Ataxin-10 Interacts with O-Linked β-N-Acetylglucosamine Transferase in the Brain*

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Modification by O-GlcNAc involves a growing number of eucaryotic nuclear and cytosolic proteins. Glycosylation of intracellular proteins is a dynamic process that in several cases competes with and acts as a reciprocal modification system to phosphorylation. O-Linked β-N-acetylglucosamine transferase (OGT) levels are highest in the brain, and neurodegenerative disorders such as Alzheimer disease have been shown to involve abnormally phosphorylated key proteins, probably as a result of hypoglycosylation. Here, we show that the neurodegenerative disease protein ataxin-10 (Atx-10) is associated with cytoplasmic OGT p110 in the brain. In PC12 cells and pancreas, this association is competed by the shorter OGT p78 splice form, which is down-regulated in brain. Overexpression of Atx-10 in PC12 cells resulted in the reconstitution of the Atx-10-OGT p110 complex and enhanced intracellular glycosylation activity. Moreover, in an in vitro enzyme assay using PC12 cell extracts, Atx-10 increased OGT activity 2-fold. These data indicate that Atx-10 might be essential for the maintenance of a critical intracellular glycosylation level and homeostasis in the brain.

Since its discovery (1), the modification of intracellular proteins by a single GlcNAc moiety has emerged as a major signaling event involving a growing number of proteins (2, 3). Changes in O-GlcNAc glycosylation levels were shown to have a regulatory effect on diverse cellular processes such as proteasome activity (4), transcription (5), and enzyme function (6). The enzyme OGT2 is encoded by a single gene, which is highly conserved among metazoans (7). Several splice forms have been described that vary in the length of the protein N terminus and subcellular localization (8). Full-length OGT is a 110-kDa polypeptide consisting of two domains. The N-terminal half contains multiple tetratricopeptide repeats (TPR) that adopt a bent superhelical fold related to the armadillo repeat motif (9). The C-terminal portion shows glycosyltransferase activity, whereas the TPR domain is responsible for substrate binding and OGT oligomerization (9–11). Originally, the enzyme was isolated from rat liver cytosol as an apparent heterotrimer consisting of two p110 subunits and one p78 subunit (12). The p110 subunit, which exerts full catalytic activity by itself, is ubiquitously expressed, whereas the p78 splice variant that lacks most of the TPR repeats appears to be restricted to certain tissues such as liver, kidney, and muscle (13).

Dysregulated OGT activity leading to hyper- or hypoglycosylation of target proteins is believed to be involved in the pathogenesis of disorders such as type II diabetes (6, 14) and Alzheimer disease (15). Protein glycosylation with GlcNAc may directly compete for serine and threonine residues with phosphorylation, thus creating a sensitive balance between positive and negative regulatory signals (16). Perturbations of this reciprocal relationship might lead to cell degeneration as in the case of hyperphosphorylated tau (17, 18). It has become evident from several observations that O-GlcNAc glycosylation is exceptionally important in neuronal tissues (17, 19). OGT expression is significantly up-regulated in the brain with the most prominent activity being localized in the cerebellar cortex (20). Interestingly, cerebellar neurons have been shown to react directly to changes of protein kinase activity with reciprocal changes in OGT activity (21).

Here, we demonstrate binding of OGT p110 to the neurodegenerative disease protein Atx-10 in the brain. Although ubiquitously expressed, both proteins show highest expression levels in the central nervous system, indicating a crucial regulatory effect of Atx-10 on intracellular glycosylation in the brain.

EXPERIMENTAL PROCEDURES

Isolation of OGT-interacting Proteins Using the Cytotrap Screen—Atx10 was isolated as an OGT-interacting protein using the CytotoTrap™ system according to the manufacturer’s protocols (Stratagene) (22, 23). For this purpose, the mitochondrial version of human OGT (7) was subcloned into the pSos
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vector (Stratagene) in-frame with human Sos protein. The screen was carried out with a mouse insulinoma (MIN6) cDNA library in the pMrY vector (Stratagene) (23, 24). After screening of about 1 × 10⁶ colonies, Atx10 was isolated as an OGT-interacting protein in three independent experiments.

