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Protective Protein/Cathepsin A Rescues N-glycosylation defects in Neuraminidase-1

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Abstract

Background—Neuraminidase-1 (NEU1) catabolizes the hydrolysis of sialic acids from sialo-glycoconjugates. NEU1 depends on its interaction with the protective protein/cathepsin A (PPCA) for lysosomal compartmentalization and catalytic activation. Murine NEU1 contains 4 N-glycosylation sites, 3 of which are conserved in the human enzyme. The expression of NEU1 gives rise to differentially glycosylated proteins.

Methods—We generated single-point mutations in mouse NEU1 at each of the 4 N-glycosylation sites. Mutant enzymes were expressed in NEU1-deficient cells in the presence and absence of PPCA.

Results—All 4 N-glycosylation variants were targeted to the lysosomal/endosomal compartment. All N-glycans, with the exception of the most C-terminal glycan, were important for maintaining stability or catalytic activity. The loss of catalytic activity caused by the deletion of the second N-glycan was rescued by increasing PPCA expression. Similar results were obtained with a human NEU1 N-glycosylation mutant identified in a sialidosis patient.

Conclusions—The N-terminal N-glycan of NEU1 is indispensable for its function, whereas the C-terminal N-glycan appears to be non-essential. The omission of the second N-glycan can be compensated for by upregulating the expression of PPCA.

General Significance—These findings could be relevant for the design of target therapies for patients carrying specific NEU1 mutations.

Keywords

Glycosylation; sialidosis; chaperone therapy; neuraminidase; sialidase; NEU1; protective protein/cathepsin A

1. Introduction

Neuraminidases or sialidases constitute a large and important family of exoglycosyl hydrolases that cleave α-glycosidically linked terminal N-acylneuraminate residues (sialic acid) from glycoproteins, glycolipids, and polysaccharides. The importance of sialic acids is reflected in their structural diversity, i.e., more than 25 variants have been identified. Sialo-glycoconjugates

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are target molecules in a variety of crucial biological processes; mainly, they serve as ligands in recognitive interactions.

The lysosomal N-acetyl-α-neuraminidase (NEU1, sialidase) initiates the catabolism of sialoglycoconjugates by hydrolyzing their terminal sialic acid residues [1,2]. Genetic mutations at the *NEU1* locus in humans are associated with sialidosis (mucolipidosis I), an autosomal-recessive lysosomal storage disease (LSD) that targets primarily the reticuloendothelial system [3]. NEU1 is active in lysosomes, exclusively when in complex with the protective protein/cathepsin A (PPCA). Targeting of most soluble lysosomal hydrolases to the lysosome is mediated via interaction with the mannose-6-phosphate (M6P) receptor, which recognizes a phosphorylated M6P moiety on N-glycans. In contrast, NEU1 is poorly phosphorylated and depends on its association with PPCA for its efficient transport to the lysosome. Once in the lysosome, NEU1 is incorporated in a multienzyme complex and then activated through its interaction with PPCA [1,4].

Human NEU1 (hNEU1) shares 91% amino acid similarity with its murine homolog and has properties identical to those of the mouse enzyme, as evidenced by the phenotype of the *neu1*-knockout mouse model, which resembles the clinical features of sialidosis patients. Murine NEU1 (mNEU1) and hNEU1 share 3 conserved N-glycosylation sites, but mNEU1 has an additional site near the C terminus of the enzyme [2]. We have previously shown that expression of NEU1 in mammalian cells gives rise to multiple protein species of different molecular weight (MW), most likely because of differential glycosylation of the enzyme [4]. Either the prevention of de novo N-glycosylation of NEU1 or the ex vivo enzymatic removal of its N-glycans converted the enzyme to a 40-kDa homogenous form, which was unstable and inactive (E.B. unpublished data). These findings indicate that N-glycosylation of NEU1 is crucial for maintaining the structural integrity and activity of the enzyme.

The difference in the number of N-glycans between mNEU1 and hNEU1 suggests that the individual sites may not be equally important for the function of the enzyme. To assess the impact of the 4 N-glycans on the biochemical properties of the enzyme, we mutated each site by substituting an aspartic acid (D) residue for an asparagine (N). The mutant enzymes were expressed in hNEU1-deficient cells and the effect(s) of the individual mutations on the MW, subcellular localization, catalytic activity and stability of mNEU1 were determined. In addition, we assessed the influence of PPCA levels on the catalytic activity of the mutant enzymes. Finally, we determined the effect of PPCA upregulation on the enzymatic properties of a naturally occurring glycosylation mutant of hNEU1, that was identified in a type I sialidosis patient.

2.1 Materials and methods

2.1 Cell culture and antibodies

Lung fibroblasts were isolated from *neu1*$^{+/+}$ (wild-type) and *neu1*$^{-/-}$ mice. GM01718 type II sialidosis fibroblasts were originally obtained from the NIGMS Human Genetic Cell Repository (Camden, NJ). All other cell lines were obtained from ATCC (Manassas, VA). Primary mouse or human fibroblasts were maintained in culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin) and maintained at 37°C (5% CO_2). HEK293T, Amphotropic (Phoenix-A), and Ecotropic (Phoenix-E) viral producer cells were grown in DMEM supplemented with 5% FBS. Antibodies against NEU1 and PPCA were produced and affinity purified as described previously [7].

2.2 Site-directed mutagenesis of mouse neu1 cDNA

Human and mouse wild-type *neu1* constructs have been described earlier [1,2]. To generate the 4 N-glycosylation mutants, we performed 2 rounds of PCR, replacing the N (AAT/AAC) residue at positions 180, 337, 346, or 372 with a D (GAT/GAC) residue. The nucleotide exchanges were confirmed by automated sequence analysis. Mutant *neu1* cDNAs where subcloned into the 5' internal ribosomal entry sequence (IRES) of the *MSCV-IRES-GFP* retroviral vector [8], and green fluorescent protein (GFP) was coexpressed as an indicator of transfection/transduction efficiency and virus titer. Human wild-type *ppca* cDNA was similarly inserted into the *MSCV-IRES-YFP* to generate a recombinant retrovirus coexpressing yellow fluorescent protein (YFP). The Human *neu1* cDNA was in vitro mutagenized to introduce a mutation (c.1034C.T; p.T345I) found in a patient of Czech origin who had type I sialidosis [9]. The mutagenized cDNA was subcloned in the mammalian expression plasmid *pSCTOP* as previously described [10].

