Expression of a novel human sialidase encoded by the NEU2 gene

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Sialidases (E.C.3.2.1.18) belong to a group of glycohydrolitic enzymes, widely distributed in nature, which remove sialic acid residues from glycoproteins and glycolipids. All of the sialidase so far characterized at the molecular level share an Asp block, repeated three to five times in the primary structure, and an F/YRIP sequence motif which is part of the active site. Using a sequence homology-based approach, we previously identified a human gene, named NEU2, mapping to chromosome 2q37. NEU2 encoded protein is a polypeptide of 380 amino acids with two Asp block consensuses and the YRIP sequence in the amino terminal part of the primary structure. Here we demonstrate that NEU2 encodes a functional sialidase. NEU2 was expressed in COS7 cells, giving rise to a dramatic increase in the sialidase activity measured in cell extracts with the artificial substrate 4-MU-NANA. Using a rabbit polyclonal antiserum, on Western blots a protein band with a molecular weight of about 42 kDa was detectable, and its cytosolic localization was demonstrated with cell fractionation experiments. These results were confirmed using immunohistochemical techniques. NEU2 expression in E.coli cells allowed purification of the recombinant protein. As already observed in the enzyme expressed in COS7 cells, NEU2 pH optimum corresponds to 5.6 and the polypeptide showed a K_m for 4-MU-NANA of 0.07 mM. In addition, based on the detectable similarities between the NEU2 amino acid sequence and bacterial sialidases, a prediction of the three-dimensional structure of the enzyme was carried out using a protein homology modeling approach.

Key words: human cytosolic sialidase/COS7 cells/E.coli / structure prediction

Introduction

Neuraminidases or sialidases (E.C.3.2.1.18) are a family of glycohydrolitic enzymes that remove N-acetyl neuraminic acid residues from various substrates such as glycolipids and glycoproteins. Viral and bacterial neuraminidases have been well characterized, and the three-dimensional structure of several of these enzymes has been determined (Crennell et al., 1993, 1994; Janakiraman et al., 1994). All of the microbial enzymes characterized so far share the F(Y)RIP domain in the amino terminal portion of the protein, followed by a series of so-called “Asp boxes” (S-X-D-X-G-X-X-T/W), which are repeated three to five times depending on the protein (Roggentin et al., 1989), but have an overall amino acid sequence identity of about 35%. Despite this low level of amino acid similarity, the overall fold of the molecules and organization of the amino acids involved in the catalysis are remarkably similar.

In mammals, several sialidase enzymes which differ in subcellular localization, substrate preferences, and pH optimum have been described. Mammalian sialidases have been involved in several cellular processes such as the catabolism of glycoconjugates, regulation of cell proliferation, clearance of plasma protein, and the developmental modeling of myelin. Detailed molecular characterization of these mammalian proteins has been hampered by both their low cellular content and their instability during the purification procedures. Only three mammalian sialidases have been cloned so far: the cytosolic sialidase from rat skeletal muscle (Miyagi et al., 1993); the soluble sialidase secreted in the culture medium by Chinese hamster ovary (CHO) cells (Ferrari et al., 1994); and recently, the human lysosomal sialidase (Bonten et al., 1996; Milner et al., 1997; Pshezhetsky et al., 1997), responsible for the lysosomal storage disorder sialidosis (Thomas and Beaudet, 1995). All these mammalian proteins share an F(Y)RIP motif in the first part of the primary structure, followed by Asp boxes, as previously described for the microbial enzymes. In addition, a multiple alignment of the amino acid sequences of these mammalian enzymes with some members of the bacterial sialidase family revealed that most of the amino acid residues which form the catalytic site of the bacterial proteins are highly conserved in all of the mammalian counterparts. Therefore, it is likely that mammalian sialidases, and bacterial and viral neuraminidases share a similar fold topology.

