An Essential Oligomannosidic Glycan Chain in the Catalytic Domain of Autotaxin, a Secreted Lysophospholipase-D*

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Autotaxin/NPP2, a secreted lysophospholipase-D, promotes cell proliferation, survival, and motility by generating the signaling molecule lysophosphatidic acid. Here we show that ectonucleotide pyrophosphatase/phosphodiesterase 2 (NPP2) is N-glycosylated on Asn-53, Asn-410, and Asn-524. Mutagenesis and deglycosylation experiments revealed that only the glycosylation of Asn-524 is essential for the expression of the catalytic and motility-stimulating activities of NPP2. The N-glycan on Asn-524 was identified as Man8/9GlcNAc2, which is rarely present on mature eukaryotic glycoproteins. Additional studies show that this Asn-524-linked glycan is not accessible to α-1,2-mannosidase, suggesting that its non-reducing termini are buried inside the folded protein. Consistent with a structural role for the Asn-524-linked glycan, only the mutation of Asn-524 augmented the sensitivity of NPP2 to proteolysis and increased its mobility during Blue Native PAGE. Asn-524 is phylogenetically conserved and maps to the catalytic domain of NPP2, but a structural model of this domain suggests that Asn-524 is remote from the catalytic site. Our study defines an essential role for the Asn-524-linked glycan chain of NPP2.

Autotaxin, also known as NPP2, is an enzyme of ~125 kDa that is secreted by various cell types in vertebrates (1, 2). It belongs to the seven-member family of ecto-nucleotide pyrophosphatase/phosphodiesterase isozymes (NPPs). Whereas some NPPs (NPP1, NPP3) preferentially hydrolyze nucleotides, others (NPP2, NPP6, NPP7) favor lipid phosphodiester substrates. NPP2 functions as the major extracellular lysophospholipase-D. It is firmly established that its catalytic domain is not yet known (2, 13, 14). NPP2 is a secreted protein that is synthesized as a pre-proenzyme, whereas NPP1 and NPP3 are largely transmembrane ectoenzymes. The activity of NPP2 is moderately increased by removal of the prepeptide by furin-like proteases (15, 16). NPP1 and NPP2 are both regulated through product inhibition by AMP and lysophosphatidic acid, respectively (17). It is also known that NPPs, including NPP2 (18), are glycoproteins and that the nature and functions of these glycosylations have not been studied in detail, except for NPP7 where an enzymatic deglycosylation was found to be associated with a loss of enzymatic activity (19). It has also been reported that NPP5 contains a high mannose-type glycan, but the exact structure and function of this glycan have not been analyzed (20). In the current study, we have mapped the glycosylation sites of NPP2 and demonstrate that the glycan chain of Asn-524, which was identified as Man8/9GlcNAc2, has a structural function and is essential for the expression of enzymatic activities by NPP2.

MATERIALS AND METHODS

NPP2 Constructs—Wild-type NPP2 (NPP2-WT) was obtained by cloning rat NPP2 (Protein Data Bank accession number AAH81747) in the EGFP-N vector (Clontech). The EGFP (enhanced green fluorescent protein) tag at the C terminus was replaced by a c-Myc tag, and a hemagglutinin (HA) tag...
was introduced at the N terminus. Site-directed mutagenesis of the putative glycosylation sites of HA-NPP2-Myc was performed using the QuikChange™ kit (Stratagene). The sequence of the primer sets used is available upon request. All constructs were verified by sequencing.

**Cell Culture**—HEK293T, A2058 melanoma cells, and NIH-3T3 cells were maintained at 37 °C under a humidified atmosphere containing 5% CO₂ in Dulbecco’s modified Eagle’s medium, supplemented with 10% (v/v) heat-inactivated fetal bovine serum, glucose (4.5 g/liter), penicillin (100 units/ml), and streptomycin (100 µg/ml). Cells were transiently transfected at 30–40% confluency using linear polyethylenimine of fected at 30–40% confluency using linear polyethylenimine of