2.3 Retrovirus preparation, transduction, and transient transfection

Ecotropic or amphotropic retroviruses were generated by transfecting the retroviral packaging cell lines Phoenix-E or -A with *MSCV-NEU1-IRES-GFP* and *MSCV-PPCA-IRES-YFP* plasmid constructs using Fugene 6 transfection reagent (Roche, Indianapolis, IN) per the manufacturer's instructions. The recombinant retroviruses were harvested from the media, filtered through 0.45-μm filters, and stored at −80°C. Mouse and human fibroblasts were either singly transduced with the wild-type or 1 of the mutant *neu1* retroviruses or were cotransduced with *ppca* and 1 of the *neu1* retroviruses. A final concentration of 8 μg/ml polybrene was used to improve transduction efficiency. Two or 3 days after transduction, GFP^+ and YFP^+ cells were sorted by Fluorescence-Activated Cell Sorting (FACS). Sorted cells were maintained in culture for an extended period of time and tested for NEU1 and PPCA expression levels, lysosomal localization, and correction of NEU1 activity. Transient transfections were performed either by singly transfecting the *MSCV-NEU1-IRES-GFP* constructs into HEK293T cells or by cotransfecting them with *MSCV-PPCA-IRES-YFP* using Fugene 6 as mentioned above.

2.4 Enzyme activity analysis, immunoblotting, and deglycosylation

Transfected or transduced cells were harvested and lysed in water containing Complete EDTA-free protease inhibitors (Roche). Neuraminidase catalytic activity was measured using the synthetic substrate 4-methylumbelliferyl-α-D-N-acetylneuraminic acid as described earlier [11], and total protein concentration was measured using the BCA assay and Bovine serum albumin as a standard (Pierce Chemical, Rockford, IL).

Cell lysate samples (10–30 μg) were resolved on 12.5% polyacrylamide gels under denaturing conditions, transferred onto PVDF membranes, and incubated with anti-NEU1 and/or anti-PPCA affinity-purified antibodies and peroxidase-conjugated goat–anti-rabbit secondary antibody (Jackson Immuno Research, West Grove, PA). NEU1 and PPCA were visualized using Western Lightning Chemiluminescence Reagents (PerkinElmer, Waltham, MA).

Deglycosylation of cell lysates was performed according to the manufacturer's protocol (New England Biolabs, Ipswich, MA). In brief, 10-μg cell lysate was denatured in 1 × glycoprotein denaturing buffer at 100 °C for 10 min, after which 1/10 (v/v) each of 10 × G7 buffer, 10% NP-40, and peptide N-Glycosidase F (PNGase F; New England Biolabs) were added. Lysates were then incubated at 37 °C for 1 h. The reactions were stopped by adding SDS-sample buffer and reduced with DTT (3–5 min, 100 °C). The samples were analyzed on immunoblots as described above.

2.5 Immunocytochemistry

FACS-sorted *neu1*$^{-/-}$ mouse lung fibroblasts were seeded directly onto Superfrost/Plus microscope glass slides (Fisher Scientific, Pittsburgh, PA) at about 60% confluence. One day later, the slides were processed according to the method of van Dongen et al [12] using affinity-purified anti-NEU1 antibody and cyanine 3–conjugated goat–anti-rabbit IgG secondary antibody (Jackson Immuno Research). The stained slides were examined using a fluorescence microscope as described earlier [1].

Confocal microscopy was performed using a Leica TCS NT SP setup. Fluorescent images of living cells were digitally recorded using a SPOT camera (Diagnostic Instruments, Sterling Heights, MI) attached to an Olympus BX50 fluorescence microscope.

2.6 Structural modeling of mouse and human NEU1

We generated structural models of mouse and human NEU1 by using the comparative modeling method [13]. Initial modeling was performed using the fold-recognition program LOOPP [14,15], which used an alignment protocol based on the scoring scheme of the structural templates of 2 bacterial sialidases: 1) sialidase from *C. perfringens* (PDB code 2BF6 [16]) for mouse protein with a sequence identity of 28%, and 2) sialidase from *M. viridifaciens* (PDB code 1EUR [17]) for human protein with a sequence identity of 30%. The highest scores were 6.6 and 9.3 for mouse NEU1 and human NEU1, respectively. Atomic models were then generated using the MODELLER program, version 9v4 [18,19].

3. Results

3.1 Each of the 4 putative N-glycosylation sites is required for complete N-glycosylation of the protein

The consensus sequence for N-glycosylation is Asn-X-Ser/Thr, where X is any amino acid except proline [20,21]. This consensus tripeptide, also called the N-glycosylation sequon, is a prerequisite for glycosylation. However, the presence of the consensus tripeptide in itself is not sufficient to secure N-glycosylation, because the folding of the protein plays an important role in the regulation of this process. We found that all mammalian NEU1 proteins present in the public DNA databases, which include NEU1 from human, chimpanzee, rhesus monkey, dog, cow, mouse, and rat, have 3 conserved N-terminal N-glycosylation sites (Fig. 1A-B). Mouse and rat are the only mammalian species in which NEU1 has acquired a fourth N-glycosylation site at the C terminus (Fig. 1A). NEU1 from zebra fish lacks the third N-glycosylation site and has only the 2 N-terminal N-glycosylation sites that are also conserved in all vertebrate species (Fig. 1A). The NetNGlyc 1.0 program is a prediction method that is based on artificial neural networks that examine the surrounding sequence context of a protein to predict whether an N-glycosylation sequon is utilized (http://www.cbs.dtu.dk/services/NetNGlyc/). Analysis of mNEU1 showed that all 4 N-glycosylation sites have an *N-glycosylation potential* that is equal to or greater than the 0.5 threshold which indicates that all 4 sites are utilized (Fig. 1C).

