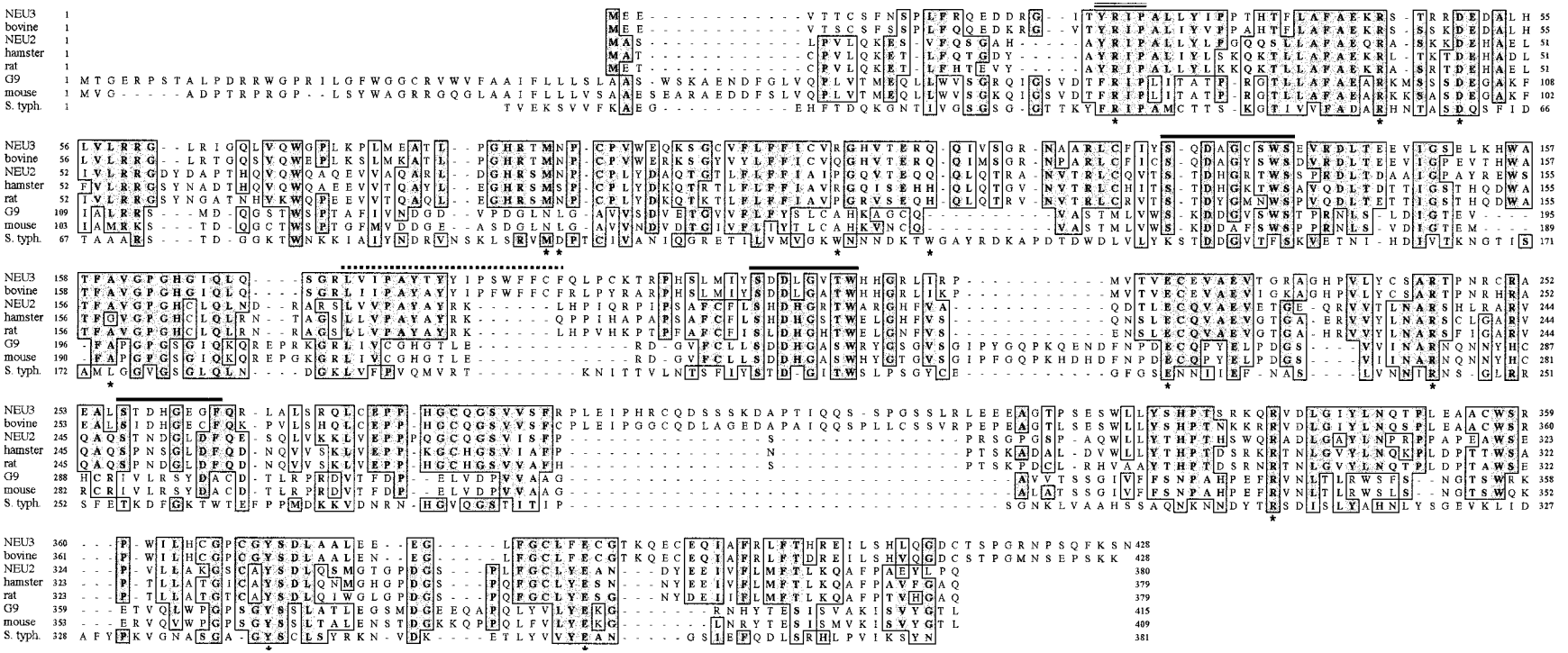


Figure 2 Multiple amino acid sequence alignment of sialidases

An alignment is shown of the amino acid sequence of human NEU3 sialidase with the sequences of the bovine plasma-membrane-associated sialidase (accession no. BAA75071.1); the cytosolic and soluble sialidases from human (NEU2) (accession no. NP-005374), hamster (accession no. A54961) and rat (accession no. A49679); the human lysosomal sialidase (G9) (accession no. NP-000425) and the mouse counterpart (accession no. CAA72215); and the *S. typhimurium* sialidase (S. typ.; accession no. P29768) as a reference. The alignment was performed with the ClustalW program. Identical residues are boxed and shown on a grey background; conservative substitutions are boxed on a white background. The conserved F(Y)RIP box, the three Asp boxes and the putative transmembrane helix are indicated by a double thin, black lines and a dotted line respectively. The active-site amino acid residues derived from crystallographic data of the enzyme from *S. typhimurium* are indicated by asterisks below the sequence.