Cell Culture and Transfection—Rat pheochromocytoma PC12 cells were obtained from ATCC (ATCC number: CRL-1721) and maintained as monolayer cultures in Dulbecco’s modified Eagle’s medium at 5% CO₂ in a humidified atmosphere at 37 °C. The medium was changed every 3 days, and cells were subcultured once a week. Transient transfection of PC12 cells with a pCDM8-Atx-10 expression plasmid was performed according to the manufacturer’s instructions using Lipofectamine 2000 (Invitrogen). On day 1 after transfection, a syringe filter. Conditioned medium was then purified using Lipofectamine 2000 (Invitrogen). On day 1 after transfection, increasing concentrations of UDP-GlcNAc substrate were added to the cells for 2 h.

Preparation and Gel Filtration Analysis of PC12 Cell and Rat Tissue Lysates—3 × 10⁷ PC12 cells were resuspended in 1 ml of phosphate-buffered saline containing protease inhibitor mix (Roche Applied Science). Cells were lysed on ice by passing several times through a 0.6-gauge syringe needle. Cell debris was removed by centrifugation, and the cytosolic fraction was passed through a 0.45-μm filter before being submitted to gel filtration. Gel filtration was performed using a prepacked Superdex S75 column (Amersham Biosciences) equilibrated with lysis buffer. 0.3 ml-fractions were collected and analyzed by Western blotting.

Preparation and Gel Filtration Analysis of PC12 Cell and Rat Tissue Lysates—For detection of Atx-10, a polyclonal antibody (Sigma) was used for the loading control. All antibodies modified by O-GlcNAc were detected using a RL2 monoclonal antibody to GlcNAc (Alexis Corp.). Monoclonal anti-β-actin antibody (Sigma) was used for the loading control. All antibodies were applied at 1:1000 dilution. For detection of primary antibodies, horseradish peroxidase-conjugated secondary antibodies were applied at 1:4000 dilution. Densitometric analysis of bands was performed using the Gel-Pro Express software (Media Cybernetics).

Recombinant Expression of Proteins—Recombinant rat Atx-10 was expressed in HEK293 cells as described previously (25). Supernatants of stably transfected cells were harvested frequently until the cells detached, pooled, and passed through a syringe filter. Conditioned medium was then purified using nickel-NTA agarose chromatography according to the manufacturer’s instructions (Qiagen). GST and OGT-GST fusion protein were expressed in Escherichia coli BL21 (DE3) using a pGex-2T vector. Cells were transformed and cultured in LB medium at 37 °C, and expression was induced at OD 0.6 by adding isopropyl-β-D-thiogalactopyranoside (0.4 mM) for 4–6 h. Cells were harvested and resuspended in Tris/HCl, pH 7.4, 2% Triton and lysed by several freeze-thaw steps and subsequent sonication. GST-OGT fusion protein was purified using glutathione-Sepharose according to the manufacturer’s instructions (Amersham Biosciences). The TPR6–7 fragment was generated by PCR using 5’-GCTCTAGACATATTTCGAGTAGCTTTGG-3’ as forward and 5’-CCGGATCTCCCTCATGCGATTGAGACTC-3’ as reverse primer and expressed together with an N-terminal polyhistidine tag in E. coli BL21 (DE3) cells using a pET19b vector (Novagen).

After expression, inclusion bodies containing TPR6–7 protein were dissolved in 6 M guanidine hydrochloride and purified under denaturing conditions using nickel-agarose chromatography. The purified His-tagged TPR6–7 protein with a molecular mass of 12 kDa was refolded while bound to the agarose by changing the buffer to physiological conditions (50 mM Tris/HCl, pH 8.0, 150 mM NaCl). The eluted protein was finally dialyzed against 50 mM Tris/HCl, pH 8.0, 150 mM NaCl.

