Purification and Structure−Function Analysis of Native, PNGase F-Treated, and Endo-β-galactosidase-Treated CHIP28 Water Channels†

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ABSTRACT: CHIP28 occurs naturally in glycosylated and nonglycosylated forms. The purpose of this study was to determine the role of glycosylation in CHIP28 structure and function. A new purification procedure based on phenylboronic acid−agarose (PBA) affinity chromatography was developed to isolate CHIP28. In purified native CHIP28 from erythrocytes, ~50% of CHIP28 molecules were glycosylated; each mole of glycosylated CHIP28 contained 5.4 kDa of monosaccharides consisting of 2 mol of Fuc, 8 mol of Gal, 1 mol of GalN, 13 mol of GlcN, 3 mol of Man, and 1 mol of Neu5Ac. The proportions of each monosaccharide and the sensitivity to endo-β-galactosidase indicated that CHIP28 contained polylactosaminyl oligosaccharides. Glycosylated and nonglycosylated CHIP28 remained tightly associated when solubilized in octyl β-D-glucoside (OG) and could not be separated by conventional chromatographic procedures. To remove the sugar moiety, CHIP28 was enzymatically deglycosylated by PNGase F and purified by Q-Sepharose anion-exchange and *Erythrina cristagalli* lectin chromatography. High-performance size-exclusion chromatography revealed that native CHIP28 eluted as an apparent dimer, whereas deglycosylated CHIP28 eluted as an apparent monomer. In reconstituted proteoliposomes, deglycosylated CHIP28 had a single channel water permeability (p_f) of 3.1×10^{-14} cm³/s (10 °C), not different from that of 3.2×10^{-14} cm³/s for native CHIP28. Circular dichroism of native and deglycosylated CHIP28 in OG revealed 45% and 48% α-helix, respectively; intrinsic tryptophan fluorescence showed no effects of glycosylation on tryptophan environment. Freeze−fracture electron microscopy with rotary shadowing indicated that native and deglycosylated CHIP28 assembled as tetramers in reconstituted proteoliposomes. The results establish a procedure to purify deglycosylated CHIP28 in functional form and indicate that glycosylation is required neither for the water transport function nor for the tetrameric assembly in membranes.

A series of integral membrane proteins that function as water transporters have been identified recently. The water channel proteins ("aquaporins") are homologous to MIP26[1] of lens fiber (Gorin *et al.,* 1984), and to date include the mammalian proteins CHIP28 (Smith & Agre, 1991), WCH-CD (Fushimi *et al.,* 1993), and MIWC (Hasegawa *et al.,* 1994b), and plant protein γ-TIP (Maurel *et al.,* 1993). CHIP28 functions as a selective water-transporting protein when reconstituted into proteoliposomes (Van Hoek & Verkman, 1992; Zeidel *et al.,* 1992), and when expressed in CHO cells (Ma *et al.,* 1993) and *Xenopus* oocytes (Preston *et al.,* 1992; Zhang *et al.,* 1993a). CHIP28 is expressed in erythrocytes and in a variety of fluid-transporting epithelia and endothelia, including kidney proximal tubule and thin descending limb of Henle, choroid plexus, ciliary body, colonic crypts, and others (Sabolic *et al.,* 1992; Hasegawa *et al.,* 1993, 1994a; Nielsen *et al.,* 1993ab).

CHIP28 has several unique biochemical features that are or may be related to its glycosylation pattern. CHIP28 is found in both glycosylated and nonglycosylated forms in

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[1] Abbreviations: CD, circular dichroism; CHIP28, channel-forming intrinsic protein of 28 kDa; DSP, dithiobis(succinimidyl propionate); ECL, *Erythrina cristagalli* lectin; Endo H_f, recombinant fusion protein of endo-β-*N*-acetylglucosaminidase H and maltose-binding protein; Fuc, L-fucose; Gal, D-galactose; GalN, D-galactosamine; GalNAc, *N*-acetyl-D-galactosamine; Glc, D-glucose; GlcN, D-glucosamine; GlcNAc, *N*-acetyl-D-glucosamine; HPAEC/PAD, high-pH anion-exchange chromatography with pulsed amperometric detection; HPSEC, high-performance size-exclusion chromatography; IMP, intramembrane particle aggregate; kDa, kilodalton(s); Man, D-mannose; MIWC, mercurial insensitive water channel; MIP26, major intrinsic protein of molecular mass 26 kDa; Neu5Ac, *N*-acetylneuraminic acid; OG, octyl β-D-glucoside; PBA, phenylboronic acid−agarose; PC, L-α-phosphatidylcholine; PE, L-α-phosphatidylethanolamine; PI, L-α-phosphatidylinositol; PNGase F, recombinant peptide-N^4-(*N*-acetyl-β-D-glucosaminyl)-asparagine amidase from *Flavobacterium meningosepticum*; PVDF, poly(vinylidene difluoride); RCA, *Ricinus communalis* agglutinin; TFA, trifluoroacetic acid; TIP, tonoplast intrinsic protein; WCH-CD, water channel-collecting duct; WGA, wheat germ agglutinin.

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native tissues and in CHIP28-expressing CHO cells and *Xenopus* oocytes. On SDS−PAGE and immunoblots probed with anti-CHIP28 antibodies, CHIP28 migrates at two positions—a sharp band at 28 kDa, believed to represent nonglycosylated CHIP28, and a glycosylated broad band with an apparent size of 45−65 kDa (Smith & Agre, 1991; Sabolic *et al.,* 1992). Another unusual feature is that CHIP28 is assembled as tetramers in native cell membranes and reconstituted proteoliposomes (Verbavatz *et al.,* 1993), and in two-dimensional crystals (Walz *et al.,* 1994; Mitra *et al.,* 1994); however, each monomer functions independently (Van Hoek *et al.,* 1991, 1992; Shi *et al.,* 1994). Residue N42, which is located in an extramembrane loop following the first transmembrane domain (Preston *et al.,* 1994; Skach *et al.,* 1994), is glycosylated in some CHIP28 molecules (Zhang *et al.,* 1993b), whereas a second consensus site for N-linked glycosylation (N205) is not modified. The molecular size and composition of the sugar moiety are not known, nor has the influence of glycosylation on CHIP28 structure and function been established.