In a previous paper we described the identification of NEU2 (GenBank accession number Y16535), a novel human gene homologous to rodent soluble sialidases (Monti et al., 1999). Here we demonstrate that NEU2 encodes a functional sialidase with cytosolic localization. In addition, a prediction of the three-dimensional structure of the enzyme was carried out using a protein homology modeling approach.
Results

The derived amino acid sequence of NEU2 and alignment similarities to bacterial and mammalian sialidases

The NEU2 gene encodes a protein of 380 amino acids (Figure 1A), just one amino acid (Pro 267) more than the two rodent enzymes. The calculated molecular weight of the NEU2 encoded protein is 42.23 kDa and the isoelectric point is 6.82. The polypeptide has one potential N-linked glycosylation site (Asn-Val-Thr) at Asn 120, exactly in the same position reported for the CHO sialidase, instead of the three present in both rat cytosolic and human lysosomal sialidase. The Kyte-Doolittle hydrophobicity plot (Figure 1B) suggests that NEU2 is a soluble protein: no hydrophobic loops consistent with transmembrane domain are detectable in its primary structure.

Database searches performed using the 2.0 version of BLAST (Altschul et al., 1997) revealed that NEU2 shares significant sequence identities with mammalian, viral, and bacterial sialidases (see Table I). As expected, the highest similarity is detectable with the hamster (73.7%) and the rat (72.4%) cytosolic sialidases. A lower but still significant level of sequence similarity was found with the G9 human lysosomal sialidase (42.5%) and the two bacterial enzymes, Salmonella typhimurium (Hoyer et al., 1992) and Micromonospora viridifaciens (Sakurada et al., 1992), along the entire length of the protein.

A multiple alignment of the amino acid sequences of these mammalian and bacterial sialidase enzymes is shown in Figure 2. The sequence motif F/YRIP, which is highly conserved in all of the sialidase enzymes described so far and occurs near the N-terminus of these polypeptides, is also found in NEU2 (amino acids 20–23). Moreover, NEU2 contains two Asp blocks, both in agreement with the consensus sequence (S^T^D^H^G^R^W, amino acids 129–136; S^H^D^H^G^R^W, amino acids 199–206). These characteristic motifs correspond to the second and third Asp blocks observed in G9 human sialidase and the bacterial enzyme from S.typhimurium LT2, being the first (amino acid 58–64) and the fourth (amino acid 247–254) Asp blocks poorly conserved in NEU2, as well as in the two rodent enzymes. NEU2 differs from G9 protein in the N-terminal region. In fact, neither a cleavage site nor a transmembrane segment is detectable in the NEU2 amino acid sequence. In addition, as already reported for G9 protein, 9 out of 12 of the amino acid residues which form the catalytic site of S.typhimurium enzyme are conserved in NEU2 sialidase. The differences concern Trp-121, Trp-128, and Leu-175 residues which in the S.typhimurium sialidase, together with the conserved Met-99, form a hydrophobic pocket which accommodates the N-acetyl group of sialic acid (Crennell et al., 1993). These amino acids are replaced in NEU2 and the two rodent enzymes by Phe-102 and Phe-157. These amino acid residues are also different in the other sialidases, suggesting their involvement in variations of substrate specificity in these enzymes.

Table I. Percentage of amino acid similarity (upper line) and identity (lower line) among sialidase proteins

<table>
<thead>
<tr>
<th></th>
<th>NEU2</th>
<th>Ham^a</th>
<th>Rat</th>
<th>G9</th>
<th>Mvir</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ham^a</td>
<td>73.7</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Rat^b</td>
<td>72.4</td>
<td>84.7</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>G9^c</td>
<td>42.5</td>
<td>40.7</td>
<td>43.2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Mvir^d</td>
<td>38.5</td>
<td>38.7</td>
<td>37.3</td>
<td>45.0</td>
<td>—</td>
</tr>
<tr>
<td>Styp^e</td>
<td>38.8</td>
<td>34.4</td>
<td>29.7</td>
<td>41.8</td>
<td>38.9</td>
</tr>
</tbody>
</table>

^aHamster cytosolic sialidase (U06143).
^bRat cytosolic sialidase (D16300).
^cHuman lysosomal sialidase (Z12650, X59730).
^dM.viridifaciens sialidase, aa 61–382 (A45244).
^eS.typhimurium sialidase (P29768).