on cells fixed with 4 % (w/v) paraformaldehyde in PBS. Cells were subsequently permeabilized with 0.2 % (v/v) Triton X-100, blocked with pig serum and incubated with anti-HA monoclonal antibody (4 μg/ml) (Boehringer Mannheim) in PBS plus pig serum and 0.1 % (v/v) Triton X-100. Staining was obtained after incubation with FITC-conjugated isotype-specific antibodies (1:100 dilution) (Dako). Fluorescence microscopy was performed with an Axioplan microscope (Zeiss).

RESULTS

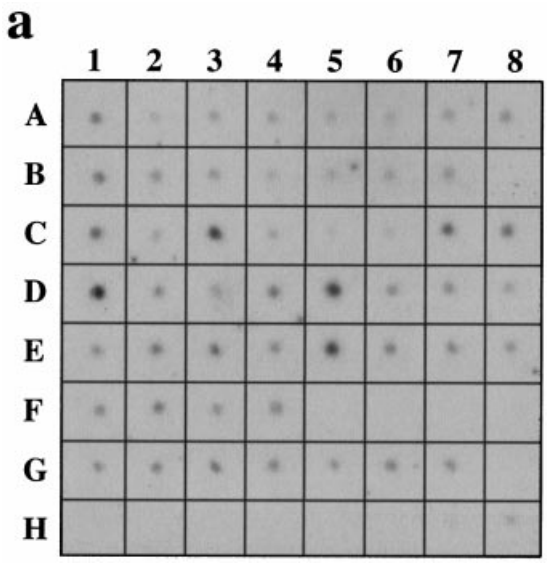
Identification and characterization of the *NEU3* gene

As a starting point for the identification of novel mammalian sialidases we searched the dbEST [37] for entries showing homology with NEU2 polypeptide. This analysis led us to the identification of a human expressed sequence tag (EST) (accession number AA578048) showing significant sequence identity with the medial part of both the query sequence and the rodent soluble sialidase. Moreover, the homology region contained an Asp box [consensus sequence S/TXD(X)GXTW/F], an amino acid motif found in all the microbial [38] and mammalian [6] sialidase enzymes cloned so far.

The sequence of the corresponding cDNA clone (IMAGE clone 1041654) revealed an insert size of 395 bp. A set of oligonucleotides (oligos) was designed on the basis of the cDNA sequence and were used for preliminary expression studies with various human RNA species as templates. Reverse-transcriptase-mediated PCR revealed a product of the expected size in heart, brain, lung and, with higher yield, in skeletal muscle (results not shown). The PCR product was used as a probe to screen a human skeletal muscle cDNA library (2×10^6 plaques); out of seven positive clones, four were characterized further and the clone containing the longest insert (HSM5) (Figure 1) was sequenced directly. A reiterated search of the EST database allowed us to identify an additional EST (IMAGE clone 744390) corresponding to the 3′ untranslated region. The cDNA consensus sequence at the 5′ end was confirmed and extended at the genomic level (as indicated in Figures 1a–1c) to build up a contig of 3540 bp. Four in-frame ATG codons were found (nt positions 424, 928, 958 and 1027 of the contig), preceded by several in-frame stop codons. Comparison of the amino acid sequence of the predicted *NEU3* protein product with its putative bovine homologue [14] revealed that the fourth ATG was the real starting codon, indicating that the human putative NEU3 protein is 428 residues long. Bioinformatic analysis of the genomic sequence upstream of the ATG, performed at http://bimas.dcrt.nih.gov/molbio/signal/, revealed the presence of several putative Sp1-binding sites and the absence of TATA and CAAT box sequences. These findings are in agreement with the expression data obtained by Northern analysis, demonstrating that *NEU3* is a ubiquitously expressed gene. During the submission of this paper a transcript corresponding to *NEU3* was isolated from human brain cDNA library and reported by Wada et al. [39].