Immunohistochemistry—C57BL/6J mice, 20–30 g in body weight (n = 6), were used. Animals were deeply anesthetized with pentobarbital (Vetanarcol®, 0.04 g/kg, applied intraperitoneally), and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The dissected brains were immersed in fixative overnight. Serial sagittal 30-μm sections were cut with a vibratome and collected in cold Tris-phosphate-buffer (0.05 M). Free-floating sections were washed thoroughly and permeabilized with 0.4% Triton X-100 in 0.1 M phosphate-buffered saline for 90 min. Nonspecific binding was blocked with normal goat serum for 2 h, and then sections were incubated with Atx-10 antibody (rabbit, 1:250 or 1:500) or OGT antibody (1:400) for 48 h at room temperature. Biotinylated goat-anti rabbit IgG (1:100; Antibodies Inc.) and the avidin-biotin complex (Vector) were applied for 90 min each. Immunoreaction was visualized with 0.05% diaminobenzidine and 0.1% H₂O₂ in Tris-phosphate-buffer. All sections were mounted on coated slides, dehydrated in graded ethanol, cleared with xylol, and coverslipped in Eukitt (Kindler, Freiburg, Germany). For control, the first antibody was omitted from the immunoreaction. Sections were viewed with an Axioskop microscope equipped with an Axiovision imaging system (Zeiss, Germany).

Immunoprecipitation—Lysates of PC12 cells and rat brain were generated as for the gel filtration experiments. After adjusting total protein concentrations, lysates were incubated for 4 h at 4 °C with an excess (10 μg) of recombinant Atx-10 protein in a total volume of 200 μl. Recombinant Atx-10 was precipitated from the mixture using Ni-NTA agarose. The precipitates were washed three times with phosphate-buffered saline and resuspended in SDS-PAGE sample buffer. Co-precipitated OGT protein was detected by Western blotting using OGT antibody. Recombinant Atx-10 was precipitated with a GST-OGT fusion protein bound to...
glutathione-Sepharose. As control, recombinant Atx-10 was incubated with GST alone.

**Mass Spectrometry**—Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis was performed in the linear, positive ion mode with blanking (<700 m/z) and pulsed (time-delayed) extraction using a Shimadzu Biotech Axima TOF² instrument (Shimadzu Biotech Deutschland, Duisburg, Germany). All reagents and protein standards were purchased from Sigma (Deisenhofen, Germany). Sinapinic acid (10 mg/ml in 50% acetonitrile, 50% 0.1% trifluoroacetic acid (all % v/v)) was used as a matrix. Sample positions on the steel 384-position sample plate were washed once with matrix solution. A 1 µl drop of the TPR6–7 preparation was allowed to dry onto the sample plate at room temperature, and the TPR6–7 preparation was then washed with a small drop of 0.1% trifluoroacetic acid, applied for ~10 s. Immediately after removal of the trifluoroacetic acid, 1 µl of matrix solution was added to the sample, and it was allowed to dry at room temperature. The standard proteins (about 1 pmol of insulin, ubiquitin, cytochrome C, myoglobin, aldolase, or β-galactosidase) were spotted onto the washed plate, and an equal volume (usually 1 µl) of matrix was immediately added to the protein drop. Each protein standard was analyzed separately, and a combined calibration of the near external standards was employed to determine the mass/charge (m/z) values.

**OGT Assay**—The OGT assay was essentially performed as described by Marshall et al. (26). 10 µl of an OGT peak fraction (0.3 mg/ml) obtained by gel filtration of PC12 cell lysate was mixed with 100 µM of casein kinase (CKII) peptide (PGGSTPVSSANMM), and the reaction was started with 20 µCi of [3H]UDP-GlcNac (PerkinElmer Life Sciences) in a final volume of 50 µl. For testing the influence of Atx-10, different concentrations of recombinant Atx-10 were added before starting the reactions. The samples were incubated at room temperature, and the reaction was stopped by adding trichloroacetic acid. [3H]Labeled glycosylated CKII peptide was separated from free [3H]UDP-GlcNac by precipitation. The pellets were washed once with ice-cold trichloroacetic acid, resuspended in scintillation solution, and counted in a liquid scintillation counter.
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RESULTS