N-lysophosphatidylcholine (14:0) at 37 °C in a total volume of 40 µl (Polysciences, Warrington, PA). The medium was collected 72 h post-transfection and cleared by centrifugation at 1000 × g for 15 min. The cells were harvested 72 h after transfection, washed once in phosphate-buffered saline, and lysed in 50 mM Tris/HCl at pH 7.5, 0.5 mM phenylmethanesulphonyl fluoride, 0.5 mM benzamidine, 150 mM NaCl, and 1% (v/v) Triton X-100. The supernatant obtained after ultracentrifugation (30 min at 100,000 × g) was used as “cell lysate.” Aliquots of the cell lysates and medium were used for immunoblot analysis and the assay of NPP2-associated activities. NPP2-Myc mutants were purified from the conditioned medium of HEK293T cells that were kept in medium that only contained 5% fetal bovine serum. 72 h after transfection the medium was collected and cleared by centrifugation (15 min at 1000 × g), and the NPP2-Myc fusions were purified by affinity chromatography on anti-c-Myc that was covalently bound to CNBr-activated Sepharose (15).

**Assay of NPP2 Activities**—Lysophospholipase-D activities were measured by incubating aliquots of medium, cell lysates, or purified NPP2-Myc fusions with 2 mM (final) of the substrate lysophosphatidylcholine (14:0) at 37 °C in a total volume of 40 µl. Subsequently, the released choline was quantified spectrophotometrically at 540 nm after incubation for 5 min with 50 µl of the peroxidase reagent (50 mM Tris at pH 9.0, 2 mM N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline, 5 units/ml peroxidase, and 0.01% (V/V) of Triton X-100) and of the choline oxidase reagent (50 mM Tris at pH 9.0, 2 mM aminoantipyrine, 5 units/ml choline oxidase, and 0.01% Triton X-100) (21).

Nucleotide phosphodiesterase activities were determined from the release of p-nitrophenolate from p-nitrophenyl thymidine 5’-monophosphate (21). Briefly, the samples were incubated at 37 °C with 5 mM substrate, 5 mM CaCl₂, 5 mM MgCl₂, and 100 mM Tris/HCl at pH 9.0 in a total volume of 35 µl. The reaction was stopped by the addition of 200 µl of 3% (v/v) trichloroacetic acid. Subsequently, the mixture was neutralized with NaOH and p-nitrophenolate was quantified colorimetrically at 405 nm.

The assay of the lysophospholipase-D and nucleotide phosphodiesterase activities of NPP2 after pretreatment with N-glycosidase F or endoglycosidase H (Figs. 1 and 5), and the corresponding controls (NPP2 with heat-inactivated enzymes), was done in the presence of 5 mM CaCl₂ and 5 mM ZnCl₂ to counteract inhibition of NPP2 by EDTA that was present in the glycosidase preparations. The cell motility-stimulating activities of affinity-purified NPP2 mutants were studied using a 48-well Boyden chemotaxis chamber (Neuroprobe). Cells were harvested using a trypsin/EDTA solution, resuspended in Dulbecco’s modified Eagle’s medium, supplemented with 0.05% (v/v) bovine serum albumin (Gentaur), and left for recovery for 1 h at 37 °C. Meanwhile, the bottom wells were filled with varying concentrations of the NPP2-Myc fusions diluted in phosphate-buffered saline supplemented with 10 µM lysophosphatidylcholine (LPC C14:0) and 0.05% (v/v) bovine serum albumin (Gentaur). The upper and lower chambers were separated by a polyvinyl-pyrrolidone-free polycarbonate membrane (8-µm pore; Neuroprobe) coated with 0.02% fibronectin (Sigma). Subsequently, 5 × 10⁴ cells were loaded into each upper well. After incubation of the assembled chambers for 240 min at 37 °C in a 5% CO₂ humidified incubator, the non-migrated cells in the upper compartments were removed and the cells that had migrated through the membrane were stained with the “Diff-Quik” procedure (Medion Diagnostics, Düdingen) and counted under the light microscope at ×200 (medium power field). For each condition, five randomly chosen fields were counted. The basal migration of the NIH-3T3 cells was determined in the absence of NPP2 and subtracted from the number of cells that migrated in response to NPP2.