The purpose of this study was to determine the composition of the oligosaccharides and to purify nonglycosylated CHIP28 for structure−function experiments. It was found that glycosylated CHIP28 from human erythrocytes could not be separated from nonglycosylated CHIP28 by conventional chromatographic techniques in nondenaturing detergents because of a tight association in heterodimers. Enzymatic deglycosylation could be accomplished by the amidase PNGase F. Endo-β-galactosidase markedly reduced the molecular size of glycosylated CHIP28, indicating polylactosaminyl structures. The reaction with PNGase F resulted in the dissociation of detergent-solubilized CHIP28 into monomers. Deglycosylated CHIP28 was purified and used for analysis of structure and function by high-performance size-exclusion chromatography, circular dichroism, intrinsic tryptophan fluorescence, freeze−fracture electron microscopy, and stopped-flow light scattering.

MATERIALS AND METHODS

Enzymes. Endo H_f and PNGase F were purchased from NEB-Laboratories (Beverly, MA). Endo-β-galactosidase from *Escherichia freundii* was kindly provided by Dr. Michiko Fukuda, La Jolla Cancer Research Foundation, Cancer Research Center, La Jolla, CA.

Protein Purification. Hemoglobin-free erythrocyte membranes were obtained from outdated human red blood cells by tangential flow filtration using a Pellicon 0.45 μm casette (Millipore). Delipidated CHIP28 in detergent micelles was prepared by stripping twice with 3% *N*-lauroylsarcosine and solubilizing in 200 mM OG as described previously (Van Hoek *et al.,* 1993), followed by chromatography on a phenylboronic acid−agarose (PBA-60, Amicon; 2.5 × 7 cm) open column equilibrated in 1 mM EDTA/50 mM sodium phosphate (pH 8.5) containing 35 mM OG (PBA−wash buffer). After sample application, the matrix was washed with 10 column volumes of PBA−wash buffer, and the protein was eluted with PBA−wash buffer containing 0.5 M sorbitol. Fractions containing CHIP28 were pooled and concentrated by ultrafiltration. PBA chromatography was performed at pH 8.5 to promote interaction of the *cis*-diols from oligosaccharides with the tetrahedral boron ion (Brena *et al.,* 1992).

To remove oligosaccharides from CHIP28, the concentrated PBA-60 eluate (10 mg/mL) was incubated with 50 IUB milliunits of PNGase F/mg of CHIP28 at 28 °C. After various incubation times, the protein sample was applied to a Q-Sepharose column (Pharmacia; 1 cm diameter, 1.2 mL of resin) equilibrated in 0.1 mM EDTA, 35 mM OG, and 10 mM sodium phosphate (pH 7.4). CHIP28 was bound to the resin whereas PNGase F protein passed in the effluent. CHIP28 was eluted with the same buffer containing 0.6 M NaCl. CHIP28 fractions were pooled, concentrated, dialyzed against the 0.6 M NaCl buffer, and applied to an ECL−agarose (Vector Laboratories, Burlingame, CA; 1 cm diameter, 2 mL of resin) column preequilibrated with the same buffer. Deglycosylated CHIP28 passed through the column, whereas unreacted glycosylated CHIP28 remained and could be recovered by elution with 0.2 M lactose in NaCl-free buffer.

High-performance size-exclusion chromatography (HPSEC) was performed as described previously with a TSK G3000SW column at a flow rate of 0.6 mL/min (Van Hoek *et al.,* 1993). SDS−PAGE was performed with minigels (Hoefer) using the discontinuous electrophoresis system of Laemmli and 10% acrylamide, Tris−glycine gels. CHIP28 samples were incubated for 1 min in SDS−PAGE application buffer without DTT. (DTT and longer incubation times in SDS resulted in aggregation of deglycosylated CHIP28.) Gels were fixed in 10% acetic acid and 25% methanol for 10 min, stained with Coomassie blue in 10% acetic acid for 5 min, and destained in 10% acetic acid. To quantify the amount of stain associated with protein, the gel was imaged by a cooled CCD camera (Photometrics) by transmitted light at a wavelength of 560 nm. Flat-field correction was carried out by recording an image with the gel removed, and background absorption was determined from unstained regions of the lane of interest. The total amount of stain associated with a region of the gel was determined from the background-subtracted absorption integrated over the specified band. Protein concentration was determined by a modified Lowry assay in the presence of SDS. Lipids were quantified by phosphorus determination after extraction with chloroform.

Protein Reconstitution. Liposomes containing PC, PI (Sigma), and cholesterol (50 mg/mL, 11:1:11 mole ratio) were prepared by reverse phase evaporation (Van Hoek *et al.,* 1993). For some experiments, liposomes also contained phosphatidylethanolamine (PC:PE:PI:cholesterol ratio = 11:5:1:11). Lipid/detergent micelles were prepared by dissolving liposomes in 1 mM EDTA, 100 mM sodium phosphate (pH 7.4) and 200 mM OG. PBA-purified CHIP28 (containing glycosylated and nonglycosylated CHIP28) and also deglycosylated CHIP28 preparations were mixed with lipid/detergent micelles at room temperature in a 1:20 (w/w) ratio and incubated for 1 h. The suspensions were dialyzed against 10 volumes of the EDTA−phosphate buffer for 3 h to decrease the OG concentration to ~20 mM, approximately at the critical micellar concentration where proteoliposomes are formed. The buffer was then diluted 10-fold with distilled water and dialyzed for 24 h with one buffer change. Vesicle diameter was determined by Coulter counting.