NEU3 protein analysis

The predicted NEU3 protein has a calculated molecular mass of 48.25 kDa and a theoretical pI of 6.78. The protein contains three canonical Asp boxes (residues 131–138, 205–212 and 256–263), an F(Y)RIP (one-letter amino acid codes) motif in the N-terminal region and one potential N-glycosylation site (Asn-348). Comparison of the amino acid sequence with protein sequence databases showed the highest degree of identity (78 %) with the bovine membrane-associated sialidase [14], followed by rat [2], NEU2 [12] and hamster [3] soluble enzymes (42 %, 42 % and 40 % sequence identities respectively) and the human [4–6] and mouse [7–9] lysosomal sialidase (31 and 30 % sequence identities respectively). As reported for the bovine protein, the analysis for putative transmembrane regions by using the TMpred and PHDhtm programs [40] at ISREC (Switzerland) and EMBL (Germany) revealed a hydrophobic stretch of 17 residues (174–190) that apparently divides the protein into two segments located on the opposite layers of the membrane. In



b

	1	2	3	4	5	6	7	8
A	whole brain	amygdala	caudate nucleus	cerebellum	cerebral cortex	frontal lobe	hippocampus	medulla oblongata
B	occipital pole	putamen	substantia nigra	temporal lobe	thalamus	subthalamic nucleus	spinal cord	
C	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
D	testis	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary gland
E	kidney	liver	small intestine	spleen	thymus	peripheral leukocyte	lymph node	bone marrow
F	appendix	lung	trachea	placenta				
G	fetal brain	fetal heart	fetal kidney	fetal liver	fetal spleen	peripheral leukocyte	fetal lung	
H	yeast total RNA	yeast tRNA	*E. coli* rRNA	*E. coli* DNA	Poly r(A)	human C0t DNA	human DNA	human DNA

Figure 3 ***NEU3*** **gene expression**

(**a**) Human mRNA master blot (Clontech) hybridized with *NEU3* probe (exposed for 4 days). (**b**) List of the types and positions of poly(A)$^+$ RNA species and control RNA and DNA species dotted on the membrane.