To determine the function of individual glycans on the biochemical properties of mNEU1 we generated 4 single-point mutants by introducing an N-to-D amino acid substitution at each of the 4 N-glycosylation sites: N180D (mut1), N337D (mut2), N346D (mut3), and N372D (mut4). For expression studies in mammalian cells, we subcloned the *neu1* mutant cDNAs into a MSCV retroviral vector, 3’ from an internal ribosome entry site (IRES) and GFP sequence. Because NEU1 depends on PPCA for both its efficient transport to the lysosome and for catalytic activation, we cotransfected the *MSCV-Neu1-IRES-GFP* constructs (wild-type, and mut1–4) with *MSCV-PPCA-IRES-YFP* into Phoenix-A cells. Transduced Phoenix-A cell lysates were analyzed on immunoblots for mNEU1 expression and assayed for catalytic activity (Fig. 2A-

B). The expression levels of the 4 mutants were similar to the level of wild-type mNEU1, with the exception of mut1 (Fig. 2A). This mutant was expressed at considerably lower levels than the others, as evidenced by the higher amount of the GAPDH loading control in this sample compared to that of the other 3 mutants and the wild-type sample. The percentage of GFP and YFP double-positive cells was similar for all samples and ranged between 69% and 77% (Fig. 2A), which indicated that the transduction efficiencies were comparable. Endogenous enzyme was not detected in the transduced cells (Fig. 2A). Overexpressed wild-type mNEU1 was resolved on an immunoblot as 4 bands with apparent MWs of 42, 44, 45, and 46 kDa (Fig. 2A). In contrast, mut1–4 lacked the lowest mobility (46-kDa) band (Fig. 2A), and mut1 and mut2 lacked the 42-kDa band (Fig. 2A).

The different migration patterns of mutant and wild-type NEU1 species were the result of differential N-glycan content because digestion of cell lysates with PNGase F, which cleaves glycans at the N residues, reduced the MW of all the NEU1 proteins to 40-kDa (Fig. 2B). This size corresponded to the size of the naked protein.

Although mut2–4 were expressed at similar levels, their catalytic activities varied dramatically. The activities of mut2-NEU1 and mut3-NEU1 were 40% and 60% that of wild-type mNEU1, while the activity of mut4-NEU1 was comparable to that of the wild-type enzyme (Fig. 2C). The activity of mut1-NEU1 was the lowest of the four, but similar to that measured in cells transduced with PPCA only. Thus, the absence of each of the N-glycosylation sites affected the enzymatic activity of the enzyme in a different way: for the acquisition of catalytic activity, N372 site (mut4) appeared to be the most dispensable, whereas N180 site (mut1) appeared to be the most essential.

3.2 NEU1 activity of N-glycosylation variants mut1–3 is differentially restored by the coexpression of PPCA

The conversion of NEU1 into a catalytically active enzyme is dependent on the following events: 1) interaction with PPCA in an early biosynthetic compartment, where it may act as a molecular chaperone, 2) PPCA-mediated transport to the lysosome, and 3) intralysosomal association with PPCA [6,22,23]. The mode of interaction between NEU1 and PPCA is not well understood, but it is clear that PPCA is essential for lysosomal compartmentalization and catalytic activation of NEU1. To test the effect of PPCA on each of the mNEU1 glycosylation mutants, we transduced *neu1*$^{-/-}$ fibroblasts with either wild-type *neu1* or mutant *neu1* constructs (*MSCV-NEU1-IRES-GFP*), and relied on the endogenous expression of PPCA [1]. GFP^{+} cells were FACS-sorted and analyzed by immunofluorescence and confocal microscopy using anti-NEU1 antibody. All 4 mNEU1 mutants showed a punctate endosomal/lysosomal staining pattern that was similar to that seen in wild-type NEU1–expressing cells, albeit the expression level of mut1-NEU1 was relatively low and somewhat perinuclear compared with that of the other mutants (Fig. 3). Thus, all 4 NEU1 mutants had apparently enough structural integrity to reach the endosomal/lysosomal compartment. The residual activity of the mutant enzymes varies considerably: Mut1 had less than 5% activity while Mut4 retained full enzymatic activity; mut2 and 3 had intermediate residual activity compared to that of the wild-type enzyme (Fig. 4A).

We then tested whether increasing the amount of the NEU1-specific chaperone PPCA may compensate for the loss of any of the 3 N-glycans (mut1–3). Therefore, we repeated the *neu1* virus-transduction experiments in the presnce of coexpressed PPCA (*MSCV-PPCA-IRES-YFP*). GFP and YFP double-positive cells were FACS-sorted and analyzed for their NEU1 activity and protein expression. Cotransduction of PPCA with mut2 increased its residual NEU1 activity to a level that was comparable to that of cells coexpressing PPCA and wild-type NEU1 (Fig. 4B). Also, the activity of mut1-NEU1 increased with the coexpression

of PPCA but remained well below the wild-type values (Fig. 4B). In contrast, PPCA had little effect on the activity of mut3-NEU1–expressing cells (Fig. 4B).

On an immunoblot probed with anti-NEU1 antibody, we observed similar expression in wild-type and mut4-NEU1 samples, intermediate expression of mut2-NEU1, and low expression of mut3-NEU1. The expression of mut1-NEU1 was below the detection limit (Fig. 4C). These results confirmed that the fourth glycosylation site, N372, is dispensable for mNEU1 function; the mutation of this site did not affect the expression level or the catalytic activity of the enzyme. In contrast, the first and third sites, N180 and N346, appeared to be the most essential for expression and activity of NEU1. Coexpression of PPCA did not compensate for the mutation of these sites. Mutation of the second site, N337, resulted in a 60% decrease in NEU1 activity, which was restored by the coexpression of PPCA.

3.3 A missense mutation identified in the NEU1 gene of a patient with type I sialidosis was rescued by the coexpression of PPCA

A homozygous *neu1* missense mutation was reported in a patient with type I sialidosis. This mutation (c.1034C.T; p.T345I) changed the threonine (T) in the second Asn-X-Ser/Thr N-glycosylation site to an isoleucine (I) [24]. The patient was reported to have residual NEU1 enzymatic activity that correlated with an attenuated phenotype [25]. We hypothesized that, as is the case for the mut2-NEU1 variant, the omission of the second N-glycan on the NEU1 enzyme was at least partially responsible for the reduced NEU1 activity in the patients' cells.

We therefore tested whether the catalytic activity of the T345I-NEU1 mutant enzyme was enhanced by the overexpression of human PPCA. A *CMV-T345I-NEU1* plasmid was transiently transfected in mNEU1-deficient macrophages and the expression level and catalytic activity of this mutant enzyme was compared to *CMV-WT-NEU1* transfected cells (Fig. 5). CMV-GFP was cotransfected with each sample as an internal control for the transfection efficiency. GFP expression varied minimally (34%–37%), as assessed by fluorescence microscopy (not shown). The NEU1 activity of T345I-NEU1–transfected macrophages was 60% lower than that of wild-type NEU1-expressing cells (Fig. 5A); this level of expression was comparable to that reported in cells from the sialidosis patient carrying this mutation [24]. The NEU1 activity of T345I-NEU1–transfected macrophages was enhanced 4 fold by coexpression of *CMV-PPCA* in these cells (Fig. 5A). Despite the lower enzymatic activity, the amount of T345I-NEU1 protein detected on the immunoblot was similar to that of wild-type NEU1, and the amounts of both enzymes increased upon coexpression of PPCA (Fig. 5B). These results demonstrate that, as is the case for the mut2-NEU1 variant, the T345I-NEU1 enzyme identified in the patient with type I sialidosis was partially rescued by increasing the level of PPCA expression.