Quantitative Monosaccharide and Amino Acid Analyses. Anionic, amino, and neutral sugars were determined using high-pH anion-exchange chromatography after acid hydrolysis with either HCl or TFA (Hardy & Townsend, 1994).

Coomassie-stained bands of purified CHIP28 from PVDF membranes were excised and hydrolyzed as described previously (Weitzhandler *et al.,* 1993). The excised bands were wetted with methanol and transferred to 1.1 mL hydrolysis tubes (Chromacol, Trumball, CT). Neutral sugars were released by hydrolysis with 2 M TFA, and amino sugars were released by hydrolysis with 6 N HCl, both at 100 °C for 3 h. Sialic acids were released by 0.1 N HCl at 80 °C for 30 min. Care was taken to ensure that the stained bands were submerged prior to hydrolysis. Hydrolysates were then centrifuged for 2 min and dried in a Speed Vac centrifuge. The dried samples were dissolved in 200 μL of water and placed in autosampler vials. Neutral and amino monosaccharides were separated isocratically (16 mM NaOH) on a Dionex GlycoStation using a CarboPac PA-1 column (4 × 250 mm) equipped with a PA1 guard column at a flow rate of 1 mL/min. The column was regenerated after 25 min with 200 mM NaOH (10 min) followed by equilibration with 16 mM NaOH. Sample injections were separated by 50 min. Sialic acids were separated with a 50−180 mM sodium acetate gradient over 20 min, using 100 mM NaOH and 0.5 M acetic acid (pH 5.5) as eluents. The chromatographic data were collected for determination of peak areas and retention times using Dionex GlycoStation Software. Amino acid analysis was performed on CHIP28 bands from PVDF membranes as described by Le Gendre and Matsudaira (1989).

Permeability Measurements. For measurement of osmotic water permeability (P_f, cm/s), vesicles were diluted in EDTA−phosphate buffer and mixed in a stopped-flow apparatus (Hi-Tech SF51, Wiltshire, England) with an equal volume of the buffer containing 250 mM sucrose to produce a 125 mOsm inwardly-directed osmotic gradient. Single-channel water permeability (p_f, cm^3/s) was calculated from P_f, liposome size, the lipid-to-protein ratio, and an assumed protein density of 1.35 g/mL as described previously (Van Hoek *et al.,* 1993).

Circular Dichroism. CD spectra were measured on a Jasco J500A spectropolarimeter (Van Hoek *et al.,* 1993). Samples of detergent-solubilized CHIP28 were prepared for CD analysis by 100-fold dilution of the OG-solubilized purified protein (10 mg of protein/mL) in 10 mM sodium phosphate (pH 7), 0.1 mM EDTA, 100 mM NaF, and 35 mM OG. Base-line spectra (for subtraction) were obtained from samples containing all components except for the protein. Secondary structure was determined from CD spectra by numerical decomposition using the basis spectra set of Chang *et al.* (1978).

Intrinsic Tryptophan Fluorescence. Steady-state tryptophan fluorescence intensity was measured on an SLM 8000c fluorometer (SLM Instruments, Urbana, IL) as described in Farinas *et al.* (1993). Excitation was at 295 nm to eliminate resonance energy transfer of tyrosines to tryptophan. The background signal was subtracted using a blank containing no protein.

Freeze−Fracture Electron Microscopy. Freeze−fracture studies of proteoliposomes reconstituted with native and deglycosylated CHIP28 were performed as described previously (Verbavatz *et al.,* 1993). Proteoliposomes were fixed in 10 mM DSP and 2% glutaraldehyde, washed with PBS, and cryoprotected in 30% glycerol for 1 h. The liposomes were mounted on copper freeze−fracture supports and frozen at −150 °C. Samples were fractured at −130 °C and rotary-shadowed with an ~1.5 nm coat of platinum at 45°, followed by 5 nm of carbon at 90°. Proteoliposomes were digested in bleach for 2 h, and the replicas were washed with methanol/chloroform and water and examined on a Philips CM10 electron microscope.

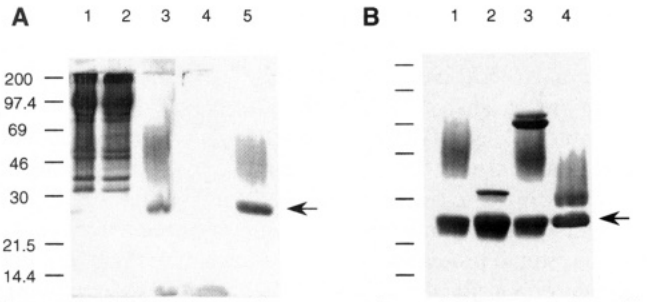

FIGURE 1: PBA affinity chromatography and deglycosylation of CHIP28. (A) Hemoglobin-free erythrocytes prepared by tangential flow filtration (lane 1); *N*-lauroylsarcosine extract (lane 2) and residue (stripped membranes, lane 3); non-retained effluent from a PBA-60 column, (lane 4) and effluent after 500 mM sorbitol (lane 5). The arrow indicates the nonglycosylated CHIP28 form. (B) PBA-purified CHIP28 (lane 1) was incubated with PNGase F (lane 2), Endo H_f (lane 3), and endo-β-galactosidase (lane 4).