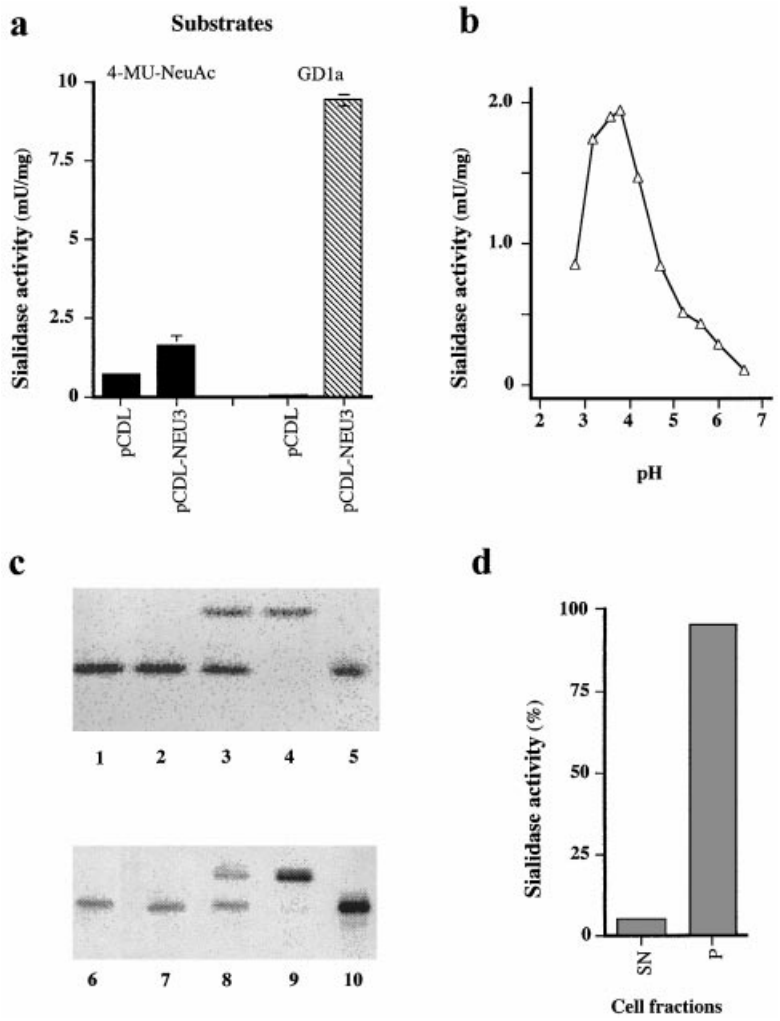


Figure 4 NEU3 expression in COS7 cells

(**a**) COS7 cells transfected overnight with pCDL vector alone or pCDL-NEU3 construct were grown for 24 h; sialidase-specific activities towards the substrate 4-MU-NeuAc and ganglioside GD1A were determined in the total cell lysate. Variation in the observed activity is indicated by the S.D. error bars ($n = 3$). (**b**) Rate of hydrolysis of 4-MU-NeuAc over the pH range 2.8–6.6 in 12.5 mM sodium citrate/phosphate buffer, with the use of aliquots of the crude cell homogenate of pCDL-NEU3-transfected cells as the enzyme source. (**c**) TLC of 10 μl of sialidase reaction mixture containing radiolabelled [^{3}H]GM3 (lanes 1–3) and [^{3}H]GD1a (lanes 6–8). Aliquots (25 μg) of the particulate material of COS7 cells transfected with pCDL (lanes 2 and 7) and pCDL-NEU3 (lanes 3 and 8) were used as enzyme sources. Standard ^{3}H-sphingolipids are loaded on the right of each TLC: GM3 (lane 5); lactosyl-ceramide (lane 4); GD1a (lane 10) and GM1 (lane 9). Detection of the ^{3}H-gangliosides was performed with a radiochromatoscanner. (**d**) Sialidase activity of the supernatant (SN) and pellet (P) obtained by ultracentrifugation at 200 000 ***g*** of the total lysate of pCDL-NEU3-transfected cells. The rates of hydrolysis of 4-MU-NeuAc are expressed as percentages of the value detectable in the total cell lysate ($n = 3$).

addition, primary structure analysis revealed a high cysteine content (21 residues, 4.9% of the total), and the secondary-structure prediction performed by the PHDsec program at EMBL (Germany) showed several β-sheet regions, a characteristic common to all the sialidase enzymes studied so far. A multiple alignment of the amino acid sequences of the cloned mammalian sialidases and the protein from *Salmonella typhimurium* is shown in Figure 2. As expected, the F(Y)RIP motif is highly conserved in all the proteins, as are the three Asp boxes. In addition, the presence of the microbial sialidase, whose three-dimensional structure has been solved [41], permitted the identification of the putative amino acid residues involved in the formation of the active site of mammalian sialidases. In fact, most of the active-site residues identified in *S. typhimurium* are conserved in NEU3 as well as in the bovine counterpart, in NEU2, in the rodent enzymes (9 out of 13) and in the two lysosomal sialidases (8 out of 13). The differences are concentrated in the hydrophobic residues (Trp-121, Trp-128 and Leu-175) that in the *S. typhimurium* enzyme form the hydrophobic pocket accommodating the *N*-acetyl group of the sialic acid [41]. These discrepancies could reflect a different organization of the hydrophobic pocket and/or differences in substrate specificity.