OGT p110 Is Associated with Atx-10 in the Brain but Not in PC12 Cells—Using the yeast cytotrap screen assay with the mitochondrial splice form of human OGT as bait, we have isolated the SCA10 gene product, Atx-10, as an interacting partner of OGT from a mouse insulinoma (MIN6) cDNA library. In rat brain cytosol, Atx-10 co-eluted with the OGT p110 subunit during gel filtration (Fig. 1A) with the peak fractions lying in a molecular mass range of about 200–300 kDa. We did not detect any dissociated OGT and Atx-10 signals in this experiment, indicating that Atx-10 binding to OGT p110 is constitutive in the brain. Recombinant Atx-10 could be co-precipitated by a GST-OGT fusion protein confirming our in vivo results (Fig. 1B). Surprisingly, in cytosol of PC12 cells, Atx-10 was not associated with OGT and eluted in a lower molecular mass region (about 150–200 kDa) (Fig. 1D), which corresponds to its homotrimeric state observed in previous experiments (25). Instead, OGT p110 co-eluted with the OGT p78 subunit, which was not detectable in brain cell lysates, and apparently prevented binding of Atx-10 to OGT p110 in PC12 cells. Co-precipitation of OGT p110 from cell lysates using recombinant Atx-10 showed higher binding activity for brain than for PC12 cell lysate (Fig. 1C), confirming the stronger association between the two OGT isoforms as compared with the OGT p110-Atx-10 binding.

Immunocytochemical Detection of Atx-10 and OGT in Mouse Brain—Colocalization of Atx-10 and OGT in neuronal tissues was also confirmed by immunocytochemistry using mouse brain sections (Fig. 2). As described previously for mouse and human brain (25), Atx-10 protein is present in the cerebellar system, i.e. the cerebellar cortex and the inner cerebellar nuclei (Fig. 2A), the inferior olive (Fig. 2E), and the pontine nuclei (not shown). OGT staining coincided with that of Atx-10 in the cerebellum (Fig. 2B), in the inferior olive (Fig. 2H), and in the pontine nuclei (not shown) with a few exceptions. In the cerebellar cortex, low levels of Atx-10 were outlining the perikarya of Purkinje cells and their dendrites (Fig. 2C). OGT protein accumulated in the soma of the Purkinje cells. In addition, inhibitory interneurons of the molecular layer labeled for OGT (Fig. 2D) but not for Atx-10, whereas Golgi cells in the granule cell layer were positive for both markers. Neurons in the cerebellar nuclei (Fig. 2, E and F) and in afferent cerebellar projection areas, as exemplified here for the inferior olive (Fig. 2, G and H), stained for Atx-10 or OGT in a similar manner. However, intracellular distribution was different in that the rather homogenous cytosolic distribution of Atx-10 was contrasted by a preferential perinuclear accumulation of OGT.

Expression of OGT Isoforms and Atx-10 in Different Rat Organs—To analyze how Atx-10, OGT p110, and OGT p78 were distributed in other tissues as compared with brain, we looked for the respective protein levels in multiple rat organs. Both OGT p110 and Atx-10 were expressed ubiquitously, although markedly up-regulated in the brain (Fig. 3, A and B). Significant expression levels of OGT p78 were restricted to liver, spleen and, most prominently, to pancreas (Fig. 3C). A basal OGT p78 signal could be detected in all organs by longer film exposition, indicating that its expression is ubiquitous, too, but generally down-regulated (not shown). Expression levels of Atx-10 protein are highest in brain and testis (Fig. 3D) and reflect the pattern of the mRNA transcript as described recently (25). Other OGT splice forms than p110 or p78 could not be detected in this experiment, probably due to their lesser abun-
dance and localization to subcellular organelles. Gel filtration of pancreas tissue cytosol showed a similar result as that of PC12 cell cytosol, confirming that the presence of OGT p78 prevents OGT p110-Atx-10 association (Fig. 3E). Interestingly, in pancreas, the cytoplasmic concentration of OGT p78 even exceeded that of OGT p110.