RESULTS

PBA Chromatography. To purify CHIP28 efficiently, hemoglobin-free ghost membranes were prepared by tangential flow filtration and used as starting material for stripping non-CHIP28 protein by the anionic detergent *N*-lauroylsarcosine. Figure 1A, lane 1, shows a faint band at 28 kDa, representing nonglycosylated CHIP28 in ghost membranes. Gels of the *N*-lauroylsarcosine extract (lane 2) and stripped membranes (lane 3) indicate that the detergent does not solubilize CHIP28. The broad stained band at the bottom of the gel arises from membrane lipids which remain associated with CHIP28. To remove lipids and in an attempt to separate the two forms of CHIP28, the stripped erythrocyte membranes were solubilized in OG and subjected to various types of chromatography. Application of the soluble material to phenylboronic acid−agarose (PBA-60) at pH 8.5 resulted in binding of glycosylated and nonglycosylated CHIP28. Impurities and lipids passed through the column (Figure 1A, lane 4). Following addition of 500 mM sorbitol to the mobile phase, glycosylated and nonglycosylated CHIP28 coeluted (Figure 1A, lane 5). Quantitative image analysis of Coomassie blue-stained gels of PBA eluates gave a molar ratio of glycosylated to nonglycosylated CHIP28 protein of 1.1 ± 0.2 (SD, $n = 5$). This result suggested that one out of two CHIP28 molecules is glycosylated, consistent with that reported for HPSEC-purified CHIP28 (Van Hoek *et al.,* 1993).

To investigate whether ionic and/or hydrophobic interactions might account for the tight association and could be exploited to separate the two forms of CHIP28, various chromatography resins and elution conditions were tested. Results obtained with PBA-30 and PBA-10 resins, which differ in the amount of phenylboronate per milliliter of agarose (30 and 10 μg/mL, respectively), were similar to those obtained for PBA-60 as described above. Varying the concentrations of OG and CHAPS (0.01−1 M) following immobilization of CHIP28 on PBA-60, PBA-30, and PBA-10 did not result in elution of nonglycosylated CHIP28.

Addition of 50% DMSO, 600 mM NaCl, 6 M urea, or 40 mM DTT, or carrying out the chromatography at 4 °C, failed to separate nonglycosylated from glycosylated CHIP28. SDS (1%) prevented binding of both forms of CHIP28. Elution from a PBA-60 column with increasing concentrations of sorbitol (50−500 mM) also failed to separate nonglycosylated from glycosylated CHIP28. Lectin chromatography was also carried out in an attempt to separate glycosylated from nonglycosylated CHIP28. Chromatography with agarose-immobilized WGA and RCA (Vector Laboratories, Burlingame, CA) showed that both forms of CHIP28 were bound to the lectins and could not be eluted separately. Finally, 40 mM DTT did not result in separation of glycosylated and nonglycosylated CHIP28, indicating that intermolecular disulfide bonds were not responsible for the tight association.

Enzymatic Deglycosylation of CHIP28. Figure 1B shows SDS−PAGE of CHIP28 after reactions with PNGase F, endo-β-galactosidase, and Endo H_f. Incubation of PBA-purified CHIP28 (lane 1) with 2 IUB milliunits of PNGase F/mg of CHIP28 resulted in removal of the carbohydrate moiety as shown by a disappearance of glycosylated CHIP28 and an increase in staining intensity of the 28 kDa band (lane 2). Densitometry of the Coomassie blue-stained gel indicated that 97% of glycosylated CHIP28 was converted by PNGase F. The sharp band just above CHIP28 arises from PNGase F (36 kDa). Incubation with 200 IUB milliunits of Endo H_f (70 kDa recombinant fusion protein of Endo H and maltose-binding protein) per milligram of CHIP28 (lane 3) at 28 °C for 24 h did not deglycosylate CHIP28. Removal of carbohydrate with endo-β-galactosidase (35 kDa; 10 IUB milliunits/mg of CHIP28, enzyme not visible on the gel) at 28 °C for 12 h gave a modified glycosylation pattern (lane 4). The broad glycosylated protein band at 45−65 kDa present in native CHIP28 (lane 1) disappeared and was converted into a band migrating at 30 kDa (lane 4). Densitometric analysis gave approximately equal intensities of the 28 and 30 kDa bands (30 kDa/28 kDa = 1.1), consistent with the results obtained with untreated CHIP28 protein.

In larger scale preparations, deglycosylated CHIP28 was prepared by incubation with 50 IUB milliunits of PNGase F/mg of CHIP28 at 28 °C for 48 h. Upon addition of SDS−PAGE application buffer, CHIP28 was quantitatively deglycosylated (Figure 2A, lanes 1 and 2). When PNGase F was removed from the incubation mixture, ~80−85% of CHIP28 was deglycosylated (not shown). Incubation at room temperature was then carried out for an additional 3 days. To separate PNGase F from CHIP28, anion-exchange chromatography with Q-Sepharose was performed. The flow-through consisted of PNGase F (lane 3), and the high-salt eluate consisted of highly purified deglycosylated CHIP28 (lane 4).

High-performance size-exclusion chromatography (HPSEC) was performed to examine CHIP28 purity and its state of association in OG. HPSEC of PBA-purified native CHIP28 in OG revealed a homogeneous CHIP28 preparation (Figure 2B, profile a). CHIP28 eluted as a single species with an apparent molecular size of 52 kDa. HPSEC of purified deglycosylated CHIP28 revealed a major peak with an apparent molecular size of 32 kDa, and a shoulder at 52 kDa, representing native CHIP28 which did not react with PNGase F (profile b). Traces of glycosylated CHIP28 were observed

