Identification of O-Linked N-Acetylglucosamine Modification of Ankyrin<sub>G</sub> Isoforms Targeted to Nodes of Ranvier<sup>*</sup>

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Xu Zhang‡ and Vann Bennett

From the Departments of Cell Biology and Biochemistry and the Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina 27710

Ankyrin<sub>G</sub> isoforms of 270 and 480 kDa are localized at nodes of Ranvier and are candidates to couple the voltage-dependent sodium channel and neurofascin to the spectrin/actin network. This study presents evidence that these ankyrins contain O-linked GlcNAc residues and identifies as the site of glycosylation a serine-rich domain that distinguishes them from other ankyrin isoforms. The 480-kDa ankyrin<sub>G</sub>, extracted from brain membranes associated with wheat germ agglutinin-affinity columns, was <sup>3</sup>H]<galactose-labeled with UDP-[<sup>3</sup>H]galactose and galactosyltransferase, and cross-reacted with an antibody against O-GlcNAc monosaccharides. Ankyrin<sub>G</sub>-associated sugars are O-linked monosaccharides based on resistance to peptide-N-glycosidase F and analysis of saccharides released by β-elimination. The serine-rich domain is the site of glycosylation based on wheat germ agglutinin binding activity of polypeptides produced by <i>in vitro</i> translation in reticulocyte lysates. Immunofluorescence revealed co-localization of ankyrin<sub>G</sub> and O-GlcNAc immunoreactivity at nodes of Ranvier. These observations suggest that ankyrin at the node of Ranvier is O-GlcNAc-glycosylated and are the first demonstration of a post-translational modification that is concentrated at the node of Ranvier and not in adjacent areas of myelinated axons.

The node of Ranvier of myelinated axons is a relatively recent adaptation of vertebrates where ion channels are enriched and the ion fluxes of action potentials occur. Understanding development and maintenance of nodes of Ranvier is likely to have immediate implications for the molecular physiology of myelinated axons and may provide general insights into the basis for membrane polarity and assembly of specialized membrane domains.

Two specific isoforms of ankyrin, 270- and 480-kDa ankyrin<sub>G</sub>, have recently been discovered to be specifically localized at nodes of Ranvier and axonal initial segments (1–3). Ankyrins are a family of spectrin-binding proteins that link the cytoplasmic domains of certain integral proteins to the spectrin/actin network (4). The 270- and 480-kDa ankyrin<sub>G</sub> isoforms colocalize with the voltage-dependent sodium channel, the ankyrin-binding cell adhesion molecules neurofascin and NrCAM at nodes of Ranvier in adult rats, as well as early intermediates in assembly of nodes of Ranvier (5, 6), and are candidates to connect the cytoplasmic domains of these membrane proteins to the spectrin/actin network at nodes of Ranvier.

Three ankyrin genes have been identified so far (4, 7): ankyrin<sub>G</sub> (8, 9), ankyrin<sub>B</sub> (10, 11), and ankyrin<sub>C</sub> (3, 12). Most products of these genes share three conserved domains: (a) a NH<sub>2</sub>-terminal 89–95-kDa membrane-binding domain; (b) a 62-kDa spectrin-binding domain; and (c) a 12-kDa “death domain” (13), which is followed by a C-terminal domain. Ankyrins also include alternatively spliced forms with either insertion or deletion of functional domains. Giant isoforms of ankyrin including the 440-kDa ankyrin<sub>B</sub> and 270- and 480-kDa ankyrin<sub>C</sub> have insertions up to 2,100 amino acids in length between the spectrin-binding and death domains, which are predicted to be configured as a random coil (3, 11).