A three-dimensional structure of the 42 kDa sialidase from S.typhimurium LT2 has been reported. It comprises six β-sheets, each composed of four antiparallel β-strands, arranged around an axis passing through the active site (Crennell et al., 1993). This folding closely resembles the crystal structure of the influenza virus neuraminidase (Burmeister et al., 1992). A similar structure has been described previously (Crennell et al.,...
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1994) for the catalytic subunit of the larger enzyme purified from *V. cholerae* (Galen et al., 1992).

The significant levels of sequence identity with both bacterial and viral sialidases allow us to further speculate on the structural organization of the NEU2 protein. Figure 3A shows the structural alignment generated by SwissPdbViewer between NEU2 and the *S. typhimurium* sialidase. The location of secondary structure elements extracted using the STRIDE software is also mapped on the alignment: the identity of this structure-based alignment corresponds to 71.1%.

The refined three-dimensional structure of the NEU2 model, obtained according to a protein homology modeling approach (Rost and Sander, 1996), is reported in Figure 3B. The predicted structure of NEU2 appears to fold into 24 β-strands, 2 α-helices, and 26 connecting segments, and closely resembles the general molecular folding found in microbial and viral enzymes. Moreover, in the NEU2 model the residues Arg21, Arg41, Asp46, Met85, Glu218, Arg237, Arg304, Tyr334, and Glu355 that are predicted to form the active site are organized in a shallow crevice in the same fashion as the *S. typhimurium* sialidase. Ramachandran plot analyses (data not shown) show that in the NEU2 model 51 out of 380 residues are in disallowed regions (13.4%), whereas in the *S. typhimurium* sialidase 25 out of 381 are in disallowed regions (6.6%).

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**Fig. 2.** Multiple sequence alignment of sialidase sequences. Alignment is shown of the amino acid sequence of human NEU2 sialidase with the sequences of the cytosolic sialidase from rat and hamster (ham), the amino acids 50–415 of the human G9 lysosomal sialidase (G9), the amino acids 61–382 of the *M. viridifaciens* sialidase (Mvir), and the *S. typhimurium* sialidase (Styp). The alignment was performed using the Pileup program of the GCG package and minor adjustments were made by hand. Residues that are identical in all six proteins are shown in white letters on black background; on dark gray background are boxed identical residues present in at least 3 out of 6 proteins, and on light gray background are boxed conservative residue substitutions. The active site amino acid residues derived from crystallographic data of *S. typhimurium* enzyme are indicated by asterisks (*) below the sequence. The canonical, and the two poorly conserved Asp boxes are underlined in black and gray, respectively. The conserved F(Y)RIP box is indicated by a square bracket.
Expression of NEU2 encoded protein in COS7 cells

To demonstrate that NEU2 encodes a sialidase enzyme, the genomic sequence from the putative ATG initiation codon to the TGA stop codon (including the 1.25 kb intron) was amplified by PCR and subcloned into a pCDL expression vector. The recombinant vector was subsequently used to transfect COS7 cells. Total lysates from cells transfected with pCDL-NEU2 or pCDL alone were tested for sialidase activity with 4MU-NANA as substrate. As shown in Figure 4A, the transfection with pCDL-NEU2 leads to a dramatic increase in the sialidase activity measured at pH 5.6. In fact, the specific activity of the enzyme measured at pH 5.6 is barely detectable in COS7 cells transfected with the pCDL vector alone (average value of 0.17 nmol/min/mg protein), whereas we measured an average activity of 51 nmol/min/mg protein in the case of pCDL-NEU2 transfected cells. To investigate the subcellular localization of NEU2 encoded sialidase, a simple fractionation of the total cell lysate into soluble and particulate cell compartments was carried out by ultracentrifugation. The results obtained clearly demonstrate the soluble nature of NEU2 (Figure 4B) because more than 80% of the activity measured in the total cell lysate was detected in the supernatant obtained by ultracentrifugation at 200,000 × g. In addition, the low level of endogenous sialidase activity detectable in COS7 cells is due to a particulate enzyme, because the enzymatic activity was measurable only in the pelleted material (not shown). The homogenization conditions do not influence these results.