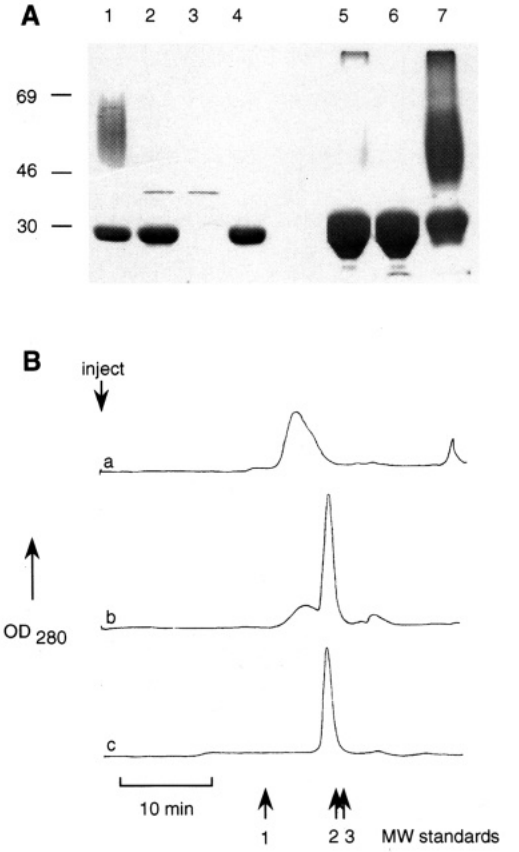

FIGURE 2: Chromatographic analysis of deglycosylated CHIP28. (A) PBA-purified CHIP28 before (lane 1) and after (lane 2) treatment with PNGase F. Q-Sepharose chromatography of PNGase F-treated CHIP28 showing non-retained PNGase F protein (lane 3), and the effluent after 600 mM NaCl (lane 4). Approximately 10-fold higher concentration of deglycosylated protein (lane 5). Further purification by ECL−lectin chromatography showing the effluent before (lane 6) and after 200 mM lactose (lane 7). (B) HPSEC of PBA-purified CHIP28 (a), PNGase F-treated CHIP28 after anion-exchange (b), and ECL−lectin (c) chromatography. Size markers, indicated by upward arrows, are (1) MIP26 (103 kDa), (2) bR (26.7 kDa), and (3) papain-digested bR (24.9 kDa).

when greater amounts of protein were applied to the gel (Figure 2A, lane 5). To remove unreacted CHIP28, lectin chromatography was performed on PNGase F-treated CHIP28 in OG with agarose-immobilized ECL as described under Materials and Methods. The effluent contained nonglycosylated and deglycosylated CHIP28 migrating as a single band on SDS−PAGE (Figure 2A, lane 6). HPSEC revealed a single species (Figure 2B, profile c). Glycosylated and nonglycosylated (unreacted) CHIP28 were eluted by 200 mM lactose in salt-free elution buffer (Figure 2A, lane 7); densitometry showed a ratio of the 45−65 kDa to 28 kDa bands of 1.1.

Monosaccharide Composition of CHIP28. To examine the fraction of CHIP28 molecules that were glycosylated and to determine the monosaccharide composition, PBA-purified CHIP28 from a PVDF blot was subjected to amino acid and sugar analyses. The alanine content of glycosylated CHIP28 in two gel lanes was 9.6 and 9.1 nmol, with the corresponding alanine content of nonglycosylated CHIP28 of 9.3 and 7.8 nmol, respectively. These results indicated that ~55% of CHIP28 monomers were glycosylated, consistent with the value of 53% from the Coomassie blue densitometry data above. Figure 3 shows the HPAEC/PAD chromatogram of

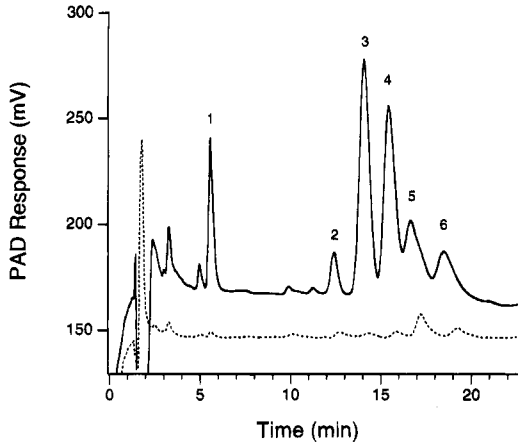

FIGURE 3: Monosaccharide analysis of CHIP28 electroeluted onto PVDF membranes. Bands representing glycosylated (45−60 kDa) and nonglycosylated CHIP28 were excised from the PVDF membrane, hydrolyzed with TFA, and subjected to HPAEC/PAD as described under Materials and Methods. The elution profile of neutral monosaccharides (solid curve) of glycosylated CHIP28 is compared with that of nonglycosylated CHIP28 (dashed curve). The elution positions of the external standards, Fuc (1), GalN (2), GlcN (3), Gal (4), Glc (5), and Man (6), are indicated by the numbered peaks.

Table 1: Monosaccharide Analysis of CHIP28

monosaccharide	mol/mol of protein[a]	molecular weight
Man	3.2	486
Gal	8.5	1458
Fuc	1.8	292
GlcN	13	2652
GalN	1.1	204
Neu5Ac	1.3	292
		total 5384

[a] Based on the Ala content of protein from parallel lanes of the same PVDF electroblot.

the broad 45−65 kDa band after acid hydrolysis with 2 M TFA. Six peaks were observed which coeluted with the external monosaccharide standards: Fuc (1), GalN (2), GlcN (3), Gal (4), Glc (5), and Man (6). The same analysis performed on the nonglycosylated band revealed two smaller peaks which coeluted with Glc and Man, but with slightly greater retention times, as shown by the dashed profile (Figure 3). These latter two peaks were judged to be contaminants because they were also found in acid hydrolysates of areas of the same PVDF membrane which did not contain protein.