Ankyrins targeted to axons include 480-kDa ankyrin<sub>B</sub> and ankyrin<sub>C</sub> isoforms of 190, 270, and 480 kDa (3, 11, 14). The 440-kDa ankyrin<sub>B</sub> and 190-kDa ankyrin<sub>C</sub> are present in unmyelinated axons, while ankyrin<sub>C</sub> of 270 and 480 kDa are expressed only in myelinated axons. The most prominent differences between 270- and 480-kDa ankyrin<sub>G</sub> of myelinated axons and other isoforms of ankyrin in unmyelinated axons is a unique 46-kDa domain, located between the spectrin-binding domain and the tail domain, which has sequence similarity to mucins and is comprised of 35% serine and threonine residues (3). The function of the serine-rich domain of nodal isoforms of ankyrin is not known, but could be involved in targeting ion channels or other specialized roles at the node of Ranvier.

This report presents evidence that 270- and 480-kDa ankyrin<sub>G</sub> polypeptides are glycosylated and 480-kDa ankyrin<sub>G</sub> is modified by single O-linked N-acetylglucosamine residues at sites in the 46-kDa serine-rich domain. Moreover, immunofluorescence using an O-GlcNAc-specific monoclonal antibody HGAC85 (15), demonstrates that O-GlcNAc-glycosylated proteins are enriched at nodes of Ranvier in sciatic nerves of adult rats and are co-localized with 480- and/or 270-kDa ankyrin<sub>G</sub>. These findings suggest a role of O-GlcNAc glycosylation in specialized functions of ankyrin and possibly other proteins at nodes of Ranvier.

**EXPERIMENTAL PROCEDURES**

Methods—3.5–17% exponential SDS-PAGE<sup>1</sup> and immunoblots were performed as described by Davis and Bennett (16). Molecular biology methods and cDNA library screening were performed essentially as described elsewhere (17). Positive clones were subcloned into pBlueScript(KS<sup>±</sup>) vector (Stratagene) and sequenced using PRISM DyeDeoxy Terminator cycle sequencing kit (ABI Applied Biosystems). Immunocytochemistry with 3-μm-thick longitudinal frozen sections of rat sciatic nerves fixed in 2% paraformaldehyde for 2 h was performed as described previously (1, 2).

Antibodies—A cDNA fragment specific for the tail domain of human 480-kDa ankyrin<sub>G</sub> (nucleotides 7930–9210) was subcloned into pGEM-MEX (Promega Inc.) expressed as a fusion protein with the viral gene.

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<sup>1</sup>The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin; HA, hemagglutinin; GFP, green fluorescent protein; PNGase F, peptide-N-glycosidase F; PVDF, polyvinylidene difluoride.
10 protein. The recombinant protein was purified and immobilized on Sepharose CL-6B (Pharmacia Biotech Inc.). To raise antibody, the recombinant protein-coated Sepharose CL-6B was sonicated and injected into rabbits. The resulting antiserum was affinity-purified using immobilized recombinant tail domain after initially depleting the serum of gene 10 antibodies.

Antibodies specific for the common tail domain of 270- and 480-kDa ankyrin<sub>G</sub> were raised in chickens against a sequence of rat 270-kDa ankyrin<sub>G</sub>, corresponding to the residues 1821–2337 of human 480-kDa ankyrin<sub>G</sub>, which contains a portion of the serine-rich domain and a complete tail domain of 270-kDa ankyrin<sub>G</sub>. Antibodies were initially purified from chicken egg yolk using an egg yolk purification kit (Pharmacia) and further affinity-purified.

Mouse monoclonal antibody HGAC85 specific for O-GlcNac residues was generously provided by Dr. N. S. Greenspan (Case Western Reserve University, Cleveland). Ankyrin<sub>G</sub>-specific antibody was prepared by J. Q. Davis (1, 18).

Fluorescein isothiocyanate-conjugated monoclonal antibody to chicken light chains and tetramethyl rhodamine isothiocyanate-conjugated wheat germ agglutinin (WGA) were purchased from Sigma. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG and tetramethyl rhodamine isothiocyanate-conjugated goat anti-rabbit IgG were obtained from Cappel.