**Electrophoresis and Immunoblotting**—Proteins were separated by SDS-PAGE using 3–8% Tricine gels, transferred to polyvinylidene difluoride membranes (Amersham Biosciences), and subjected to 60 V for 2 h in 192 mM glycine, 25 mM Tris, and 10% methanol (pH 8.6). After blotting, nonspecific binding sites were blocked with 3% bovine serum albumin (Serva) and 0.1% Tween 20 in phosphate-buffered saline. Following overnight incubation with anti-Myc (clone 9E10) monoclonal antibodies, the Myc-tagged fusion proteins were visualized using horseradish peroxidase goat anti-mouse IgG (Dako) and enhanced chemiluminescence (PerkinElmer).

Blue Native PAGE was performed according to the method of Schägger and von Jagow (22) with the following modifications. A 6% polyacrylamide gel containing 66.7 mM 6-aminocaproic acid and 50 mM Bis-tris at pH 7.0 was run for 30 min at 80 V. Subsequently, the electrophoresis was continued at 200 V until the tracking line of Coomassie Blue G-250 reached the edge of the gel. As cathode buffer 50 mM Tricine, 15 mM Bis-tris, and 0.02% Coomassie Blue G-250 (pH 7.0) was used. The anode buffer contained 50 mM Bis-tris at pH 7.0. Medium samples were loaded in Blue Native sample buffer containing 25 mM 6-aminocaproic acid, 5 mM Bis-tris, and 5% glycerol at pH 7.0. Immunoblotting was performed as described above, except that 0.01% SDS was added to the blotting buffer.

**Purification and Enzymatic Treatments of Melanoma NPP2 and NPP2 Fusions**—The conditioned medium of HEK293T cells was concentrated 10-fold by ultrafiltration (Vivaspin) and incubated overnight with anti-c-Myc-Sepharose at 4 °C. After washing of the beads with 20 mM Tris/HCl, pH 7.5, and 0.5 mM NaCl, the retained proteins were eluted with 100 mM triethanolamine at pH 12.0. N-linked glycans were removed by overnight incubation of 30 µl of the NPP2 fusions or melanoma NPP2 at 37 °C with 50 milliuunits of N-glycosidase F (10 units/ml; Roche Applied Science), 0.5% (v/v) Triton X-100, 1.3 mM phenylmethanesulphonyl fluoride, and 1.3 mM benzamidine, either in the absence (non-denaturing) or presence (denaturating) of 0.1% (v/v) SDS and 50 mM mercaptoethanol. For degly-
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cosylation with α-1,2-mannosidase (23) or endoglycosidase H (5 units/ml; Roche Applied Science), the NPP2 fusions or melanoma NPP2 were incubated overnight with 10 milliunits of the glycosidase in the presence of 20 mM sodium acetate at pH 5.5. For trypsinolysis, affinity-purified NPP2 fusions were incubated for 30 min at 30 °C with the indicated concentrations of trypsin. The treatment was stopped by boiling the samples for 5 min in the presence of 100 mM Tris, 4% (v/v) mercaptoethanol, 8% (w/v) SDS, 24% (v/v) glycerol, and 0.02% (w/v) Serva blue. Subsequently, the NPP2 fusions were visualized by immunoblotting.

Reverse Transcription PCR—RNA was isolated from NIH-3T3 cells using the Mammalian Total RNA extraction kit (Sigma). Following reverse transcription with the M-MuLV reverse transcriptase (Fermentas), the cDNA was PCR-amplified using primers for the LPA1/Edg-2 receptor with TTTCACCTCACTCTAGTCTGTGGCA being the forward and the reverse primer, respectively, and for the LPA2/Edg-4 receptor with TGGGCTACCTCTTGTCATAGTTC and ATTGACCAGTGAAGTTGCC as sense and antisense primer, respectively. The PCR product was separated on an agarose gel and visualized with ethidium bromide. Actin was used as a positive control. The specificity of the PCR reactions was evaluated by parallel incubations in the absence of reverse transcription polymerase.