The rate of hydrolysis of 4-MU-NANA to 4-MU by the soluble fraction of pCDL-NEU2 transfected cells was measured over the pH range 3.6–6.6 in citrate/phosphate buffer (Figure 4C). The enzyme exhibited considerable activity over a broad pH range, from 4.2 to 6.6, with a plateau at pH 5.6–6.0. The same pH curve was obtained with the recombinant enzyme purified by E.coli cell extracts (not shown).

These data were confirmed by Western blot analysis of COS 7 cells transfected with pCDL-NEU2 or HA-NEU2 (Figure 4D). The anti-NEU2 antiserum recognized a band of about 42 kDa, as expected from the calculated molecular weight of NEU2. Moreover, the band was lightly enriched in the supernatant and barely detectable in particulate fraction (lanes 2–4, anti-NEU2). A superimposable pattern was observed with anti-HA monoclonal antibody, with a recognized band of about 46 kDa, corresponding to the predicted molecular weight of the HA-NEU2 chimera (lanes 2–4, anti-HA). An additional light band of about 32 kDa was detectable in the fractions obtained by ultracentrifugation using the latter antibody reagent, a signal probably related to proteolytic degradation of HA-NEU2. This 32 kDa band appears enriched in the particulate fraction (lane 4, anti-HA), suggesting a trapping effect and/or low solubility of the polypeptide. Moreover, with longer posttransfection times a larger amount of the 32 kDa band was detectable, together with the appearance of a novel 30 kDa band, both showing roughly the same signal intensity of the 46 kDa main product (not shown). By Western blot analysis, both the antibodies detected additional protein bands of about 72–75 kDa. The presence of these bands in cells transfected with the expression vector alone (lanes 1) indicates that they are unrelated to NEU2.

No evidence of glycosylation of the polypeptide was detectable, as demonstrated by transfecting cells in the presence of tunicamycin or by N-glycosidase F treatment of the cell extracts (not shown). Moreover, no protein was detected in the cultured media, even after 6× concentration of the media by ultrafiltration, indicating that NEU2 is not secreted from COS7 cells (not shown).

Immunofluorescence localization of NEU2 in COS7 cells

Immunofluorescence staining was carried out in COS7 cells transfected with HA-NEU2 and pCDL-NEU2. Transient expression of NEU2 up to 72 h posttransfection yielded an extensive fluorescence labeling, a staining pattern associated
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with soluble protein (Figure 5). These results were obtained by detecting the chimera HA-NEU2 and the wild type protein (Figure 5, panels A,B and C,D, respectively), and by using a different procedure of cell fixation and permeabilization (Figure 5C,D). In some cells overexpressing HA-NEU2, an extensive nuclear staining was detectable (Figure 5B). This could be due to the high expression level of the polypeptide. No crystal-like structures described in the case of lysosomal sialidase overexpression (Bonten et al., 1996; Milner et al., 1997) were recognized by our antibodies, even in cells with very high production of NEU2 (Figure 5C).