**Wheat Germ Agglutinin-Affinity Chromatography—** Denyelinated membranes were isolated from 100 g of frozen bovine brain (16). Peripheral membrane proteins were extracted with 150 ml of 0.1 N ice-cold NaOH for 15 min at 4 °C, neutralized by addition of 30 ml of 1 M NaHCO<sub>3</sub> and centrifuged at 30,000 × g for 1 h. The supernatant was dialyzed against 2 liters of 0.2 M NaCl, 50 mM sodium phosphate buffer (pH 7.4), 2 mM β-mercaptoethanol, and then 2 liters of WGA-affinity column washing buffer (0.2 M NaCl, 10 mM sodium phosphate buffer (pH 7.4), 2 mM β-mercaptoethanol). The dialyzed NaOH extract was applied at 3–4 ml/h to a WGA-affinity column (1.2 cm × 8 cm). After extensive washing with 35 × bed volumes of the washing buffer, the column was eluted by 0.2 M GlcNAc in the washing buffer.

**Immunoprecipitation, Galactosyltransferase Labeling, and Galactose Filling—** The WGA-affinity column eluate was incubated in 0.2% SDS for 20 min at 65 °C and diluted by 2% Triton X-100 before immunoprecipitation using affinity-purified rabbit polyclonal antibody specific for the 480-kDa ankyrin<sub>G</sub> tail domain. The final volume of the washed protein A agarose beads was adjusted to 25 μl and subjected to galactosyltransferase treatment according to the protocol from O-GlcNAc detection kit (Oxford Glycosystem) except for using UDP-[<sup>3</sup>H]galactose (40 Ci/mmol, from DuPont NEN). The final [H]-labeled products were either boiled in SDS-PAGE loading buffer and resolved in SDS-PAGE for β-elimination or subjected to PNGase F treatment. Hen egg ovalbumin was labeled using UDP-[<sup>3</sup>H]galactose (30 mCi/mmol) under the same conditions. [H] signals in SDS-PAGE were enhanced by Amplify (Amersham Corp.) before exposure. In galactosyl-filling experiments, the immunoprecipitated 480-kDa ankyrin<sub>G</sub> was added by galactosyltransferase using 0.5 mM UDP-[<sup>3</sup>H]galactose and the incubation was performed at room temperature for 1 h. Immunoprecipitation using rabbit nonimmune serum and the affinity-purified chicken antibody specific for both 480- and 270-kDa ankyrin<sub>G</sub> were performed in nondon dissociation conditions without adding SDS.

**PNGase F Treatment, β-Elimination, and Thin Layer Chromatography Analysis—** Peptide-N-glycosidase F (PNGase F) treatment of the immunoprecipitation mixture was performed according to the protocol from O-GlcNAc detection kit (Oxford Glycosystem). After treatment, an aliquot was applied to the PVDF membrane and counted. A control experiment was carried out in parallel except without adding PNGase F.

In β-elimination experiments, the [<sup>3</sup>H]galactose-labeled immunoprecipitated 480-kDa ankyrin<sub>G</sub> or hen egg ovalbumin were resolved in SDS-PAGE and transferred to a PVDF membrane. The membrane area corresponding to the position of 480-kDa ankyrin<sub>G</sub>, identified by immunoblot in a parallel experiment, was cut out and treated with 0.5 ml of 1 M NaBH<sub>4</sub> and 0.1 N NaOH at 37 °C for 18 h (β-elimination). The PVDF membrane strip containing [<sup>3</sup>H]-labeled hen egg ovalbumin was also isolated by comparing with the position of [<sup>3</sup>H]-labeled-ovalbumin in a SDS-PAGE gel. After the β-elimination reaction, membranes were counted and the β-elimination reaction solution of 480-kDa ankyrin<sub>G</sub> was subjected to thin layer chromatography (TLC) to analyze the released saccharides using ethyl acetate/pyridine/H<sub>2</sub>O (10:4:3) and a plastic cellulose plate (19). Reduced disaccharide standards were prepared exactly as the β-elimination reaction of ankyrin<sub>G</sub>, 480 kDa and evaluated by AgNO<sub>3</sub> staining (20).