OGT p78 and Atx-10 Compete for OGT p110 Binding—To verify whether OGT p78 directly competes with Atx-10 for OGT p110 binding, we incubated PC12 cell cytosol with an excess of recombinant Atx-10 protein (−5 μM) prior to gel filtration. As shown in Fig. 4A, recombinant Atx-10 protein applied at high concentrations inhibited OGT p78/p110 association (compare Fig. 1D) by displacing OGT p78 and binding to OGT p110. As the cytosolic concentration of OGT p78 does not apparently exceed that of endogenous Atx-10 in PC12 cells, we conclude that OGT p110 binds to OGT p78 with a significantly higher affinity than to Atx-10. Jinek et al. (9) have demonstrated that the TPR repeats 6 and 7 function as the dimer contact region of OGT. As Atx-10 and the OGT TPR domain share a common structural motif, we speculated that Atx-10 might associate via the TPR6–7 region with OGT. We have expressed the TPR6–7 fragment in E. coli to analyze whether it was able to compete the Atx-10-OGT p110 interaction. MALDI-TOF analysis of the TPR6–7 preparation showed two major peaks: one at an m/z of 10,555 and the other at m/z of 21,100. The m/z value of 10,555 is smaller than that expected for the full-length TPR6–7 protein (12, 25), which is suggestive of proteolysis during purification. As laser power was incrementally increased, the first peak to appear above background was the 21,100 m/z peak followed by the 10,555 m/z peak. At higher laser powers, the 10,555 peak predominated. These observations confirm that the TPR6–7 protein forms stable dimers in solution as predicted by the structural data (9). Incubation of rat brain lysate with an excess of recombinant TPR6–7 protein (−20 μM) led to a clear dissociation of the Atx-10 and OGT p110 signals during gel filtration, indicating that Atx-10 uses the homo-oligomerization domain of OGT p110 for binding (Fig. 4B). The TPR6–7 protein was detected in fractions containing both OGT p110 and Atx-10, indicating that it associated with both proteins. The same experiment performed with PC12 cell lysate led to the formation of a second OGT p110/p78 peak in a lower molecular mass region (as compared with Fig. 1D) but not to a dissociation of the complex (Fig. 4C). This result suggests that the TPR6–7 fragment does not compete with OGT p78 for binding sites and that it can associate with OGT p110 in addition to OGT p78, probably by competing homo-oligomerization domain of OGT p110 for binding (Fig. 4B). The TPR6–7 protein was detectable in this experiment throughout the OGT and Atx-10 peaks with some signal being found also in the void volume of the column (not shown), indicative of self-aggregation.

Influence of Atx-10 Binding on Glycosyltransferase Activity—How does binding of OGT p78 or Atx-10 influence the enzymatic activity of OGT p110? As the expression levels of OGT

![Image](image-url)
p110 and Atx-10 are markedly up-regulated in the brain, we assumed that the OGT p110-Atx-10 complex corresponds to high glycosyltransferase activity, whereas dissociation of this interaction by OGT p78 might induce a down-regulation of intracellular glycosylation. To answer this question, we transfected PC12 cells with a cytoplasmic expression vector for Atx-10 and monitored intracellular glycosylation in the presence of different UDP-GlcNAc substrate concentrations. As control, cells were incubated without UDP-GlcNAc. Cells lysates were submitted to Western blot analysis using RL2 antibody. Note that the upper parts (>82 kDa) of both blots were exposed and developed separately to prevent overexposition of the lower parts.