Expression of NEU2 encoded protein in E.coli

To obtain a large amount of NEU2 we subcloned the coding region of the gene in the pGEX-2T bacterial expression vector as described in Materials and methods. The polypeptide was expressed in transformed E.coli cells as glutathione S-transferase fusion protein. As expected, a new protein band with an apparent molecular mass of about 65 kDa appeared on SDS–PAGE of the transformed cell extracts, indicating expression of the fusion protein (Figure 6, lane 3). The sialidase activity toward 4MU-NANA substrate was detectable only in lysate of bacterial cells harboring pGEX-2T-NEU2, while cells transformed with pGEX-2T alone did not show any sialidase activity (see Table II). The observed specific activity was roughly four times higher than the reported value for the recombinant rat cytosolic enzyme expressed in E.coli with the same vector (Miyagi et al., 1993), and about half of the value detectable in crude homogenate of insect cells expressing the soluble enzyme from Chinese hamster ovary cells (Ferrari et al., 1994). Batch purification of glutathione S-transferase-NEU2 fusion protein, followed by thrombin cleavage, leads to selective enrichment of a protein band with a molecular mass of about 43 kDa, and a roughly comparable amount of uncleaved fusion protein (Figure 6, lanes 4 and 5). The purified preparation showed sialidase activity at pH 5.6 of 3.12 nmol/min/mg protein and a K_m value toward 4MU-NANA of 0.07 mM. Puriﬁcation with glutathione-Sepharose-4B led to a 376-fold increase in the enzymatic speciﬁc activity.
these data, it is not surprising that no ESTs encoding NEU2 gene is only poorly transcribed in skeletal muscle. From able to isolate cDNA clones from a large set of cDNA libraries. hamster sialidase cDNA. Using the same probe, we were not ing a human genomic library using as probe a portion of the 1318 (Monti et al., 1999). Briefly, the gene was isolated by screen- ing a human genomic library using as probe a portion of the hamster sialidase cDNA. Using the same probe, we were not able to isolate cDNA clones from a large set of cDNA libraries. In fact, expression studies in adult tissues demonstrate that the NEU2 gene is only poorly transcribed in skeletal muscle. From these data, it is not surprising that no ESTs encoding NEU2 could be found among more than 1,200,000 human and 400,000 mouse partial cDNA sequences.

Primary structure analysis revealed that NEU2 has significant homology with bacterial and mammalian components of the sialidase family. Moreover, the secondary structure prediction of NEU2 indicates a high β-sheet content as already reported for the microbial enzymes purified from S.typhimurium and V.cholerae. Thus, we decided to predict the three dimensional structure of the polypeptide by homology modeling, using as template the solved structure of S.typhimurium sialidase. It should be noted that protein models based on homology modeling are fully reliable when the known protein structure shares at least 40% sequence identity with the unknown protein structure (Blundell, 1991). Although in our case the sequence identity corresponds to 28.4%, the high structural similarities between viral and bacterial sialidases crystallized so far give a strong rationale support to this prediction. The model obtained shows the same fold topology of the S.typhimurium enzyme and allows the conservation in topologically equivalent positions of both the two conserved Asp box and the nine amino acid residues involved in the active site. The role of the Asp box structural motifs is still unknown, although their presence on the surface of the sialidase enzymes may suggest a potential role as functional and/or recognition site. In addition, a recent study demonstrates that the recom- binant hamster enzyme and the S.typhimurium sialidase share the same stereoselectivity of catalysis (Kao et al., 1997). Since a strong correlation of this catalytic behavior with active site architecture has been demonstrated (Gebler et al., 1992), these data strongly suggest that microbial and mammalian sialidases have similar active site topology even though the proteins do not share high amino acid sequence similarities. These features further support the accuracy of the NEU2 three-dimensional structure model.

Expression of the NEU2 in COS7 cells and E.coli demon- strates that this new human gene encodes a sialidase. Moreo- ver, the molecular weight of the protein detected by specific antiserum in COS7 cells, and the sialidase activity measured in E.coli cell extracts showed that the transfected NEU2 gene was spliced as predicted from the genomic sequence (Monti et al., 1999).