**Preparation of cDNA Constructs—** A 4.8-kilobase pair 270-kDa ankyrin<sub>G</sub> cDNA fragment (clone no. 9) was isolated from adult rat brain by stretch plus cDNA library (Clontech) and ligated with another rat ankyrin<sub>G</sub> cDNA fragment (clone no. 2A, courtesy of Dr. Mark Steele) isolated from rat testicle cdNA library. This ligated product, which represented a nearly full-length 270-kDa ankyrin<sub>G</sub> and contained half of the membrane-binding domain, a complete spinin-binding, serine-rich, tail and C-terminal domain, and a 1-kilobase pair 3′-untranslated region of 270-kDa ankyrin<sub>G</sub>, was constructed into EcoRI site of pBlue-
The 480-kDa ankyrin$_G$ was isolated by WGA-affinity chromatography (Fig. 1) and immunoprecipitated as described under "Experimental Procedures." The final washed protein A-agarose beads with the immunoprecipitated products were labeled by galactosyltransferase and UDP-[3H]galactose (40 Ci/mmol) according to the protocol from O-GlcNAc detection kit (Oxford Glycosystem). A, immunoprecipitation was performed using 480-kDa ankyrin$_G$, specific antibody. Coomassie Blue-staining of the immunoprecipitated 480-kDa ankyrin$_G$ could not detect a visible band at 480 kDa (lane 1). Immunoblot confirmed the existence of the immunoprecipitated 480-kDa ankyrin$_G$ (lane 2). A strong [3H]galactose-labeled band at 480 kDa was revealed in lane 3. B, immunoprecipitation was performed using the affinity-purified chicken antibody specific for both 480- and 270-kDa ankyrin$_G$. Lane 1 was the immunoblot of the immunoprecipitated products. Lane 2 showed [3H]galactose-labeled bands at both 480 and 270 kDa. The arrow in lane 2 suggested a possible contaminated band. C, the immunoprecipitation products using the nonimmune serum were treated by UDP-[3H]galactose and galactosyltransferase and showed no detectable [3H]-labeled bands at either 480 or 270 kDa.

The 480- and 270-kDa Ankyrin$_G$ Are [3H]Galactose-Labeled by Galactosyltransferase—The possibility that ankyrin$_G$ contains GlcNAc residues was evaluated using galactosyltransferase which transfers [3H]galactose from UDP-[3H]galactose to terminal GlcNAc residues of N-or O-linked carbohydrate structures (21). 480-kDa ankyrin$_G$ was immunoprecipitated with two different affinity-purified antibodies from the eluate of the WGA-affinity column under either dissociation conditions, to prevent potential interactions between 480-kDa ankyrin$_G$ and other glycosylated components (Fig. 2A), or nondissociation conditions (Fig. 2B). Immunoprecipitated 480-kDa ankyrin$_G$ was easily detectable in the immunoblot (Fig. 2, A, lane 2, and B, lane 1) and heavily labeled by galactosyltransferase and UDP-[3H]galactose (Fig. 2A, lane 3 and B, lane 2) but still could not be recognized by Coomassie Blue staining (Fig. 2A lane 1). No [3H]-labeling at the position of 480 kDa was detectable when immunoprecipitation was performed with nonimmune rabbit serum (Fig. 2C). These data suggested that the [3H]galactose-labeled band in Fig. 2A, lane 3, was not due to contaminating glycosylated proteins co-migrating with the 480-kDa ankyrin$_G$. [3H]Galactose labeling by galactosyltransferase suggested the existence of terminal GlcNAc residues on the 480-kDa ankyrin$_G$.

The 270-kDa ankyrin$_G$, which also bound to WGA-affinity column, was immunoprecipitated from the WGA eluate using an antibody against determinants shared with 480-kDa anky-
rende (Fig. 2B, lane 1) and was $^3$H-labeled by galactosyltransferase (Fig. 2B, lane 2). These results suggested that 270-kDa ankyrin$_G$ also contained terminal GlcNAc residues. However, further analysis of the carbohydrate moieties of 270-kDa ankyrin$_G$ was hindered by the low yield of immunoprecipitation experiments and the existence of a contaminating $^3$H-labeled band (Fig. 2B, lane 2, arrow).