Sequencing of the Glycan Chains—Determination of the glycan chains at position 524 was performed as described by Laroy et al. (24). Briefly, 10 μg of affinity-purified NPP2-m1,2,3,5 was denatured by incubation for 1 h at 50 °C with 50 μl of a buffer containing 8 m urea, 360 mM Tris at pH 8.6 and 3.2 mM EDTA. Subsequently, the protein was bound to the polyvinylidene difluoride membrane of a Multiscreen-IP plate (Millipore) by a gentle vacuum. Next, the bound protein was reduced and iodoalkylated, followed by the release of the glycan chains by addition of 1.25 IU MB (International Union of Biochemistry and Molecular Biology) milliunit of N-glycosidase F in 20 μl of 10 mM Tris acetate, pH 8.3, for 3 h at 37 °C. The glycan mixture was evaporated to dryness and a 1:1 mixture of 8-amino-1,3,6-pyrenetrisulfonic acid (Molecular Probes, Eugene, OR) in 1.2 M citric acid and 1 M NaCNBH3 in dimethyl sulfoxide was added. The derivatization was allowed for 18 h at 37 °C. After this, the reaction was quenched by the addition of 10 μl of deionized water. Excess 8-amino-1,3,6-pyrenetrisulfonic acid was removed using a bed of Sephadex G10 packed in a Multiscreen filter plate (Millipore). After sample application, the resin beds were eluted three times by addition of 10 μl of water and centrifugation for 10 s at 750 × g. The eluate was evaporated to dryness and reconstituted in 5 μl of deionized water. 0.4-μl aliquots of the cleaned-up derivatized glycan were incubated overnight at 37 °C in 2 μl of 10 mM sodium phosphate, pH 5.0, in the presence of Arthrobacter ureafaciens sialidase (2 units/ml; Roche Applied Science), jack bean β-N-acetylhexosaminidase, Diplodocus pneumoniae β-1-4-galactosidase (1 unit/ml; Roche Applied Science), jack bean β-N-acetylhexosaminidase (30 units/ml; Glyko), bovine epididymis α-fucosidase (0.5 units/ml; Glyko), almond fucosidase (3 milliunits/ml; Glyko), and recombinant Trichoderma reesei α-1,2-mannosidase, as indicated (23). Unit definitions are as specified by the enzyme suppliers. The native as well as the digested glycan chains were analyzed using an Applied Biosystems 377A DNA sequencer (PerkinElmer).

Modeling—The macromolecular model (Fig. 7A) was built with DeepView 3.7SP5 (25).

RESULTS

Mapping of the Glycosylation Sites of NPP2—Stracke et al. (26) showed that NPP2 binds to concanavalin A, which is initial evidence that it is an N-glycosylated protein. Consistent with this notion, we found that the deglycosylation of Myc-tagged rat NPP2 (Fig. 1A) or human teratocarcinoma NPP2 (not shown) with N-glycosidase F increased their mobility during SDS-PAGE. The same mobility shift was detected when the N-glycosidase F treatment was performed under non-denaturing conditions (Fig. 1A), enabling us to examine the effect of deglycosylation on the enzymatic activity of NPP2. Both the lysoosphospholipase-D activity and the nucleotide phosphodiesterase activity of rat (Fig. 1B) and human NPP2 (not shown) were largely abolished by deglycosylation. The remaining activity could be sedimented with concanavalin A-Sepharose (not illustrated), suggesting that it stemmed from incomplete deglycosylation. The above data indicated that the N-glycosylation of NPP2 is essential for the expression of its catalytic activity. The program NetGlyc (www.Cbs.dtu.dk/services/) predicts that rat NPP2 contains five potential N-glycosylation sites, namely Asn-53, Asn-398, Asn-410, Asn-524, and Asn-806, further denoted as sites 1, 2, 3, 4, and 5, respectively (Fig. 2A). These residues are
only the mutation of site 4 abolished the nucleotide and lysophospholipid phosphodiesterase activities of NPP2, showing that the glycosylation of Asn-524 is required for the expression of catalytic activity. Next, we used the reverse mutational approach and mutated either all or all but one of the five predicted N-glycosylation sites. Compared with the mobility of NPP2 with all five sites mutated, a decreased mobility during SDS-PAGE was only noted when sites 1, 3, or 4 were mutated to an Asn (Fig. 2B). This is consistent with the previous conclusion that these are the only N-glycosylated residues. Furthermore, of the latter mutants only the one with an intact site 4 was catalytically active (Fig. 2B), confirming that only the glycosylation of this site is required for the expression of catalytic activity. It is also worth noting that the mutation of site 4 caused an accumulation of NPP2 in the cells (Fig. 2C), amounting to 160 ± 3% (n = 3) of the level of NPP2-WT, suggesting that the glycosylation of Asn-524 also contributes to the maturation and/or trafficking of NPP2. However, this represents a relatively minor effect because the accumulation of NPP2 in the culture medium, which contains more than 95% of the total pool of NPP2, was at most marginally (86 ± 24% of the level of NPP2-WT; n = 3) affected by mutation of site 4, as detected by immunoblotting (Fig. 2C). A combination of the mutation of site 4 with that of three other sites also resulted in an increased cellular retention of NPP2, except for NPP2-m1,2,3,4, which, however, also accumulated somewhat less in the medium, indicating that it represents a mutant that is less well expressed (Fig. 2C).