A comparison of the specific activity of the purified recombinant NEU2 with the rodent counterparts is possible only by considering the values reported for the originally purified enzymes, since no data on the corresponding recombinant pro- teins are available. NEU2 measured specific activity toward 4MU-NANA (3.12 μmol/min mg) is very similar to the reported values in the case of the hamster (10.1 μmol/min mg) and rat liver (5.1 μmol/min mg) enzymes. The observed $K_m$ value of 0.07 mM is about 5.7 and 9.6 times lower than the reported value for hamster and rat protein, respectively. In addition, the pH optimum at pH 5.6, very similar to the values of the ham- ster (5.9) and rat (5.5) soluble sialidase, together with the high amino acid sequence similarities of these proteins, strongly suggest a similar kinetic behavior for the three polypeptides. No glycosylation is detectable in either hamster or human sialidase, but NEU2 is not released in the culture media, at least in COS7 cells, whereas the hamster enzyme was originally puri- fied from culture media of Chinese hamster ovary cells (Warner et al., 1993).

**Discussion**

We report the characterization of a second human sialidase encoded by the NEU2 gene, mapping to chromosome 2q37 (Monti et al., 1999). Briefly, the gene was isolated by screen- ing a human genomic library using as probe a portion of the hamster sialidase cDNA. Using the same probe, we were not able to isolate cDNA clones from a large set of cDNA libraries. In fact, expression studies in adult tissues demonstrate that the NEU2 gene is only poorly transcribed in skeletal muscle. From these data, it is not surprising that no ESTs encoding NEU2

![Fig. 6. NEU2 expression in E.coli cells. Transfected cells, after 36 h of growth, were collected and homogenized, and the proteins were subjected to SDS-PAGE under reducing conditions as described in Materials and methods. The 10% (w/v) polyacrylamide gel was stained for protein detection with Coomassie brilliant blue R-250. Lane 1, Cells transformed with pGEX-2T. Lane 2, Cells transformed with pGEX-NEU2 without IPTG induction. Lane 3, Cells transformed with pGEX-2T with IPTG induction (0.1 mM). Lanes 4 and 5, purified NEU2 sialidase (1.0 and 0.5 μg, respectively).](https://academic.oup.com/glycob/article-abstract/9/12/1313/612319)
NEU2 appears to be a soluble protein, as demonstrated by both subcellular fractionation and Western blot analysis. A simple fractionation into soluble and membrane associated proteins was carried out and the fractions assayed for sialidase activity. The enzymatic activity was detectable mainly in the soluble material, and these results were confirmed by the amount of antigen detected by specific antibodies in the two subcellular fractions. Moreover, immunofluorescence localization of both the wild type protein or the HA-NEU2 chimera, using different cell-fixation and permeabilization procedures to avoid artifacts, indicates a cytosolic localization of the enzyme.

The localization in adult tissue of the rat skeletal muscle cytosolic sialidase was carried out by immunohistochemical techniques (Akita et al., 1997). The enzyme seems to be widely distributed inside the muscle fiber, but is also detectable in axons, Schwann cells, and cells of endomysium and blood vessels. These observations clearly indicate that this cytosolic sialidase is also present in cells other than skeletal muscle fibers. The cytosolic localization of a sialidase enzyme is rather puzzling, and still unknown is its physiological role.

An intriguing paper by Tokuyama et al. describes the suppression of pulmonary metastasis in murine melanoma cells by transfection with the rat cytosolic sialidase cDNA (Tokuyama et al., 1997). Surprisingly, this change in melanoma cell behavior appears to be related to a variation of intracellular glycolipid levels. In fact, a decrease in ganglioside GM3 and an increase in lactosylceramide were the major changes in glycolipid levels. In fact, a decrease in ganglioside GM3 and an increase in lactosylceramide were the major changes in glycolipid levels.

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Amino acid sequences were compared to the non redundant sequence databases present at the NCBI (National Center for Biotechnology Information) using the BLAST network service (Altschul et al., 1997). Pairwise and multiple amino acid sequence alignments were performed using the Bestfit and PileUp programs, respectively (Genetics Computer Group, Wisconsin Package, Version 9, Madison, WI). Secondary structure prediction was carried out with the PeptideStructure program of GCG package.