The monosaccharide analysis of the glycosylated CHIP28 is given in Table 1. Neutral and amino sugars and Neu5Ac were analyzed using the three different hydrolysis conditions described under Materials and Methods. The molar ratio of monosaccharides-to-protein was based on the alanine content. The presence of three Man residues is consistent with the protein having a single glycosylated Asn residue. Resistance to Endo H_f cleavage indicates the absence of oligomannosidic-type structure. The absence of GalN in the 28 kDa band suggests that CHIP28 is not O-linked glycosylated but that the N-linked oligosaccharides apparently contain GalN. The ratio of Gal to GlcN is consistent with the presence of polylactosamine structures, as supported by its susceptibility to endo-β-galactosidase.

Functional Analysis of Deglycosylated CHIP28. Water permeability was measured in proteoliposomes reconstituted

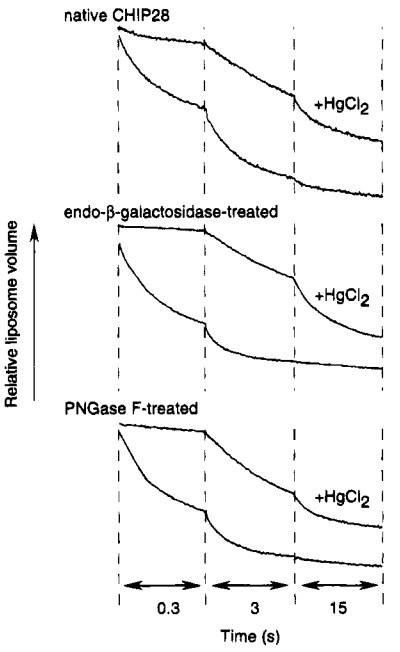

FIGURE 4: Osmotic water permeability of CHIP28 in proteoliposomes. PBA-purified CHIP28 (upper panel), endo-β-galactosidase-trimmed CHIP28 (middle panel), and PNGase F-deglycosylated CHIP28 (lower panel) were reconstituted into proteoliposomes and mixed with a hypertonic sucrose solution in the stopped-flow apparatus at 10 °C. The change in scattered-light intensity was followed until completion, using three consecutive time scales as indicated. Where indicated, proteoliposomes were incubated with 0.1 mM $HgCl_2$.

with native (A, top) endo-β-galactosidase-treated (B, middle), and PNGase F-treated CHIP28 (C, bottom) (Figure 4). The stopped-flow light-scattering curves showed similar and high water permeabilities in proteoliposomes containing native and modified CHIP28 with strong inhibition by $HgCl_2$. P_f values were 0.011 (A), 0.015 (B), and 0.013 (C) cm/s, after correction for vesicle diameters of 190, 226, and 219 nm, respectively. Lipid-to-protein ratios in these proteoliposomes ranged from 20 to 30 (w/w). Calculated single-channel water permeabilities (at 10 °C) were 3.2×10^{-14}, 3.3×10^{-14}, and 3.1×10^{-14} cm^3/s, respectively, consistent with previous results on native CHIP28 (Van Hoek *et al.*, 1993). These results indicate that neither deglycosylation nor removal of polylactosamine chains affected CHIP28 water transport function. Proteoliposomes reconstituted with deglycosylated CHIP28 were impermeable to urea and protons (not shown), similar to results reported previously for proteoliposomes containing native CHIP28 (Van Hoek *et al.*, 1993).

Structural Analysis of Deglycosylated CHIP28. CHIP28 secondary structure was examined by CD spectroscopy and intrinsic tryptophan fluorescence, and oligomeric assembly in membranes was studied by freeze−fracture electron microscopy. CD spectra of the native and deglycosylated CHIP28 were qualitatively similar and contained the characteristic absorption maxima and minima indicative of moderate α-helical content (Figure 5). By nonconstrained spectral deconvolution using the basis spectra set of Chang *et al.* (1978), α-helical contents were 45 and 48% for native and deglycosylated CHIP28, respectively; the corresponding β-sheet contents were 39% and 32%. The microenvironment of tryptophans was assessed in solubilized and membrane-associated CHIP28 by fluorescence measurements as described by Farinas *et al.* (1993). The emission maximum

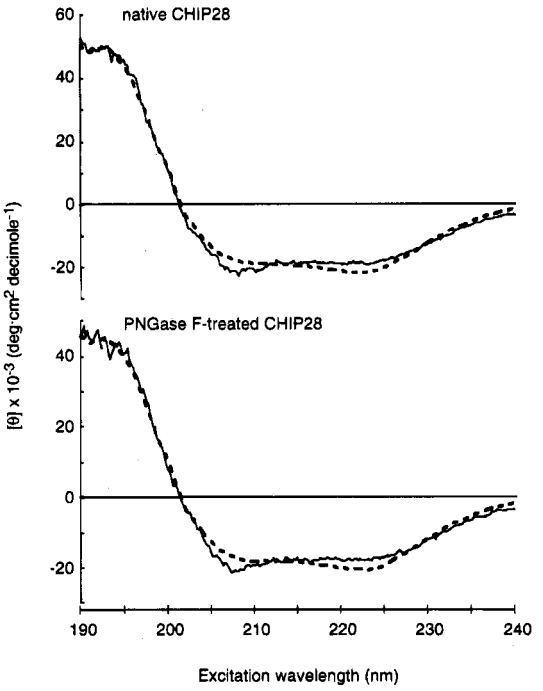

FIGURE 5: Circular dichroism (CD) analysis of soluble CHIP28 fractions. CD spectra (solid curves) of PBA-purified (upper panel) and PNGase F-deglycosylated CHIP28 (lower panel). Spectra were decomposed (dashed curves) using the Chang *et al.* (1978) basis set. Values for secondary structure content are given in the text.

of native CHIP28 in OG was at 332 nm (excitation 295 nm), which was slightly blue-shifted after treatment with PNGase F (331 nm). Similar maxima were determined in reconstituted proteoliposomes. These results indicate no significant effect of deglycosylation on the secondary structure of CHIP28 and on the microenvironment of the four tryptophans.