DISCUSSION

Nucleocytoplasmic modification with O-GlcNAc is an abundant and dynamic process involving a growing number of target proteins (19). Unlike N- and O-linked glycosylation in the
endoplasmic reticulum and Golgi apparatus, intracellular glycosylation is uniform and may directly change the functional state of a protein by competing with phosphorylation for the same serine and threonine side chains (27). Furthermore, it is catalyzed by the activity of a single enzyme, OGT, which shows a broad substrate spectrum (9) and thus transmits extracellular stress and nutrient signals to diverse cellular processes. OGT p110 has been shown to reflect the main regulatory organ for glucose metabolism, exhibits the most pronounced OGT p78 expression level. Furthermore, our data suggest that reconstitution of the OGT p110-Atx-10 complex in a cell type with high OGT p78 expression restores the capacity to respond to higher levels of UDP-GlcNac. On the kinetic level, Atx-10 induced a 2-fold increase of OGT activity at saturating concentrations. Interestingly, Kreppel and Hart (11) have shown that the truncated 2-TPR domain of OGT p78, although still being capable of competing for UDP-GlcNac binding by the catalytic domain, does not allow for efficient substrate processing.

Our study demonstrates that Atx-10 and the OGT p78 splice form are competitors for OGT p110 binding. Atx-10 binding to OGT p110 appears to be constitutive in tissues where OGT p78 is absent, whereas OGT p78 binding to OGT p110 is constitutive in tissues even in the presence of Atx-10. Structural analysis and competition assays suggest a heterodimer formation of OGT p110 and Atx-10 by mimicry of OGT p110 homodimerization. As Atx-10 shows a similar structural motif as the OGT TPR domain, it may be discussed that Atx-10 serves as an adapter molecule, which is able to alter and broaden the substrate binding groove in the concave surface of the superhelix, whereas the dimerization site lies at the convex surface (9). Several groups (11, 31) have shown that the removal of a significant number of TPR repeats (>5) dramatically decreases glycosylation efficiency. This implies that the truncated 2-TPR domain of OGT p78, although still being capable of competing for UDP-GlcNAc binding by the catalytic domain, does not allow for efficient substrate processing. In a recent publication, Lazarus et al. (32) have shown that recombinantly expressed OGT p78 does not show any glycosylation activity. We therefore speculate that OGT p78 expression might correspond to a negative regulatory signal, probably in response to high intracellular glucose levels in the respective tissues. This assumption is strengthened by the fact that neuronal tissues, which show the highest levels of OGT activity, lack OGT p78 expression, whereas pancreas, a regulatory organ for glucose metabolism, exhibits the most pronounced OGT p78 expression level. Furthermore, our data suggest that reconstitution of the OGT p110-Atx-10 complex in a cell type with high OGT p78 expression restores the capacity to respond to higher levels of UDP-GlcNAc. On the kinetic level, Atx-10 induced a 2-fold increase of OGT activity at saturating concentrations. Interestingly, Kreppel and Hart (11) have reported that different subunit compositions can modulate the transferase activity of OGT p110.

Our study demonstrates that Atx-10 and the OGT p78 splice form are competitors for OGT p110 binding. Atx-10 binding to OGT p110 appears to be constitutive in tissues where OGT p78 is absent, whereas OGT p78 binding to OGT p110 is constitutive in tissues even in the presence of Atx-10. Structural analysis and competition assays suggest a heterodimer formation of OGT p110 and Atx-10 by mimicry of OGT p110 homodimerization. As Atx-10 shows a similar structural motif as the OGT TPR domain, it may be discussed that Atx-10 serves as an adapter molecule, which is able to alter and broaden the substrate spectrum of the enzyme. A dysregulated intracellular glycosylation resulting from a mutated SCA10 gene would therefore offer an explanation for the selective neurodegeneration of cerebellar cells in SCA10 patients.
sion by RNA interference, although inducing apoptosis, did not significantly alter intracellular glycosylation levels in primary cerebellar neurons (not shown), indicating that Atx-10 has additional cellular functions. This is strengthened by the observation that Atx-10 levels are not down-regulated in tissues in which Atx-10 and OGT p110 do not interact. We assume that the control of the OGT p78 isoform expression is a key mechanism regulating tissue-specific intracellular glycosylation activity. The challenge for future studies will be to unravel the molecular basis for the strong OGT p110-p78 interaction and to address the question how OGT p78 expression is controlled on the genetic level.

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