Tertiary structure prediction

A first model of the three-dimensional structure of NEU2 was predicted through the program Modeller 4 (Sali and Blundell, 1993), using as a template the sialidase from Salmonella typhimurium LT2 (Protein Data Bank accession number 2SIM). Modeller 4 copied the atomic coordinates from 2SIM to NEU2 and minimized the obtained structure. The model-default.top script was employed. A refined model was obtained through the program SwissPdbViewer 3.0 (Peitsch, 1996), generating a sequence alignment from a structural superposition between NEU2 and 2SIM atomic coordinates. This sequence alignment was used as an input file for Modeller 4 to produce a refined model. Location of secondary structure elements for both NEU2 and 2SIM structures was performed by the STRIDE program available at http://www.embl-heidelberg.de/args/stride/down_stride.html. It extracts from the atomic coordinates of a protein the position of secondary structure elements and maps them on the amino acid sequence. The structural quality of the NEU2 model was evaluated comparing the Ramachandran plots, obtained through SwissPdbViewer 3.0, for both NEU2 and 2SIM structures. Amino acids taking part in the active site were predicted from the multiple sequence alignment of sialidases and mapped on the NEU2 structure.

Three-dimensional structures of model and template were visualized using the Rasmol 2.6 program available at http://www.umass.edu/microbio/rasmol/. A first model of the three-dimensional structure of NEU2 was predicted through the program Modeller 4 (Sali and Blundell, 1993), using as a template the sialidase from Salmonella typhimurium LT2 (Protein Data Bank accession number 2SIM). Modeller 4 copied the atomic coordinates from 2SIM to NEU2 and minimized the obtained structure. The model-default.top script was employed. A refined model was obtained through the program SwissPdbViewer 3.0 (Peitsch, 1996), generating a sequence alignment from a structural superposition between NEU2 and 2SIM atomic coordinates. This sequence alignment was used as an input file for Modeller 4 to produce a refined model. Location of secondary structure elements for both NEU2 and 2SIM structures was performed by the STRIDE program available at http://www.embl-heidelberg.de/args/stride/down_stride.html. It extracts from the atomic coordinates of a protein the position of secondary structure elements and maps them on the amino acid sequence. The structural quality of the NEU2 model was evaluated comparing the Ramachandran plots, obtained through SwissPdbViewer 3.0, for both NEU2 and 2SIM structures. Amino acids taking part in the active site were predicted from the multiple sequence alignment of sialidases and mapped on the NEU2 structure.

Expression of NEU2 encoded protein in COS7 cells

The genomic region of about 2.4 kb containing the entire NEU2 ORF, including the two predicted exons and a single intron of about 1.25 kb (Monti et al., 1999). The amplified insert was also cloned into an RSV promoter construct (Monti et al., 1999). The PCR product was cloned in different expression vectors. HA-NEU2 was constructed by cloning the amplified insert in-frame with the hemagglutinin (HA) epitope into plasmid pCDNA1 (Invitrogen). The amplified insert was also cloned into an RSV promoter vector (Takebe et al., 1988), yielding the pCDL-NEU2 construct. COS7 cells were grown in petri dishes (100 mm diameter) using Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum. Transfections were performed overnight using 8 µg of plasmid DNA and LipofectAMINE reagent, according to the manufacturer’s

Materials and methods

In general, standard molecular biology techniques were carried out as described by Sambrook et al. (Sambrook et al., 1989). DNA restriction and modifying enzymes were from Boehringer unless otherwise indicated.
Preparation of anti-NEU2 antiserum and Western blot analysis

To generate anti-NEU2 serum, two rabbits were immunized with an E.coli expressed and purified NEU2 protein, following the standard immunization protocol. Immunoreactivity of the obtained anti-NEU2 antiserum was determined by ELISA; the serum was used without further purification. Protein samples corresponding to 20 µg of each fraction were electrophoresed on SDS-10% (w/v) polyacrylamide gels (Laemmli, 1970) and subsequently transferred onto nitrocellulose extra blotting membrane (Schleicher & Schuell) 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, either anti-NEU2 rabbit antisem or anti-HA monoclonal antibody (Boehringer), was added at appropriate dilution in BB and blots were incubated for 1 h. After washing in TTBS, the membranes were incubated for 1 h with the appropriate horseradish peroxidase-conjugated IgG (Amersham) diluted in BB. After final washing in TTBS, visualization of the antibody binding was carried out with ECL (Amersham) according to the manufacturer’s instructions. All incubations were carried out at room temperature under constant shaking.