The 480-kDa Ankyrin$_G$ Contains O-Linked Single GlcNAc Residues—Evidence that 480-kDa ankyrin$_G$ contains single O-linked GlcNAc residues was provided by enzymatic and chemical deglycosylation. PNGase F specifically releases N-linked sugars such as those in hen egg ovalbumin. The $^3$H-labeled immunoprecipitation mixture including 480-kDa ankyrin$_G$, the antibody, and protein A-Sepharose beads was subjected to PNGase F treatment. 90% of the $^3$H-labeling of galactosyltransferase-treated hen egg ovalbumin was released by PNGase F, while only 25% of the $^3$H-labeling was released from the 480-kDa ankyrin$_G$ (Fig. 2B, lane 2, arrow). The lane of 480-kDa ankyrin$_G$ was cut into 0.5-cm wide strips and counted. Disaccharide standards were evaluated by AgNO$_3$ staining, and their positions are indicated by arrows.

FIG. 3. The 480-kDa ankyrin$_G$ contains O-linked single N-acetylgalactosamine residues. A, $[^3]$Hgalactose-labeled 480-kDa ankyrin$_G$ was treated by PNGase F as described under “Experimental Procedures.” An aliquot was loaded to a PVDF membrane before or after PNGase F treatment and counted. As a control, hen egg ovalbumin was also $^3$H-labeled using 30–40 mCi/mmol and applied to a PVDF membrane before or after PNGase F treatment and counted. B, $^3$H-labeled 480-kDa ankyrin$_G$ was resolved by SDS-PAGE and transferred to a PVDF membrane. The membrane strip corresponding to the position of 480-kDa ankyrin$_G$ was cut out and treated with 0.5 ml of 1 N NaBH$_4$ and 0.1 N NaOH for 18 h at 37°C ($\beta$-elimination). The membrane strip was counted after $\beta$-elimination. As a control, $^3$H-labeled hen egg ovalbumin was also transferred to the PVDF membrane, treated by $\beta$-elimination, and counted. C, saccharides released by $\beta$-elimination were analyzed by thin layer chromatography using ethyl acetate/pyridine/H$_2$O (10:4:3) and a cellulose-coated plastic plate. Reduced disaccharide standards (a, b, and c) were prepared as for the 480-kDa ankyrin$_G$. The lane of 480-kDa ankyrin$_G$ was cut into 0.5-cm wide strips and counted. Disaccharide standards were evaluated by AgNO$_3$ staining, and their positions are indicated by arrows.

The Serine-rich Domain of Ankyrin$_G$ Isoforms Contains O-GlcNAc Glycosylation Sites—Rabbit reticulocyte lysates contain enough O-GlcNAc transferase and UDP-GlcNAc to add GlcNAc to potential O-GlcNAc-glycosylated proteins (22). In vitro translation of ankyrin$_G$ transcripts in rabbit reticulocyte lysates was used to confirm O-glycosylation and to identify the domain responsible for O-glycosylation. Criteria for O-GlcNAc glycosylation of in vitro translated proteins was their ability to
O-GlcNAc Glycosylation of AnkyrinG at Nodes of Ranvier

A schematic diagram of constructs used in experiments to determine domains responsible for glycosylation of the 480-kDa ankyrinG. Constructs were prepared as described under “Experimental Procedures.” The full-length construct was named construct M-Sb-Sr-T-C, indicating that it contains half of the membrane-binding domain (M), a complete spectrin-binding domain (Sb), a serine-rich domain (Sr), a tail domain (T), a C-terminal domain (C), and a 1-kilobase pair 3′-untranslated region (3′-UTR). Construct Sr-T-C lacks the membrane-binding domain and the spectrin-binding domain. Construct T-C contains only about 200 base pairs of the serine-rich domain and a complete tail and the C-terminal domain, while construct C contains only the C-terminal domain. Construct M-Sb-C was constructed from one of the cDNA fragments isolated from rat testicle cDNA library and represents a kidney isoform of ankyrinG, which lacks the serine-rich domain and the tail domain. Construct Sr was prepared using a PCR-amplified serine-rich domain and contained only a HA-GFP tag, the serine-rich domain, and 3′-UTR.