Expression analysis of the *NEU3* gene

To ascertain the expression level of the *NEU3* gene, a dot-blot analysis was performed with a PCR product spanning the entire putative *NEU3* coding region, by using a dot-blot containing poly(A)$^+$ RNA extracted from 50 different human tissues. After exposure overnight, expression was detectable in testis, skeletal muscle, adrenal gland and thymus; several other tissues gave faint signals (Figure 3). A 4-day exposure of the membrane revealed a ubiquitous expression of the gene in all the human tissues tested, with the lowest expression detectable in bladder

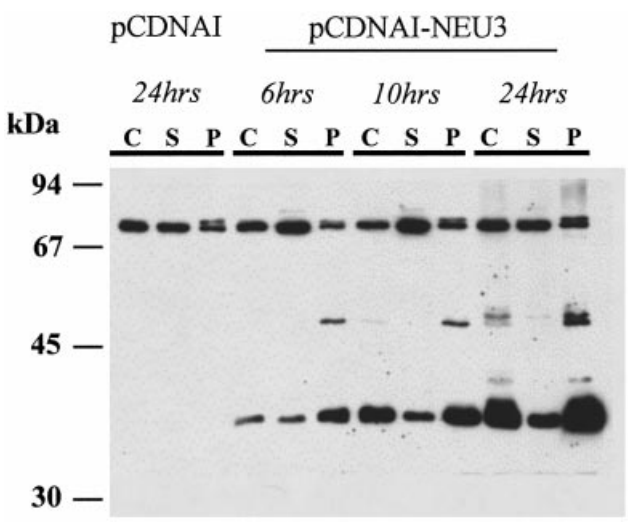


Figure 5 Western blot analysis of COS7 cells transiently expressing NEU3-tagged protein

Western blot analysis of protein samples (10 μg) obtained by cells transfected with pCDNAI-NEU3 construct at different times after transfection. pCDNAI vector alone was used as a control. For each time after transfection, aliquots of crude homogenate (C), and of supernatant (S) and pellet (P) obtained by ultracentrifugation, were analysed. The blot was with anti-HA monoclonal antibody. Detection of antibodies bound to HA epitope was performed with peroxidase-conjugated isotype-specific antibodies, followed by enhanced chemiluminescence developing reagents. The positions of molecular mass markers are indicated at the left.

Table 1 Kinetic constants of NEU3 expressed in COS7 cells

Aliquots corresponding to 25 μg of protein from the crude particulate fraction obtained by ultracentrifugation were used as enzyme sources. Abbreviation: n.d., not determined.

Substrate	Presence of Triton X-100	V_{max} (m-units/mg)	K_m (μM)
GM1	−	n.d.	
	+	n.d.	
GM3	−	12.5	166
	+	92.4	158
GD1a	−	9.4	98
	+	61.6	95
GD1b	−	1.2	85
	+	3.1	81
GT1b	−	6.7	130
	+	17.9	125
4-MU-NeuAc	−	7.6	143

and uterus. In addition, *NEU3* was expressed in human fetal tissues.

Expression of *NEU3* in COS7 cells

To demonstrate that *NEU3* encodes a sialidase, the entire ORF deduced from the cDNA sequence was amplified by PCR and subcloned into a mammalian expression vector (pCDL). The recombinant vector (pCDL-NEU3) was used to transfect COS7 cells transiently. Crude homogenates from cells transfected with pCDL-NEU3 or pCDL alone were tested for sialidase activity by using the artificial substrate 4-MU-NeuAc and the ganglioside GD1a. Transfection with pCDL-NEU3 gave rise to a 2.5-fold increase in the enzymic activity detected in the crude cell extract with the artificial substrate (Figure 4a). The enzyme had an extremely low pH optimum: the pH curve revealed detectable activity from pH 2.8 and a maximum at pH 3.8 (Figure 4b). NEU3 showed a higher activity towards ganglioside GD1a than 4-MU-NeuAc (Figure 4a). In fact, in the presence of GD1a ganglioside micelles, the detected increase in sialidase activity corresponded to approx. 90-fold that observed in the pCDL-transfected cells.