3.4 Structural models of mouse and human NEU1

Structural models of human and mouse NEU1 show a high degree of spatial similarity (Fig. 6A-B), and they could be superimposed with the rmsd deviation of 1.5 Å for C-alpha atoms (Fig. 6C). The first 3 N-linked glycosylation sites occupy homologous positions in the structures at the periphery of the molecule. The fourth N-glycosylation site appears to be less structurally conserved than the other 3 sites. In addition, site 4 is localized on an extended loop (Fig. 6A-B), and small perturbations in the vicinity of this site would probably have no effect on the conformation of the NEU1 molecule. These models support the observation that the difference in sequence and structure of site 4 between mNEU1 and hNEU1 has no effect on structural integrity, protein stability, or catalytic activity. A dramatic effect of mut1 (N180D) on the catalytic activity could be the result of conformational changes in the molecule and shifts in the positions of the catalytic residues caused by the breakdown of the hydrogen-bonding network between glycans and the side chains of the protein. A similar scenario may explain

the effect of the T345I mutation on the activity of hNEU1 (Fig. 6D). The breakdown of the hydrogen-bonding network between glycans and the neighboring side chains of site 2 would affect the loop conformation and position of important catalytic residues R341 and Y370 (Fig. 6D). Thus, it is plausible that mutations of N-glycosylation sites that lead to the omission of N-glycans affect the catalytic activity and structural integrity by removing the structural constraints made by these glycans. Lacking an interaction model of NEU1 with PPCA, we are unable to explain how PPCA restores the structural integrity and catalytic activity of NEU1. We hypothesize that the binding of PPCA may exert structural constraints on NEU1, thereby preventing the movement of catalytic site residues, even when the second N-glycan is omitted.

4. Discussion

Glycosylation is an important posttranslational modification that plays a crucial role in several physiological properties of a glycoprotein, including interaction with chaperones and the ER quality machinery, folding, stability, transport, catalytic activity, solubility or viscosity, membrane orientation, and protein-protein interaction [26–32]. However, N-glycosylation does not occur at every potential N-glycosylation consensus site, and the function of N-glycans varies among proteins.

We have previously shown that NEU1 is a heterogeneously N-glycosylated enzyme [1]. The in vitro removal of all the N-glycans on the protein resulted in a complete loss of enzymatic activity and stability (E.B. unpublished data). We therefore argued that the N-glycans on NEU1 may be crucial for its activity, stability, and lysosomal targeting. However, whether all 4 N-glycosylation sites were involved in this process and equally important for NEU1 function remained unknown. To affect N-glycosylation, the N residue of the N-glycosylation sequon is expected to be at the surface of the protein structure. Analysis of the structural models of mouse and human NEU1 predicts that each of the 4 N-glycosylation sites are located at the surface of the protein, which correlates with all 4 sites being occupied by glycans.

The most N-terminal N-glycosylation site N180 1 of 2 sites that is conserved in NEU1 of all vertebrate species, which underlies its importance. By performing limited in vitro trypsin proteolysis of purified wild-type and mutant NEU1 proteins, we found that mut1 (N180D) was the only mutant enzyme with substantially decreased stability; that of the mut2–4 was comparable to the stability of the wild-type protein (not shown). Thus, the N180-linked glycan is most likely important for protein stability.

Omission of the most C-terminal NEU1 glycosylation site N372 had no effect on the expression level or catalytic activity of mNEU1. Interestingly, this glycosylation site has evolved in rodents but is absent in all other vertebrate species. On the basis of this fact and our data, we deduce that this N-glycan is not essential for NEU1 function. In expression experiments with mNEU1 N-glycosylation mutants, the presence of the fourth N-glycan was unable to compensate for the loss of any of the other individual N-glycans. Thus, for protein stability and enzyme function, NEU1 does not simply require a minimum of 3 N-glycans; instead, the location of each N-glycan is important for maintaining the biochemical properties of the enzyme.

The second glycosylation site of NEU1 (N337) is conserved in all vertebrate species. Mutation of this site also decreases the enzyme activity. However, the loss of catalytic activity was restored to near normal values by the coexpression of PPCA. On the basis of these results, we hypothesize that the second N-glycan protects NEU1 against premature intralysosomal degradation. It has been well established that PPCA acts as a molecular chaperone for NEU1 and that the 2 proteins associate in an early biosynthetic compartment [22]. We postulate that

PPCA binding occurs near the second N-glycan of NEU1 and has a stabilizing effect similar to that of the N-glycan itself.

The identification of a mutation affecting the second N-glycosylation site of NEU1 in a patient with type I sialidosis with an attenuated phenotype underscores its importance for NEU1 function. The PPCA-induced increase of the residual activity of this mutant enzyme offers the potential for chaperone therapy in certain type I sialidosis patients. Substantial improvements of the LSD phenotype, or delay of the onset may be achieved by relatively small increases in the level of the deficient lysosomal hydrolase. In the case of sialidosis, besides the possible therapeutic administration of exogenous PPCA, it may be possible to identify small molecules that could potentially increase the level of expression of endogenous PPCA, and thereby indirectly also enhance the levels of residual NEU1 activity. Besides the therapeutic benefits to the systemic disease, these small molecules may potentially cross the blood-brain barrier which could also aid the correction of CNS disease pathology.