Next, we examined whether the N-glycosylation of NPP2 is required for its cell motility-stimulating capability. Because the latter activity of NPP2 is mainly mediated by the LPA₁ receptor, and to a lesser extent by the LPA₂ receptor (5), we first identified cells that express these LPA receptors. Consistent with a report by Fischer et al. (27), we found by reverse transcription PCR that NIH-3T3 fibroblasts express both the LPA₁ and LPA₂ receptor (not illustrated), making these cells suitable for our motility studies. Subsequently, we have used affinity-purified NPP2 (mutants) as chemoattractants for NIH-3T3 cells in Boyden chambers (Fig. 3, A and B). Although NPP2-WT, NPP2-N53A, and NPP2-N410A were equally efficient chemoattractants, NPP2 with sites 1–5 mutated (NPP2-(m1–5)) and NPP2-N524A did not measurably stimulate cell motility, demonstrating that the N-glycosylation of Asn-524 is essential for NPP2 to act as a chemoattractant.

**Asn-524 of NPP2 Contains an Oligomannosidic Side Chain—**
To examine the structure of the essential glycan side chain of site 4, we used affinity-purified NPP2-m1,2,3,5, which is only glycosylated on Asn-524 (site 4). After an incubation of this NPP2 mutant with N-glycosidase F, the released glycans were labeled fluorescently and analyzed by DNA sequencing equipment (24). This analysis revealed that the native glycans had the structures Man₅GlcNAc₂ and Man₆GlcNAc₂ (Fig. 4A, panel 2), which were also detected in the reference protein RNase B (panel 10). Additional glycan structures were released by the N-glycosidase F treatment of the NPP2-m1,2,3,5 preparations, but these could be shown to be derived from the monoclonal antibodies that were used for purification and are minor contaminants of the NPP2 preparation, and sialylated glycans on the first of the motif NXS/T, where X can be any residue and the last residue can be either Ser or Thr. Of the predicted glycosylation sites, all but site 2 are conserved in human and mouse NPP2 (not illustrated). To examine which of the predicted glycosylation sites of rat NPP2 were actually glycosylated, we first mutated each of the corresponding asparagines individually into an alanine and examined the effects of these mutations on the mobility of NPP2 during SDS-PAGE and on its enzymatic activities (Fig. 2B). An increased mobility during SDS-PAGE was only noted following the mutation of sites 1, 3, and 4, indicating that these are the only true glycosylation sites. Moreover,
contaminating proteins deriving from the culture medium (panel 11 and not shown). The identity of the Asn-524-linked glycan as Man\textsubscript{8}GlcNAc\textsubscript{2} and Man\textsubscript{9}GlcNAc\textsubscript{2} was confirmed by the sensitivity of these glycans to digestion by /H9251-1,2-mannosidase (panel 4).