To analyse the subcellular localization of *NEU3*-encoded sialidase, fractionation into soluble and particulate cell materials was performed by centrifugation, using as a starting material the crude homogenate of COS7 cells transfected with pCDL-NEU3. The experiments clearly demonstrated the particulate nature of NEU3 (Figure 4d): more than 95% of the activity detectable in the homogenate was recovered in the pelleted material.

These results were confirmed by a Western blot analysis of COS7 cells transiently transfected with pCDNAI-NEU3. In these experiments, *NEU3* cDNA ORF was subcloned into a pCDNAI mammalian expression vector, giving rise in transfected cells to a fusion protein (HA-NEU3) carrying the HA epitope in the N-terminal region. This tagged chimaera protein was easily revealed with a commercial monoclonal antibody against the HA epitope. A protein band with a molecular mass of approx. 51 kDa, corresponding to the expected molecular mass of the tagged sialidase, was detectable in the 200000 ***g*** pelleted material of the transfected cells; its amount increased with the time after transfection (Figure 5). An additional band at 37 kDa was detected by the anti-HA antibody, with a much higher signal than the original transcribed chimaera protein. The finding that a longer transfection time led to an increase in the 37 kDa signal suggested that this protein band was related to a strong proteolytic degradation of the HA-NEU3. The presence of the degradation product in the soluble material might suggest a labile binding of the shortened polypeptide to the cell membranes. In addition, at 24 h after transfection, a novel band with a molecular mass of approx. 52 kDa was detectable in the crude homogenate and in the particulate fraction, possibly representing a modified form of the 51 kDa NEU3. Additional protein bands of approx. 75 kDa were detected by the monoclonal antibody. The presence of the same bands in the cell fractions obtained from COS7 cells transfected with pCDNAI vector alone (Figure 5, left three lanes) indicated that they were unrelated to NEU3.

Aliquots of the crude particulate fraction obtained from pCDL-NEU3-transfected cells were used to analyse the substrate specificity of the protein enzyme. Under the experimental conditions used to measure the kinetic parameters towards ganglioside substrates, the endogenous sialidase activity detected in the particulate material of control COS7 cells transfected with pCDL vector was undetectable, as shown with the radiolabelled substrates GM3 and GD1a, and a radiochromatoscanner as detection system (Figure 4c). The results are summarized in Table 1. NEU3 hydrolysed all the gangliosides tested except GM1, which seemed to be resistant, with similar K_m values. However, a comparison between the observed values of apparent V_{max} showed that the enzyme acted preferentially on terminal sialic acid residues, linked to the oligosaccharide moiety with an α2-3 sialyl linkage. In addition, the presence of Triton X-100 led to a strong increase in the enzymic activity, as demonstrated by the increase in apparent V_{max} observed with all ganglioside substrates.

Mapping and gene structure

Mapping assignment of *NEU3* was performed with a set of primers (HS1F and HS1R) located in the coding region (see the Experimental section) on the Radiation Hybrid mapping panel

Table 2 Splice-site junctions of *NEU3*

Capital letters denote exonic sequences and lower-case letters intronic sequences.