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Abbreviations

D	aspartic acid
DMEM	Dulbecco's modified Eagle's medium
DTT	dl-dithio-threitol
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
GFP	green fluorescent protein
LSD	lysosomal storage disease
N	asparagine NEU1, lysosomal neuraminidase-1
PCR	polymerase chain reaction
PPCA	protective protein/cathepsin A
YFP	yellow fluorescent protein

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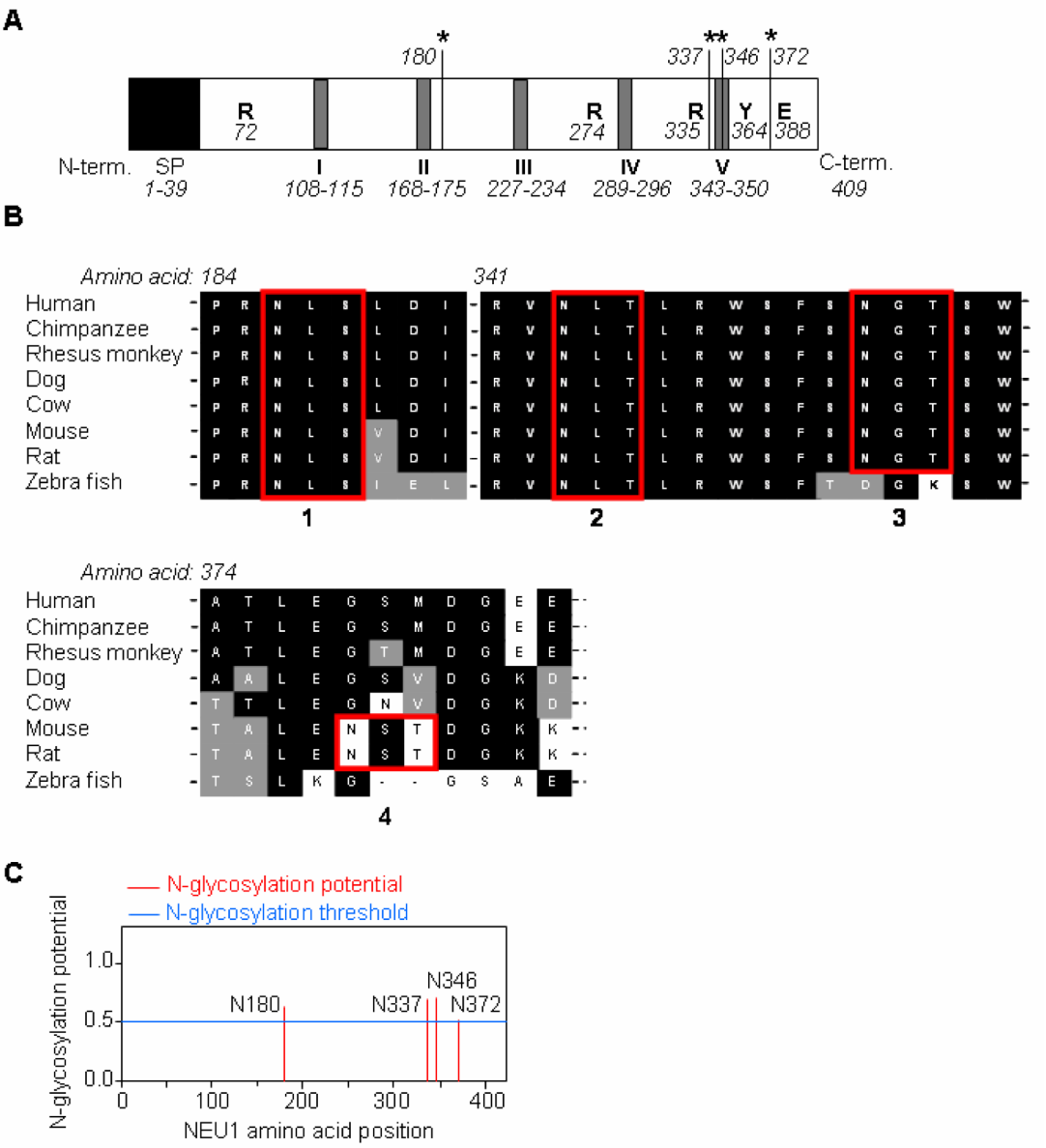

Fig. 1.

Comparison of NEU1 from different vertebrate species by sequence homology. (A) A diagram of the primary structure of murine NEU1 protein indicating the positions of conserved amino acid residues. SP, signal peptide; grey box, Asp-box motif I-IV; asterisk, N-glycosylation site; amino acid positions are indicated in italics; the 5 active-site residues that are conserved in all neuraminidases are indicated in bold. (B) Alignment (CLUSTALW) of selected regions containing N-glycosylation sites of NEU1 from different vertebrate species. N-glycosylation sites 1–4 are boxed in red; identical amino acids are shaded in black, and similar residues are shaded in grey; amino acid positions of human NEU1 are indicated in italics. (C) N-glycosylation sites on murine NEU1 predicted by the NetNGlyc 1.0 online analysis. All 4 potential N-glycosylation sites (Asn-X-Ser/Thr) are indicated and have a potential of at least 0.5, which is the threshold for N-glycosylation site utilization.