As a final line of evidence for the presence of an oligomannosidic side chain on Asn-524, Fig. 5A shows that treatment of affinity-purified NPP2-m1,2,3,5 from the conditioned medium of HEK293T cells with the hybrid/high mannose-specific endoglycosidase H or with N-glycosidase F resulted in an increased mobility during SDS-PAGE and a highly deficient catalytic activity. Again, the remaining activity could be sedimented by incubation with concanavalin A-Sepharose (not shown), indicating that this activity was derived from incompletely deglycosylated NPP2. To rule out the possibility that the essential side chain is an artifact resulting from the transient overexpression of NPP2, the N-glycosidase F treatment was
repeated with the conditioned medium of NPP2-secreting A2058 melanoma cells (18). Both the nucleotide phosphodiesterase activity and the lysophospholipase-D activity were nearly completely abolished after enzymatic deglycosylation (Fig. 5B), and the remaining activity was retained by concanavalin A-Sepharose (not shown).

The Asn-524-linked Glycan Chain Is a Structural Determinant of NPP2—Oligomannosidic glycans are usually transiently present on glycoproteins in the endoplasmic reticulum before they get further processed in the Golgi to complex or hybrid-type glycans (28). The presence of an oligomannosidic side chain in a mature mammalian glycoprotein indicates that this chain might contribute to the structure of the protein by forming non-covalent contacts with surrounding residues. As a result, this side chain may no longer be accessible for the processing enzymes in the Golgi. That this applies to the glycan chain on Asn-524 of NPP2 was initially suggested by the observation that treatment of NPP2-m1,2,3,5 with \( \alpha\)-1,2-mannosidase affected neither the mobility during SDS-PAGE nor the catalytic activity of this mutant (Fig. 5A), indicating that the oligomannose side chain on Asn-524 is not accessible to this glycosidase. The same results were obtained for melanoma NPP2 (Fig. 5B).

In further agreement with the notion that the glycan chain on Asn-524 is a structural determinant, we found that NPP2-N524A was degraded by trypsin concentrations that were \( \sim 10 \) times lower than those needed for the degradation of wild-type NPP2 (Fig. 6A). Interestingly, an increased sensitivity to trypsin was not seen when the other N-glycosylation sites of NPP2 were mutated, as in NPP2-m1,2,3,5, indicating that these sites do not contribute to the structure of NPP2. We also noted that the mutation of site 4 (Asn-524), unlike the mutation of sites 1, 2, 3, and/or 5, resulted in an increased mobility of NPP2 during Blue Native PAGE (Fig. 6B). Because this mobility is determined by the extent of binding of the negatively charged Coomassie Blue stain (22), which is affected by the surface to volume ratio of the protein, these results suggest that NPP2-N524A has a less compact structure than the wild-type protein, accounting for the better binding of Coomassie Blue and the increased electrophoretic mobility.
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**A.**

**DISCUSSION**

Glycosylation of NPP2 on Asn-524 Is Functionally Important—Glycosylation is an important post-translational modification that can affect the intracellular trafficking, stability, and/or function of glycoproteins. We show here that NPP2 is N-glycosylated on three sites, two of which were mapped to the catalytic domain (Figs. 1 and 2). Of these, only the glycosylation of Asn-524 appears to contribute to some extent to the trafficking of NPP2 (Fig. 2C). Similarly, the routing of NPP3 (30) and NPP7 (19) to the plasma membrane also appears to be controlled by N-glycosylation. Mutagenesis studies revealed that the Asn-524-linked glycan of NPP2 is also required for the expression of its lysophospholipase-D as well as for its nucleotide phosphodiesterase activities (Fig. 2B). Because the lysophospholipase-D activity of NPP2 mediates most, if not all, of its biological functions, it comes as no surprise that the Asn-524 glycan was also required for the cell motility-stimulating activity of NPP2 (Fig. 3). It is important to note that mature NPP2 (Fig. 1A) and NPP2-m1,2,3,5 (Fig. 5A) are also inactivated by enzymatic N-deglycosylation, indicating that the Asn-524-linked glycan is required to maintain NPP2 in an active conformation. These data do not rule out, however, that this glycan chain also contributes to the folding of NPP2 during maturation. NPP7 is also inactivated by deglycosylation or mutagenesis of N-glycosylation sites (19). However, the activity of NPP7 is regulated by multiple N-glycosylation sites and none of these corresponds to Asn-524 of NPP2. Our results are at variance with an earlier study that did not find an effect of pretreatment of NPP2 with N-glycosidase F on its motility-stimulating activity (18). However, no dose-response curves were done in the latter study, and it is therefore possible that effects of N-deglycosylation were missed because the motility-stimulating effects were measured at supersaturating concentrations.