Exon number	5′ splice site	3′ splice site	Exon size (bp)
1	–	TGCAGAgtgagcgggg	–
2	AtccttgcagAGGTCA	GTACAGgtgactcttc	211
3	CcttgtctagTGGGGG	–	–

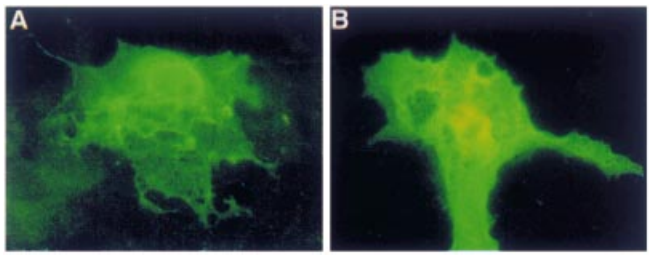


Figure 6 Immunofluorescence localization of NEU3

COS7 cells were transfected with pCDNAI-NEU3 and grown for 24 h (**A**) and 48 h (**B**) before fixation and immunofluorescence staining. Cells were fixed with paraformaldehyde and permeabilized with Triton X-100, then treated with anti-HA monoclonal antibody. Staining was performed with FITC-conjugated isotype-specific antibodies. Magn. × 230.

Genebridge 4. The results obtained indicated that *NEU3* maps to chromosome 11q13 between markers D11S916 and D11S3966.

To determine the genomic structure of *NEU3*, the RPCI-5 PAC library was screened by PCR with the same set of primers as those used for the mapping. Two positive clones (919A14 and 1039I21) were identified and then used for direct sequencing with cDNA-derived oligonucleotide primers. The locations and sequences of all exon–intron boundaries (Table 2) were therefore determined.

NEU3 gene is organized in three exons, with the ATG codon located in the second one. All the exon–intron junctions agree with the GT--AG consensus sequence. *NEU3* and *NEU2* [12] share a similar gene structure with the ORF spanning only two exons, whereas the lysosomal enzyme seems to be more complex, with the gene organized into five exons [6].

Immunofluorescence localization of NEU3

Immunofluorescence localization was studied in COS7 cells transiently transfected with pCDNAI-NEU3. Expression tested up to 72 h after transfection gave an extensive cell-surface labelling, a staining pattern typically associated with plasma membrane proteins (Figure 6). The membrane structures detected inside the cells can be related to *NEU3*-encoded protein travelling from the rough endoplasmic reticulum to the cell surface, as a consequence of the high expression level driven by the pCDNAI-NEU3 vector.

DISCUSSION

So far, three different types of mammalian sialidase have been characterized at the molecular level. They are the cytosolic, or soluble, forms [2,3,12,13], the lysosomal form [4–9] and a plasma-membrane-associated protein from *Bos taurus* [14]. Here we describe the molecular cloning and expression of a novel human sialidase, encoded by the *NEU3* gene, that is highly similar to the bovine protein.

Expression of the polypeptide in COS7 cells demonstrates the particulate nature of the enzyme. In fact, the enzymic activity is found associated with the membrane structures pelleted by ultracentrifugation. These results were confirmed by Western blot analysis, with a protein chimaera tagged at the N-terminus with the HA epitope. As already observed for HA-NEU2 [13], the chimaera is catalytically inactive, suggesting a critical role of the N-terminus in correct protein folding and/or in active-site formation. The 51 kDa HA-NEU3 chimaera is detectable only in the particulate fractions. HA-NEU3 undergoes proteolytic degradation with high efficiency, as demonstrated by the detection of a large amount of a 37 kDa protein band. This phenomenon could be explained by the high content of foreign proteins induced by the expression vector promoter that could also result in a cytotoxic effect, and/or by the presence of the HA epitope on the protein chimaera. Immunofluorescence localization of the tagged polypeptide further demonstrates the distribution of NEU3 at the cell surface.