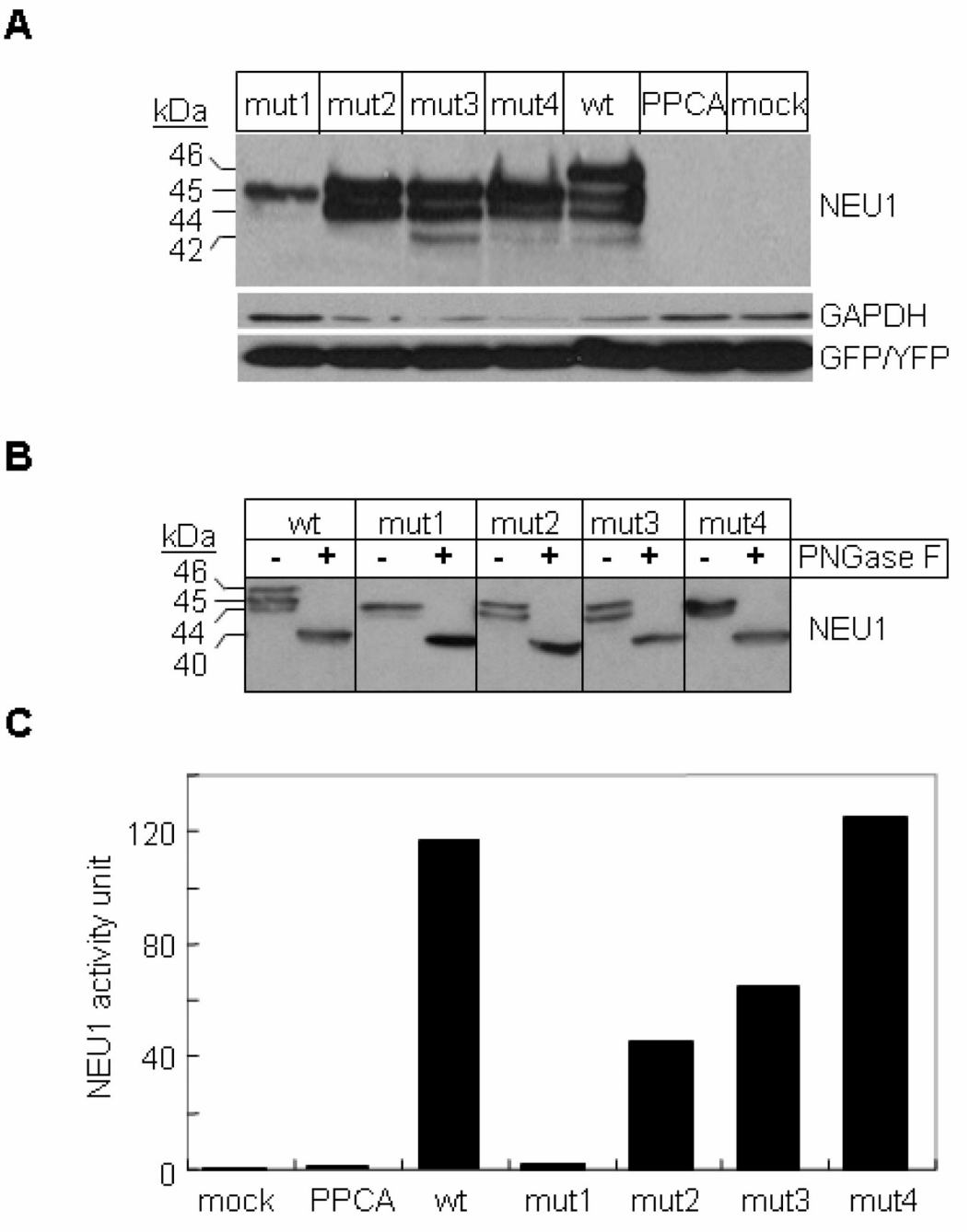

Fig. 2.

All 4 N-glycosylation sites of Neu1 are used during glycosylation of the protein. (A) Immunoblot analysis with anti-NEU1 antibody of MSCV-transduced Phoenix-A cells expressing 1 of 4 Neu1 glycosylation mutants (mut 1–4), wild-type Neu1 (wt), or PPCA. GAPDH was used as a loading control, and GFP/YFP was used as a control for transduction efficiency. (B) Immunoblot analysis with anti-NEU1 antibody of peptide N-glycosidase F (PNGase F)-digested, MSCV-transduced Phoenix-A cell lysates. (C). NEU1 catalytic activity measured in lysates from MSCV-transduced Phoenix-A cells. Neu1 activity unit was defined as nmol substrate per h per mg protein.

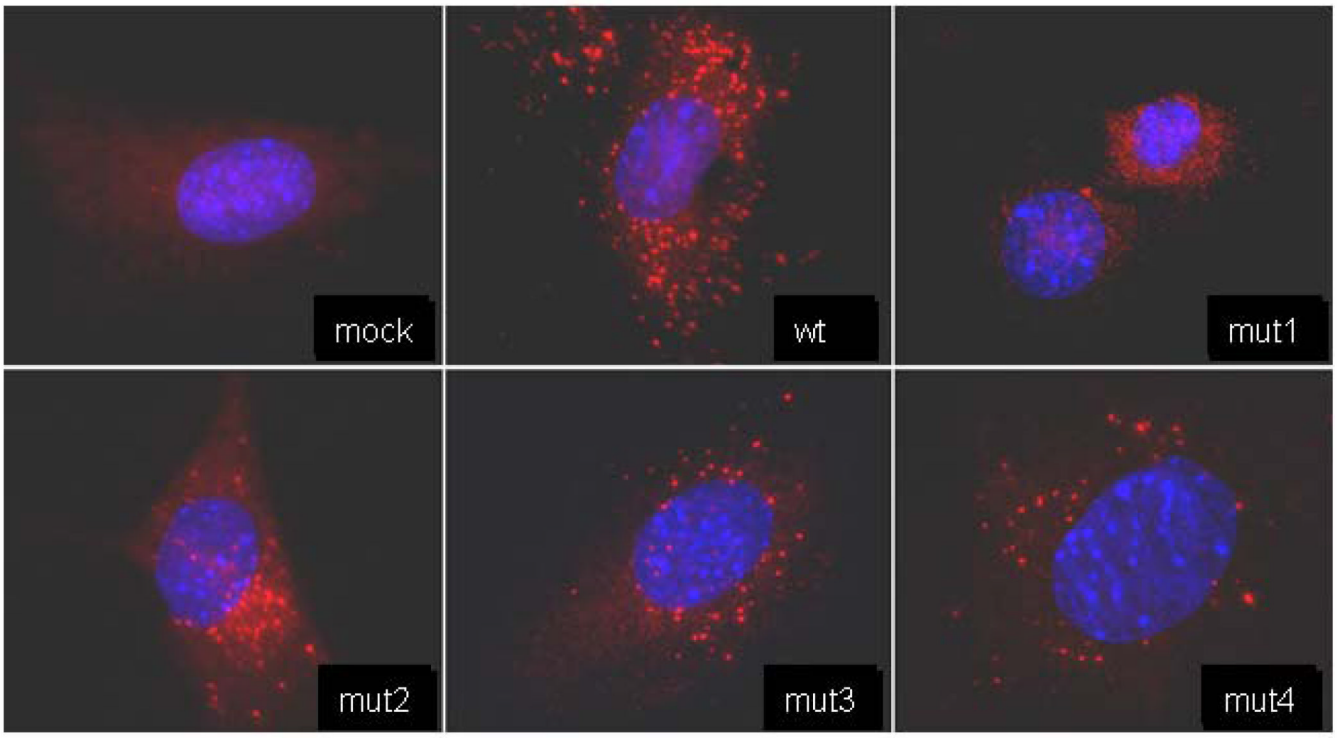

Fig. 3.

NEU1 glycosylation mutants show lysosomal/endosomal localization similar to that of wild-type NEU1. MSCV-transduced n*eu1*$^{-/-}$ mouse lung fibroblasts were maintained in culture on super-frost slides and fluorescently stained with anti-NEU1 primary antibody and anti–rabbit-IgG-Texas red secondary antibody (red). Nuclei were stained with DAPI (blue). Cells were imaged by confocal microscopy. Magnification, 40x.