**Structure and Function of the Asn-524-linked Glycan**—We have identified the Asn-524-linked glycan chain by classical sequence analysis, before and after treatment with a host of endoglycosidases, as Man$_{7-9}$GlcNAc$_{2}$ by exoglycosidase array sequencing (Fig. 4). Interestingly, NPP5 from brain membranes is retained by the mannose-specific Galanthus nivalis lectin, suggesting that it may also contain an oligomannosidonic glycan chain (20).

High mannose glycan chains are relatively rare in mature eukaryotic proteins because they are usually processed in the Golgi to complex or hybrid glycan chains. Nevertheless, there are some proteins known to contain this type of immature glycan chain, including the neural adhesion molecule L1 (31), a neural glycoprotein (32), and the β-subunit of rabbit gastric H,K-ATPase (33). The presence of some Man$_{7-9}$GlcNAc$_{2}$, but not smaller high mannose glycans, in mature NPP2 is suggestive for partial trimming by the endoplasmic reticulum mannosidase I without further processing by the Golgi mannosidases (33). We suggest that the Asn-524-linked glycan of NPP2 does not undergo additional processing in the Golgi because it is no longer accessible, probably because it is a structural determinant of NPP2 and becomes (partially) buried during the folding of NPP2. Consistent with this view we find that this glycan is accessible for hydrolysis by N-glycosidase F and endoglycosidase H but not by α-1,2-mannosidase (Fig. 5A), indicating that the non-reducing termini of the glycan are shielded.

It is intriguing that Asn-524 is mapped to the hinge region between the catalytic and nuclease-like domains (Fig. 2A). It is
therefore tempting to speculate that the Asn-524-linked glycan stabilizes the interaction between these two domains, which may be essential for the expression of catalytic activity. In accordance with this interpretation we found that NPP2 showed an increased sensitivity to trypsinolysis following the mutation of Asn-524 (Fig. 6A). In contrast, the mutation of sites 1, 2, 3, and 5 did not have such an effect, suggesting that the N-glycans of NPP2 do not merely sterically protect trypsin-susceptible sites. Likewise, only the mutation of Asn-524 increased the mobility of NPP2 in Blue Native PAGE, indicating that it causes a major conformational change. This mobility shift was reversed by the addition of metals.

We noted that EDTA also increases the mobility of NPP2 during Blue-Native PAGE, indicating that it causes a major conformational change. This mobility shift was reversed by the addition of metals. These data add further support to our proposal that the Asn-524 moiety on Asn-524, a site that is phylogenetically conserved, not only in NPP2 but also in six of seven NPP isozymes. We suggest that this glycan chain has a structural function and is involved in the interaction between the catalytic and nuclease-like domains of NPP2.

Note Added in Proof—Following the acceptance of our manuscript we have found that the removal of the essential glycan chain at Asn-524 of NPP2 by N-glycosidase F and endoglycosidase H requires the interaction between the catalytic and nuclease-like domains of NPP2.