The primary structure of these mammalian proteins reveals the presence of amino acid motifs, such as the F(Y)RIP motif and Asp boxes, which are conserved in all sialidases cloned so far. Interestingly, the plasma-membrane-associated sialidase seems to be more similar to the cytosolic sialidases than to the lysosomal forms. This can be related to the substrate specificities of these three forms of sialidase. In fact, whereas the soluble forms showed high activity towards various gangliosides [42,43], G9 is apparently unable to hydrolyse mixed gangliosides [6]. Recently, a lysosomal sialidase-mediated depletion of GM2 ganglioside in Tay–Sachs neuroglial cells was reported, demonstrating the activity of this lysosomal protein *in vivo* towards ganglioside GM2 [44]. This last finding suggests that the different degrees of sequence similarity between these forms of sialidases might be related to one or more different factors, not only to substrate specificities. Overall, a multiple alignment of the amino acid sequences of these mammalian polypeptides demonstrates that they belong to a unique protein family with highly conserved amino acid blocks throughout the primary structure. In addition, the presence in topologically equivalent positions of the F(Y)RIP motif, the Asp boxes and nine amino acid residues that are possibly involved in the active site suggests the existence of a common folding topology similar to the typical superbarrel or β-propeller described for the viral and microbial sialidases [1]. This picture strongly supports the hypothesis of a monophyletic origin of the sialidase enzymes.

By using protease digestion of a protein chimaera tagged at the C-terminus with different antibodies, it was demonstrated that the bovine plasma membrane sialidase is a classical type I membrane protein, with the N-terminus exposed to the extracellular side of the lipid bilayer [14]. In this spatial arrangement the conserved Arg-25 residue, which is located within the YRIP motif, Arg-45, Asp-50, Met-87 and the other amino acids involved in the formation of the active site hydrophobic pocket are exposed at the cell surface and thus isolated from Glu-225, Arg-245, Arg-340, Tyr-370 and Glu-387, which are located in the portion of the protein exposed to the cytosol. From the structural and functional data collected so far, it is unlikely that a sialidase with these characteristics would show catalytic activity. In addition, the detection of the 37 kDa HA-NEU3 proteolytic degradation product in the soluble fractions obtained by ultracentrifugation suggests a labile interaction of this polypeptide with the membranes. Therefore alternative model(s) must be found. One possibility is that NEU3, and its bovine counterpart,

are associated with the plasma membrane via a single hydrophobic segment of 16–20 residues located within the 50 residues at the C-terminus and can either span the phospholipid bilayer or dip into the membrane in a loop structure. The Kyte–Doolittle hydrophobicity plot of the protein showed several hydrophobic regions; although those that are more consistent are located at the N-terminus and in the middle of the primary structure, a short segment can be found at the C-terminus (residues 369–387). Further experiments will be performed to elucidate this aspect of NEU3 biology.

It is noteworthy that, despite the high similarity between NEU3 and the corresponding bovine protein [14], the results obtained by transfecting mammalian cells with the corresponding cDNA suggest different biochemical behaviours of the two enzymes. In fact, whereas the activity towards the artificial substrate 4-MU-NeuAc was practically identical in COS7 cells expressing the bovine membrane sialidase, under the same experimental conditions we observed a 2–3-fold increase on NEU3 expression with a more acidic pH optimum. However, the main differences are detectable on ganglioside substrates. The observed values of apparent V_{max} indicate that the human enzyme hydrolysed ganglioside GM3 with higher efficiency, as reported for the bovine enzyme, but seemed to be much less active toward the gangliosides GT1b. Moreover, whereas the bovine enzyme showed a hydrolysis rate for the GD1b intermediate between those observed for GD1a and GT1b, NEU3 hardly acted on the α2-8 sialyl linkage of this ganglioside substrate. NEU3 activity towards the ganglioside substrate was increased by Triton X-100, demonstrating that further disaggregation of the membrane is important for substrate recognition. The detergent activation of membrane-bound sialidase has been already reported in human fibroblasts [45], neuroblastoma cells [21] and human brain tissue [46]. A rapid comparison of the substrate preferences of these different membrane-bound enzymes suggests a great molecular heterogeneity of these proteins. NEU3 is, for example, the enzyme with the most acidic pH optimum. The great variety of results available on membrane sialidase enzymes, together with the several biological roles of the gangliosides, makes the detailed characterization of these proteins an attractive field of study.

Overall, the identification of the cDNA encoding NEU3 might contribute to a better understanding of the biological functions of the different members of the sialidase enzyme family.

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