These tagged constructs were in vitro translated in rabbit reticulocyte lysate and labeled by [35S]methionine (Fig. 5). Products of transcripts that contain the serine-rich domain (constructs M-Sb-Sr-T-C and Sr-T-C) exhibited WGA-binding activity and were eluted by GlcNAc (Fig. 5A, lanes 1, 1′, 3, and 3′). Other in vitro translated products of transcripts lacking the serine-rich domain (constructs M-Sb-C and T-C) did not exhibit any affinity to WGA (Fig. 5A, lanes 2, 2′, 4, and 4′).

The in vitro translated serine-rich domain (construct Sr) alone also showed the WGA-binding activity and was eluted by GlcNAc (Fig. 5B, lanes 2 and 2′). In contrast, the serine-rich domain translated in wheat germ extracts, which do not contain O-GlcNAc transferase activity (22), lacked the WGA binding affinity (Fig. 5B, lanes 1 and 1′). Lack of WGA binding activity of the serine-rich domain translated in wheat germ extracts demonstrates that the WGA binding affinity of the serine-rich domain translated in rabbit reticulocyte lysate was due to posttranslational modification rather than the polypeptide per se.

The serine-rich domain translated in a wheat germ extract exhibited a faster gel mobility in SDS-PAGE than that translated in rabbit reticulocyte lysate (compare Fig. 5B, lanes 1 and 2). The difference between their gel mobility in SDS-PAGE corresponded to an apparent difference in a size of 3 kDa, or about 10 GlcNAc residues attached to each serine-rich domain translated in rabbit reticulocyte lysate (22, 24). Treatment with N-acetylgalactosaminidase shifted the gel mobility of the serine-rich domain translated in rabbit reticulocyte lysate to a similar position as that translated in wheat germ extract (data not shown). As a control, the in vitro translated C-terminal domain (construct C), which has a similar gel mobility as that of the serine-rich domain, did not exhibit either WGA binding affinity or apparent gel mobility difference in either in vitro translation system (Fig. 5B, lanes 3, 4, 3′, and 4′).

O-GlcNAc-glycosylated Proteins Are Enriched at Nodes of Ranvier and Co-localize with 480-kDa AnkyrinG—Monoclonal antibody HGAC85 recognizes a single GlcNAc residue with O-linkage to serine/threonine residues independent of the primary structure of glycosylated polypeptides (15). The immunoprecipitated 480-kDa ankyrinG was recognized by HGAC85 in the presence of 100 mM galactose (Fig. 6, lane 2), suggesting that 480-kDa ankyrinG bears O-linked single GlcNAc residues. Recognition of 480-kDa ankyrinG by HGAC85 was totally abolished in the presence of 100 mM GlcNAc (Fig. 6, lane 3). Moreover, immunoprecipitated 480-kDa ankyrinG lost immunoreactivity to HGAC85 after being extensively modified by adding galactose to its terminal GlcNAc residues (galactose filling) (Fig. 6, lane 5). Fig. 6, lane 4, is an immunoblot against the 480-kDa ankyrinG after galactose filling, indicating that the loss of immunoreactivity to HGAC85 was not due to degradation of 480-kDa ankyrinG during the galactose-filling reaction. HGAC85 specifically stained nodes of Ranvier in sciatic nerves of adult rats in the presence of free galactose (Fig. 7A and A′). Nodal staining by HGAC85 was entirely abolished in the presence of free GlcNAc (Fig. 7B). Some nodes of Ranvier displayed a clear nodal cytoplasmic staining (Fig. 7A′ arrow). Double-staining using HGAC85 and an antibody specific for 480-kDa ankyrinG revealed co-localization of O-GlcNAc-glycosylated proteins and 480-kDa ankyrinG at nodes of Ranvier (yellow color in Fig. 7E). Double-staining using rhodamine-conjugated WGA and the chicken antibody specific for both 270- and 480-kDa ankyrinG was also performed (Fig. 7, F–H).
O-GlcNAc Glycosylation of Ankyrin<sub>G</sub> at Nodes of Ranvier

Although 270- and 480-kDa ankyrin<sub>G</sub> were localized inside the WGA staining at nodes of Ranvier (yellow color in Fig. 7H), WGA also strongly stained areas external to the axon at nodes of Ranvier (red color in Fig. 7H). Lack of the external staining by HGAC85 suggested its specificity for the intracellular O-linked single GlcNAc residues.