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REFERENCES

1. Moolenaar, W. H., van Meeteren, L. A., and Giepmans, B. N. (2004) BioEssays 26, 870–881.
2. Stefan, C., Jansen, S., and Bollen, M. (2005) Trends Biochem. Sci. 30, 542–550.
3. Tokumura, A., Majima, E., Kariya, Y., Tominaga, K., Kogure, K., Yasuda, W., Atlas, A., Coons, S. W., and Berens, M. E. (2005) Neoplasia 7, 7–16.
4. Fox, M. A., Colello, R. J., Macklin, W. B., and Fuss, B. (2003) Mol. Cell. Neurosci. 23, 507–519.
5. Fox, M. A., Alexander, J. K., Afschari, F. S., Colello, R. J., and Fuss, B. (2004) Mol. Cell. Neurosci. 27, 140–150.
6. Gijsbers, R., Ceulemans, H., Stalmans, W., and Bollen, M. (2001) J. Biol. Chem. 276, 1361–1368.
7. Gijsbers, R., Ceulemans, H., and Bollen, M. (2003) Biochem. J. 371, 321–330.
8. Jansen, S., Stefan, C., Creemers, J. W. M., Waelkens, E., Van Eynde, A., Stalmans, W., and Bollen, M. (2005) J. Cell Sci. 118, 3081–3089.
9. Koike, S., Keino-Masu, K., Ohito, T., and Masu, M. (2006) Genes Cells 11, 133–142.
10. van Meeteren, L. A., Ruurs, P., Christodoulou, E., Goding, J. W., Takakusa, H., Kikuchi, K., Perrakis, A., Nagano, T., and Moolenaar, W. H. (2005) J. Biol. Chem. 280, 21155–21161.
11. Stracke, M. L., Árestad, A., Levine, M., Krutzsch, H. C., and Liotta, L. A. (1995) Melanoma Res. 5, 203–209.
12. Wu, J., Hansen, G. H., Nilsson, Å., and Duan, R. (2005) Biochem. J. 386, 153–160.
13. Ohe, Y., Ohnishi, H., Okazawa, H., Tomizawa, K., Kobayashi, H., Okawa, K., and Matozaki, T. (2003) Biochem. Biophys. Res. Commun. 308, 719–725.
14. Cimpean, A., Stefan, C., Gijsbers, R., Stalmans, W., and Bollen, M. (2004) Biochem. J. 381, 71–77.
15. Schägger, H., and von Jagow, G. (1991) Anal. Biochem. 199, 223–231.
16. Moras, M., Callewaert, N., Pius, K., Claeysens, M., Martinet, W., Dewaele, S., Contreras, H., Dewiere, I., Penttila, M., and Contreras, R. (2000) J. Biotechnol. 77, 255–263.
17. Leroy, W., Contreras, R., and Callewaert, N. (2006) Nat. Protocols 1, 397–405.
18. Guex, N., and Peitsch, M. C. (1997) Electrophoresis 18, 2714–2723.
19. Stracke, M. L., Krutzsch, H. C., Unsworth, E. J., Árestad, A., Cioce, V., Schifflmann, E., and Liotta, L. A. (1992) J. Biol. Chem. 267, 2524–2529.
20. Fischer, D. J., Nusser, N., Viraq, T., Yokoyama, K., Wang, D., Baker, D. L., Bautista, D., Parrill, A. L., and Tsigy, G. (2001) Mol. Pharmacol. 60, 776–784.
21. Roth, J. (2002) Chem. Rev. 102, 285–303.
22. Zalatan, J. G., Fenn, T. D., Brunger, A. T., and Herschlag, D. (2006) Biochemistry 45, 9788–9803.
23. Meerson, N. R., Bello, V., Delauney, I., Slimane, T. A., Delauntier, D., Lenoir, C., Trugnan, G., and Maurice, M. (2000) J. Cell Sci. 113, 4193–4202.
24. Horstkorte, R., Schachner, M., Magyar, J. P., Vorherr, T., and Schmitz, B. (1993) J. Cell Biol. 121, 1409–1421.
25. Clark, R. A. C., Gurd, J. W., Bissonoon, N., Trucad, N., Molnar, E., Zamze, S. E., Dwek, R. A., McIlhinney, R. A. J., and Wing, D. R. (1998) J. Neurochem. 70, 2594–2605.
26. Tyagarajan, K., Lipniunas, P. H., Townsend, R. R., and Forte, J. G. (1997) Biochemistry 36, 10200–10212.
27. Skipper, R., and DeStephano, D. (1989) J. Histotechnol. 12, 303.