The effects of deglycosylation on CHIP28 assembly in membranes were studied by freeze−fracture electron microscopy with rotary shadowing. Representative micrographs are given in Figure 6. Intramembrane particles (IMPs) with an average diameter of ~8.5 nm were observed in all samples. Many of the IMPs had a clear tetrameric substructure as found previously in proteoliposomes reconstituted with CHIP28 and in cell membranes (Verbavatz *et al.*, 1993). In >10 micrographs examined for each sample, the IMPs had similar size and appearance without evidence of clusters or the presence of CHIP28 monomers or dimers. These results indicate that deglycosylation by PNGase F or oligosaccharide trimming by endo-β-galactosidase does not affect the tetrameric assembly of CHIP28 in membranes.

DISCUSSION

Unlike many other membrane proteins, CHIP28 occurs naturally in glycosylated and nonglycosylated forms. The purpose of this study was to determine the role of glycosylation in CHIP28 structure and function. Phenylboronic acid affinity chromatography was utilized as a rapid procedure to purify and delipidate CHIP28 from *N*-lauroylsarcosine-stripped erythrocyte membranes. It was not possible to separate glycosylated from nonglycosylated CHIP28 under nondenaturing conditions by a series of chromatographic procedures and elution conditions. Susceptibility to endo-β-galactosidase and quantitative monosaccharide analysis of glycosylated CHIP28 indicated the presence of a polylactosamine-type structure. Removal of the sugar moiety or trimming of the oligosaccharide structure was accomplished by enzymatic deglycosylation with recombinant PNGase F or with endo-β-galactosidase. The purified, deglycosylated CHIP28 was fully functional in reconstituted proteoliposomes and had the same secondary structure and tetrameric assembly in the membrane as native CHIP28. Interestingly, dimeric native CHIP28 in OG became monomeric after deglycosylation with PNGase F.

The inability to separate nonglycosylated from glycosylated CHIP28 in nondenaturing detergents indicates a very tight association. This was first observed by Smith and Agre (1991), and we have extended this observation using PBA chromatography. Major concerns were the choice of elution conditions to promote specific interaction of tetrahedral borate and the *cis*-diols of the oligosaccharide moiety, and to exclude hydrophobic and $\pi-\pi$ interactions with the phenyl group (Brena *et al.*, 1992). Since lectin chromatography also failed to separate the two CHIP28 protein forms, it was unlikely that nonglycosylated CHIP28 was bound directly to the PBA resin by any of the interactions described above, but indirectly through glycosylated CHIP28. Carbohydrate analysis indicated that the inability to separate the two CHIP28 protein forms could not be due to the interaction of O-linked oligosaccharides on the 28 kDa "nonglycosylated" CHIP28.

PBA chromatography was found to effectively remove aggregated CHIP28, cholesterol/lipids, and lower molecular weight protein impurities. Previously, the isolation of CHIP28 of similar purity required anion-exchange chromatography followed by preparative size-exclusion chromatography (Van Hoek *et al.*, 1993). In reconstituted proteoliposomes, PBA-purified CHIP28 had similar oligomeric size and functional properties to that prepared by our original procedure (Van Hoek *et al.*, 1993; Verbavatz *et al.*, 1993). The secondary structure content and the degree of glycosylation also did not differ. Notwithstanding the inability to separate nonglycosylated from glycosylated CHIP28, these findings demonstrate that PBA chromatography can be used to obtain a highly purified and homogeneous CHIP28 preparation.

Because nonglycosylated and glycosylated CHIP28 could not be separated under nondenaturing conditions by conventional chromatographic procedures, enzymatic deglycosylation was utilized. The ability of PNGase F to react with glycosylated CHIP28 suggests both N-linked glycosylation and the absence of a Fuc-α(1−3) substitution on the GlcNAc (Maley *et al.*, 1989), whereas the lack of effect of Endo H_f indicates the absence of a chitobiose core of high mannose. The reaction with endo-β-galactosidase, which cleaves at internal galactose residues linked to glucosamine and releases linear and branched GlcNAcβ(1−3)Gal oligosaccharides, indicates a polylactosaminyl oligosaccharide structure. This structure is also consistent with our observations that ECL and tomato lectin (data not shown) bind glycosylated CHIP28. Two other erythrocyte membrane glycoproteins have been described which contain polylactosamine-type oligosaccharides, band 3 and band 4.5 [for a review, see Fukuda (1985)]. On SDS-gels, the CHIP28 migrated to 28 kDa after deglycosylation with PNGase F, and to 30 kDa after treatment with endo-β-galactosidase, initially suggesting oligosaccharide chains of 15−35 kDa size. However, the