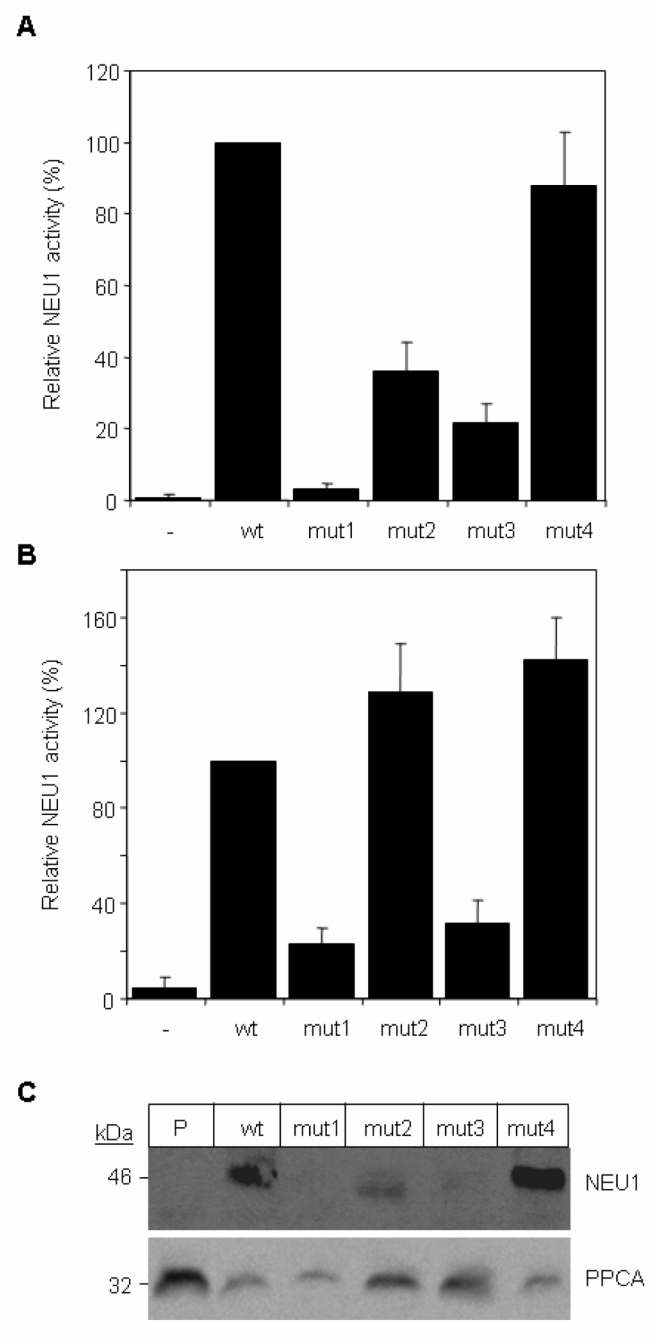

Fig. 4.

Expression of mNEU1 and mNEU1-glycosylation mutants in NEU1-deficient fibroblasts. (A-B) The relative Neu1 catalytic activity of wild-type (wt) and the 4 N-glycosylation mutants (mut1–4) transiently expressed in primary NEU1-deficient fibroblasts with (A) or without (B) coexpression of PPCA. MSCV-transduced cells were sorted by FACS for GFP and YFP double-positive cells. The catalytic activity of the wild-type samples was set at 100%, and all other activities are relative to that value. Error bars, ± standard deviation. (C) Immunoblot analysis of MSCV-transduced NEU1-deficient fibroblasts with anti-NEU1 and anti-PPCA antibodies.