**DISCUSSION**

Glycosylation by single GlcNAc residues O-linked to serine or threonine residues has been discovered in diverse intracellular proteins including nuclear pore proteins, transcription regulatory proteins, RNA polymerase II, and cytoskeletal, oncopogene, heat shock, and viral proteins (21, 25), as well as neuronal proteins such as clathrin assembly protein AP3 (26), synapsin I (27), neurofilament L and M (28), an 83-kDa protein from the axoplasm of Aplysia axons (29), and β-amyloid precursor protein (30). This report presents evidence that 480- and 270-kDa isoforms of ankyrin<sub>G</sub>, which are enriched at nodes of Ranvier, contain O-linked GlcNAc residues and identifies as the site of glycosylation a serine-rich domain which distinguishes these ankryins from other members of the ankyrin family. The 480- and 270-kDa ankyrin<sub>G</sub> polypeptides extracted from bovine brain membranes associate with wheat germ agglutinin-affinity columns, are H<sup>3</sup>galactose-labeled with UDP-[H]<sup>3</sup>galactose and galactosyltransferase, and cross-react with an antibody that recognizes O-GlcNAc monosaccharides (Figs. 1, 2, and 6). Ankyrin<sub>G</sub>-associated sugars are O-linked monosaccharides based on resistance to enzymatic digestion with PNGase F, susceptibility to chemical cleavage by β-elimination, and analysis of the saccharides released by β-elimination (Fig. 3). The serine-rich domain was identified as the domain modified by glycosylation based on WGA binding activity of polypeptides produced by in vitro translation in rabbit reticulocyte lysates, which are competent to perform O-GlcNAc glycosylation of cytoplasmic proteins (22, 31).

Immunofluorescence studies using a monoclonal antibody which recognizes single O-linked GlcNAc residues independent of the primary structure (15) revealed co-localization of ankyrin<sub>G</sub> and O-GlcNAc-glycosylated proteins at nodes of Ranvier in sciatic nerves of adult rats (Fig. 7). Since most of the 480-kDa ankyrin<sub>G</sub> extracted from bovine brain membranes is O-GlcNAc-glycosylated, it is likely that the O-GlcNAc-glycosylated 480-kDa ankyrin<sub>G</sub> is localized at nodes of Ranvier. However direct evidence for localization of O-GlcNAc-glycosylated ankyrin at nodes of Ranvier will require development of antibody specific for O-GlcNAc-glycosylated ankyrin<sub>G</sub> and remains to be determined.

One proposed function for cytoplasmic O-GlcNAc glycosylation was to cap potential phosphorylation sites based on the similarity in sequence preference between protein kinases and protein GlcNAc transferases (25, 32, 33). This hypothesis is supported from studies on RNA polymerase II (34) and c-Myc oncogene protein (35). Interestingly, neurofilaments, which represent the major cytoskeletal components in axons, have been demonstrated to be less phosphorylated at nodes of Ranvier and axonal initial segments than in the internodal areas (36). These observations considered together with our discovery that O-GlcNAc-glycosylated proteins are enriched at nodes of Ranvier, suggests the possibility that at nodes of Ranvier O-GlcNAc glycosylation replaces phosphorylation for some proteins. Potential consequences of a reduced phosphorylation...
could be interference with the normal axonal transport and result in delivery of nodal components as proposed by Brady and colleagues (37–39). It is of interest that the serine-rich domain of ankyrinG isoforms contains potential phosphorylation sites, although in vivo phosphorylation has not yet been evaluated. It will be important in future experiments to determine if the 480/270-kDa ankyrinG is phosphorylated at sites overlapping with O-GlcNAc glycosylation, and to determine effects of phosphorylation on axonal transport of ankyrinG.