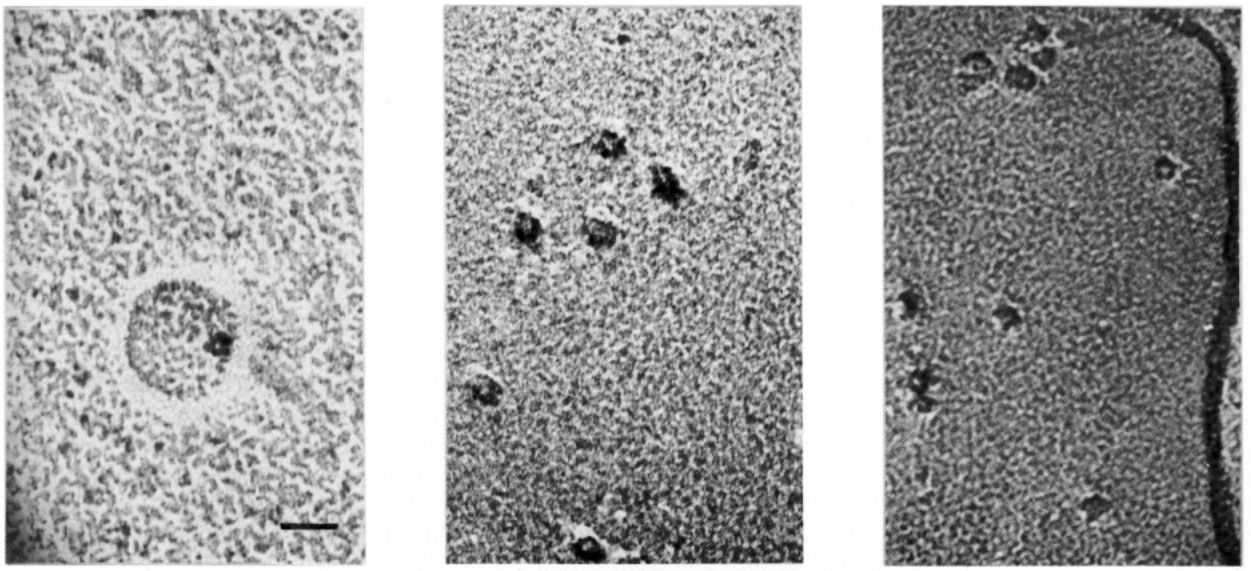

FIGURE 6: Freeze−fracture electron micrographs of reconstituted CHIP28 fractions. Rotary-shadowed freeze−fracture of native CHIP28 (left panel), endo-β-galactosidase-trimmed CHIP28 (middle panel), and PNGase F-deglycosylated CHIP28 (right panel). Bar denotes 20 nm.

average molecular mass determined from the molar ratio of monosaccharide units to protein was only ~5.4 kDa. The retarded migrational behavior of unmodified glycosylated CHIP28 in SDS−PAGE is probably related to relatively inefficient binding of SDS (Segrest & Jackson, 1972).

PNGase F was utilized for deglycosylation of CHIP28 because the deglycosylated protein was free of residual modified N-linked oligosaccharides. In addition, PNGase F does not bind to anion-exchange resins (Elder & Alexander, 1982) and thus could be separated easily from CHIP28. An important technical concern was the effective removal of unreacted CHIP28 and free carbohydrate. Because native CHIP28 adsorbs selectively to lectin resins, the CHIP28 protein which did not react with PNGase F could be removed by lectin chromatography. HPSEC and SDS−PAGE indicated that ECL−lectin chromatography in the presence of high concentrations of salt effectively removed unreacted native CHIP28 protein and free oligosaccharides.

The formation of CHIP28 monomers in OG following enzymatic deglycosylation by PNGase F was observed by the relatively large shift in peak position in HPSEC from an apparent molecular size of 52 to 32 kDa. The shift cannot be due solely to the removal of the 5.4 kDa oligosaccharide moiety. This conclusion is supported by studies of band 3, also containing polylactosamine oligosaccharides (Fukuda *et al.,* 1984), in which the HPSEC chromatogram underwent a small shift upon deglycosylation (Casey *et al.,* 1992). The mechanism by which monomers are formed after deglycosylation is unclear. Glycosylation might influence the monomer−monomer interaction by altering protein conformation, or, possibly, the polylactosamine chains from the glycosylated form of CHIP28 might associate directly with nonglycosylated CHIP28. In addition, the negative charge produced by PNGase treatment (asparagine is converted to aspartate) on the modified CHIP28 may have a role in the monomer−monomer interaction.

The proportion of CHIP28 which is glycosylated determined here differs from the initial observation that 10−25% of CHIP28 is glycosylated as estimated from the reactivity of glycosylated CHIP28 on immunoblots (Denker *et al.,* 1988). Our finding that ~53% of CHIP28 molecules are glycosylated was based on amino acid analysis from electroblots and densitometry of gel lanes, containing 5−80 μg of protein. The 1.1 ratio may represent a slight overestimate, because a small amount of nonglycosylated CHIP28 migrated as a dimer in SDS (Denker *et al.,* 1988) and would contribute to the amount of glycosylated CHIP28 measured by amino acid analysis and densitometry. Using purified deglycosylated CHIP28, 2−5% of dimeric CHIP28 was formed in SDS as determined by densitometry. These considerations suggest that one out of two CHIP28 monomers is glycosylated, consistent with the tight association of glycosylated (glyCHIP) and nonglycosylated CHIP28 (CHIP) as dimers in OG. Theoretically, three forms of homo- and heterodimers are possible, CHIP−CHIP, glyCHIP−glyCHIP, and glyCHIP−CHIP. The existence of CHIP−CHIP dimers is *inconsistent* with the results from PBA and lectin chromatography because dimeric CHIP28 was bound specifically through the carbohydrate moiety. The existence of glyCHIP−glyCHIP and glyCHIP−CHIP is *consistent* with a carbohydrate-mediated interaction. The conclusion that one out of two CHIP28 monomers is glycosylated excludes glyCHIP−glyCHIP in native CHIP28 as a major form.

The determination of the oligomer size as a dimer in OG differs from previous data reporting that CHIP28 formed tetramers in Triton X-100 (Smith & Agre, 1991). This difference might be due to the detergent or may be related to the size calibration procedure. The tetrameric protein size from nonequilibrium sedimentation analysis and gel filtration in the Smith and Agre study was determined using water-soluble proteins with known Stokes' radii and sedimentation coefficients. The determination of protein sizes of native CHIP28 and deglycosylated CHIP28 by HPSEC reported here was based on the relative mobilities of membrane proteins, including MIP26, bacteriorhodopsin (bR), and papain-digested bR (Van Hoek *et al.,* 1993). To determine the size of solubilized membrane proteins, column calibration should preferably be performed with membrane proteins. As was pointed out by Le Maire *et al.* (1986), solubilized membrane proteins behave differently, and their Stokes radii

are often overestimated when the column calibration is performed with water-soluble globular proteins.

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