Immunofluorescence staining of NEU2 in COS7 cells

For immunofluorescence, COS7 were grown in 8-well chamber slide culture chambers (Nunc). Transfections were carried out as previously described, using 0.2 µg DNA for each well. Indirect immunostaining of HA-NEU2 and NEU2 was performed on 4% (w/v) paraformaldehyde in PBS or methanol/acetone 1:1 (v/v) fixed cells. Cells fixed with the former reagent were permeabilized with Triton X-100 or saponin 0.2% (v/v) in PBS, blocked with porcine serum and incubated with anti-HEA monoclonal antibody (10 µg/ml) or anti-NEU2 rabbit antisem (1:500 dilution) in PBS plus porcine serum alone or containing the permeabilizing reagents. Staining was obtained after incubation with fluorescein 5-isothiocyanated conjugated isotype-specific antibodies (1:100 dilution) (DAKO). Fluorescence microscopy was carried out using an Axioskan microscope (Zeiss).

Expression of NEU2 encoded protein in E.coli

The entire coding region of NEU2 was obtained by PCR amplification using the isolated genomic clone HG7 as template. The DNA sequence from the Met to the Gln in position 67 (exon 1) was amplified using Neu2-Nt (see above) with a 5′-BamHI restriction site and a 5′-phosphorylated antisense primer Neu2-67R (5′-CTGAACCTGGTTGGGTTGTCG-3′). The DNA sequence from the Trp in position 68 to the stop codon (exon 2) was amplified using Neu2-68F (5′-TGGCAAGCTCAGGAGGTGTTG-3′) and Neu2-Ct (see above) with a 5′-EcoRI restriction site. The two PCR products, obtained using cloned pfu polymerase (Stratagene), were ligated using T4 ligase and the resulting fragment of about 1.15 kb was subcloned into BamHI–EcoRI sites of pGEX-2T expression vector (Pharmacia Biotech). The recombinant plasmid, pGEX-2T-NEU2, was completely sequenced using both vector- and gene-specific primers. E.coli DH5 alpha cells were transformed with the recombinant plasmid pGEX-2T-NEU2 or the expression vector pGEX-2T alone, and the transformed cells grown in LB-ampicillin medium at 37°C to mid-log phase before addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 0.1 mM. After overnight growth at room temperature, cells were pelleted and suspended in 1/50 volume of phosphate-buffered saline (PBS). Cells lysed by sonication and supernatants were assayed for sialidase activity. Purification of glutathione-S-transferase-NEU2 fusion protein was carried out according to the manufacturer’s instructions (Pharmacia Biotech, GST Gene Fusion System, 18–1115–20). Samples of E.coli crude lysates (20 µg) and affinity purified NEU2 (0.5–1 µg) were subjected to SDS–PAGE under denaturing conditions (Laemmli, 1970).

Sialidase assay

The enzymatic activity of NEU2 in total cell lysates and in cellular subfractions toward the artificial substrate 2-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (4MU-NANA) (Sigma) was determined by fluorimetric assay (Venerando et al., 1994). Reactions were set up in duplicate using up to 50 µg of total protein in 0.1 M Na citrate/phosphate buffer pH 5.6, in the presence of 400 µg bovine serum albumin, with 0.2 mM 4MU-NANA in a final volume of 100 µl, and incubated at 37°C for 5–10 min. Reactions were stopped by addition of 1 ml of 0.2 M glycine/NaOH pH 10.2. Fluorescence emission was measured on a Jasco FP-770 fluorometer with excitation at 365 nm and emission at 445 nm, using 4-methylumbelliferone (4-MU) to obtain a calibration curve.

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References

Expression of a human cytosolic sialidase