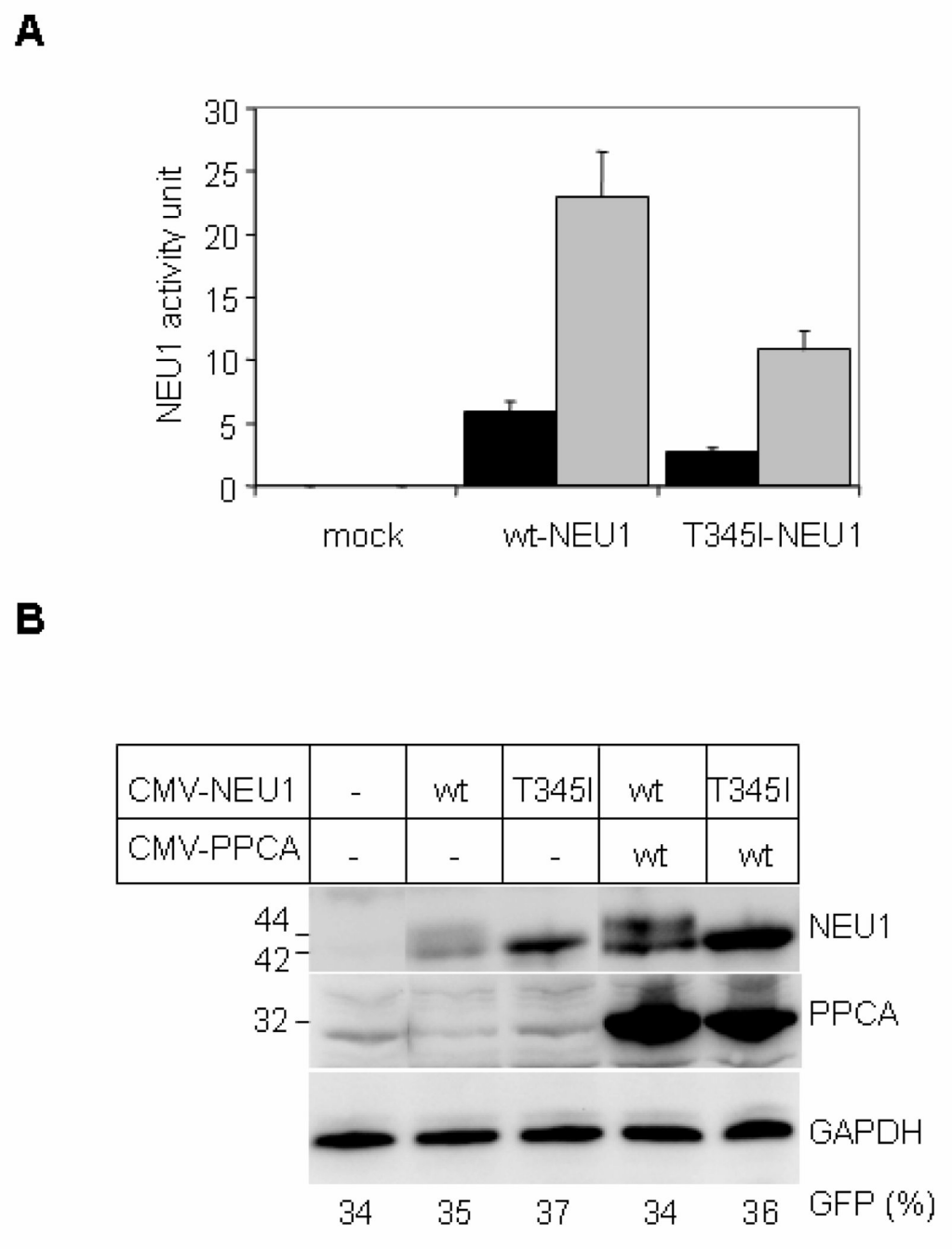

Fig. 5.

Analysis of NEU1 N-glycosylation mutant T345I in a patient with type I sialidosis. (A) NEU1 activity was measured in NEU1-deficient macrophages transiently expressing human wild-type NEU1 or the T345I-NEU1 glycosylation mutant derived from a patient with type I sialidosis. Cells were transiently transfected with wild-type NEU1 (wt) or T345I-NEU1 alone (black bars) or with coexpression of human PPCA (gray bars). GFP expression was used as an internal control for the transfection efficiency, which was similar for all samples. NEU1 activity unit was defined as nmol substrate per h per mg protein; error bars, ± standard deviation. (B) Immunoblot analysis with anti-NEU1 and anti-PPCA antibodies of transfected NEU1-deficient macrophages. GAPDH was used as a loading control.

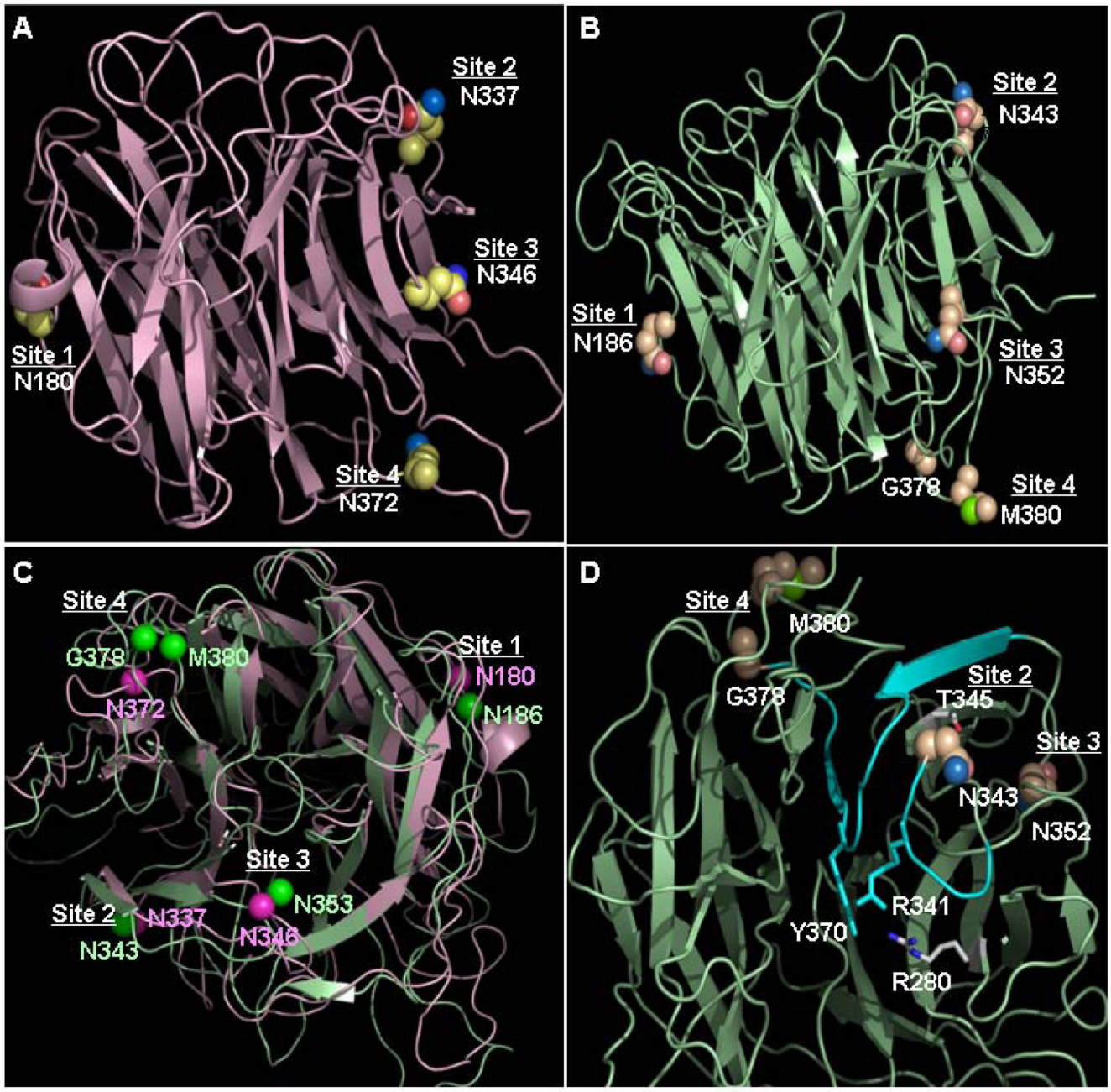

Fig. 6.

Structural models of mouse and human NEU1. (A) Mouse NEU1 N-glycosylation sites 1–4 are indicated. (B) Human NEU1 N-glycosylation sites 1–3 are indicated; site 4 corresponds to the fourth N-glycosylation site in mouse NEU1, which is absent in humans. (C) Superimposition of mouse Neu1 (pink) and human NEU1 (green) structures. (D) The proposed effect of the T345I mutation (site 2) on the catalytic machinery of human NEU1. The breakdown of the hydrogen-bond network between glycans (not shown) and the neighboring side chains of site 2 would affect the loop conformation and positions of the important catalytic residues R341 and Y370.