A unifying feature of O-GlcNAc-glycosylated proteins is participation in reversible multimeric complexes. One function of the O-GlcNAc-glycosylated serine-rich domain of the 480-kDa ankyrinG could be to mediate homophilic interactions, which would result in clustering of ankyrin and thus ankyrin-associated integral proteins such as the voltage-dependent sodium channel. Preliminary evidence in support of this idea is that the serine-rich domain exhibits a larger Stokes radius when expressed in glycosylation-competent rabbit reticulocyte lysates than in wheat germ lysates which cannot O-GlcNAc glycosylate expressed proteins.2 It is also possible that O-GlcNAc glycosylation could promote interactions of ankyrin with other proteins.

The serine-rich domain of ankyrinG distinguishes those isoforms which are concentrated at the node of Ranvier from other forms of ankyrin present in unmyelinated axons. The observations in this study that the serine-rich domain is O-GlcNAc-glycosylated and that O-GlcNAc-glycosylated epitopes are concentrated at the node of Ranvier is the first demonstration of a post-translational modification that occurs at the node of Ranvier and not in adjacent areas of myelinated axons. This finding could be related to observations that the extent of phosphorylation, in contrast, is reduced at the nodal and axonal initial segmental areas of axons (36, 40). It is anticipated that understanding structural and functional consequences of O-GlcNAc glycosylation of this domain will contribute to understanding of nodes of Ranvier at a molecular level.

Fig. 7. O-GlcNAc-glycosylated proteins are enriched at the node of Ranvier and co-localize with 480-kDa ankyrinG. 3-μm thick cryosections of sciatic nerves of adult rats were prepared and permeabilized by 0.5% Triton X-100, 10% normal goat serum and 1% bovine serum albumin in phosphate-buffered saline for 30 min at room temperature. Incubation with primary antibodies (15 μg/ml) were performed in 0.05% Triton X-100, 1% NGS and 0.1% bovine serum albumin in phosphate-buffered saline, overnight at 4 °C. Secondary antibodies were applied in the same buffer at 4 °C for 2 h. Between incubations with different antibodies, sections were washed there to four times in phosphate-buffered saline for 20 min at room temperature. Immunostaining using monoclonal antibody HGAC85 which recognizes the O-linked single GlcNAc residue was carried out in the presence of 50 mM galactose or 50 mM GlcNAc as indicated. All images were obtained from a Zeiss confocal microscope. Monoclonal antibody HGAC85 clearly stained the node of Ranvier in the presence of 50 mM galactose (A). In some cases, nodal cytoplasm was also stained (A’, arrowhead). Immunostaining of the node of Ranvier by HGAC85 was totally abolished by the presence of 50 mM GlcNAc (B). Double-staining using HGAC85 and antibody specific for the tail domain of the 480-kDa ankyrinG revealed co-localization of O-GlcNAc-glycosylated proteins and the 480-kDa ankyrinG at the node of Ranvier (yellow color in E). C and D are individual channels of image in E. C was the fluorescein channel representing an individual staining pattern of HGAC85, while D was the rhodamine channel showing the immunostaining pattern using the antibody specific for the 480-kDa ankyrinG. Double-staining using the rhodamine-conjugated WGA (10 μg/ml) and the chicken antibody specific for the 270- and 480-kDa ankyrinG suggested a partial overlap (yellow color in H). F and G are individual channels representing the individual immunostaining patterns of the rhodamine-conjugated WGA and the chicken antibody, respectively. The node of Ranvier is indicated by a straight arrow. Axons are indicated by a curved arrowhead. The curved arrowhead suggests the surface of the Schwann cell. Each panel has a same magnification. Scale bar, 3 μm.

2 X. Zhang and V. Bennett, unpublished data.
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