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Research Article

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Human Red Cell Aquaporin CHIP

I. Molecular Characterization of ABH and Colton Blood Group Antigens

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Abstract

Blood group antigens are structural variants in surface carbohydrate or amino acid polymorphisms on extracellular domains of membrane proteins. The red cell water channel-forming integral protein (Aquaporin CHIP) is a homotetramer with only one *N*-glycosylated subunit, however no CHIP-associated blood group antigens have yet been identified. Immunoblotting, monosaccharide composition analysis, and selective glycosidase digestions revealed that the CHIP-associated oligosaccharide contains ABH determinants and resembles a band 3-type glycan that cannot be cleaved from intact membranes by Peptide:*N*-glycosidase F. The molecular structure of the Colton antigens was previously unknown, but CHIP was selectively immunoprecipitated with anti-Co^a or anti-Co^b. The DNA sequence from Colton-typed individuals predicted that residue 45 is alanine in the Co(a+b-) phenotype and valine in the Co(a-b+) phenotype. The nucleotide polymorphism corresponds to a PflMI endonuclease digestion site in the DNA from Co(a-b+) individuals. These studies have defined antigens within two blood group systems on CHIP: (a) an ABH-bearing polylectosaminoglycan attached to a poorly accessible site in the native membrane; and (b) the Colton antigen polymorphism which may permit the identification of rare individuals with defective water channel expression. (*J. Clin. Invest.* 1994. 94:1043-1049.) Key words: erythrocyte membrane • blood group antigens • water channels • membrane protein glycosylation

Introduction

The field of human genetics originated from the study of human blood group antigens. Approximately 250 antigens have been described serologically and are members of 22 different blood group systems (1). The ABH antigens and a few other blood group antigens are known to be specific oligosaccharide structures formed by glycosyltransferases with altered monosaccharide specificities caused by polymorphisms in their genes (2).

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As seen within the MNSs system, the structures of many blood group antigens may result from amino acid substitutions in surface domains of red cell membrane proteins (for reviews see references 3 and 4), whereas other antigens such as Wr^b may result from the juxtaposition of two known membrane structures (5). Search for the molecular structures of blood groups Rh (6, 7), Kell (8), and Duffy (9) led to the identification of new red cell membrane proteins. Nevertheless, the molecular identities of several blood group antigens remain unknown.

Channel-forming integral protein (CHIP)¹ is an abundant membrane protein of red cells and renal proximal tubules (10-13). CHIP functions as a water-selective pore (14, 15) and is the archetypal member of the Aquaporin family of water transporters found in animals and plants (16, 17). The structure of CHIP has been partially characterized; the amino acid sequence deduced from the cDNA encodes a protein with six presumed bilayer-spanning domains, internal amino and carboxy termini, and two potential *N*-glycosylation sites (18). CHIP exists as a homotetramer comprised of identical, noncovalently associated subunits, although only one of four subunits contains an N-linked glycan (11). Asparagine-42 was shown to be the glycosylation site, and the proposed membrane topology of biologically active CHIP molecules was confirmed by insertional mutagenesis (19). The tetrameric organization is supported by freeze fracture of CHIP proteoliposomes (20, 21) and by high resolution electron microscopic analysis of two-dimensional membrane crystals which retained full biological activity (22).

CHIP was first identified because it contaminated preparations of the Rh membrane proteins (23, 24), however no CHIP-associated red cell antigens are known. Although its electrophoretic mobility is similar to the band 3 polylectosaminoglycan (25), the structure of CHIP-glycan is undefined, and the substoichiometric glycosylation remains unexplained. The locus of *Aquaporin-1*, the gene encoding CHIP, was identified at human chromosome 7p14 (26). This location is coincident with the location of the Colton blood group locus on 7p (27). The present study was conducted to characterize the CHIP-glycan and to determine if CHIP is the molecular site of the Colton polymorphism.

Methods

Materials. Polyclonal, affinity-purified rabbit antibodies to CHIP were previously described (11, 13). Anti-glyRh was obtained from rabbits immunized with partially purified Rh protein (23, 24); anti-CHIP immunoglobulin was removed by CHIP affinity chromatography (10, 11). Typed red cells and antisera were obtained through the American Red

1. *Abbreviations used in this paper:* CHIP, channel-forming integral protein; CHIP28, nonglycosylated 28-kD subunit; glyCHIP, glycosylated 40-60-kD subunit.

Cross, Maryland Affiliate, the Johns Hopkins Hospital Blood Bank, or the International Blood Group Reference Laboratory. Red cells from two donors of type Co(a-b+) were obtained from the National Blood Transfusion Service (Cardiff, Wales). Human anti-Co3 was a gift from J. Moulds (Gamma Biologicals, Houston, TX). Molecular biologic enzymes and reagents were from GIBCO BRL (Gaithersburg, MD). Glycosidases were from Genzyme Corp. (Cambridge, MA). Carrier free ^{125}I was from New England Nuclear (Boston, MA). Other reagents and supplies were from Bio-Rad (Melville, NY), Schwarz/Mann (Spring Valley, NY), Sigma Immunochemicals (St. Louis, MO), Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ), Eastman Kodak Co. (Rochester, NY), or J. T. Baker, Inc. (Phillipsburg, NJ).

Electrophoresis and immunoblot methods. SDS-PAGE was performed with the buffer system of Laemmli (28), and immunoblotting was performed with visualization by ^{125}I -protein A autoradiography (11) or enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) as described (13).

Carbohydrate studies, A and B antigens. CHIP protein was purified from group A, B, AB, and O red cells by hydroxylapatite chromatography in SDS (11). Nonglycosylated CHIP (28 kD) and glyCHIP (40–60 kD) were separated by preparative SDS-PAGE and eluted into 0.1% SDS, 10 mM sodium phosphate (pH 7.2), 1 mM Na_2EDTA and concentrated over a Centricon-10 (Amicon, Beverly, MA). The samples were analyzed by SDS-PAGE immunoblot by reaction with human primary antisera (anti-A or anti-B [Immucor, Norcross, GA] or anti-A,B [Ortho Pharmaceutical, Raritan, NJ]) and rabbit anti-human IgM secondary antiserum, with visualization by ^{125}I -protein A autoradiography.

Glycosidase studies. CHIP protein was purified in Triton X-100 by Mono Q chromatography, and $\sim 20 \mu\text{g}$ of protein was precipitated with -20°C acetone, resuspended in 6% SDS, 50 mM sodium phosphate (pH 7.4), and labeled with carrier free ^{125}I by chloramine T oxidation as described (11). The ^{125}I -CHIP and ^{125}I -glyCHIP subunits were separated by SDS-PAGE. The gel slices were washed extensively in 10% methanol, freeze dried, and digested with 100 $\mu\text{g}/\text{ml}$ of α -chymotrypsin in 50 mM NH_4HCO_3 at 37°C for 12 h and again with fresh protease for 6 h. Fractions corresponding to the ^{125}I -glycopeptides were injected onto a Superose 12 column equilibrated with 20 mM Tris-HCl (pH 7.5), 0.2 mM NaCl running at 1 ml/min. ^{125}I -glycopeptide fractions were suspended in deionized water, and aliquots were digested for 20 h at 37°C with the following glycosidases as specified (Genzyme Corp.): (a) endoglycosidase H, 10 mU in 50 μl 50 mM sodium phosphate (pH 6), 1 mM phenylmethylsulfonyl fluoride (activity confirmed by digestion of ovalbumin); (b) endo- β -galactosidase, 50 mU in 50 μl sodium acetate (pH 5.8), 0.2 mg/ml bovine serum albumin; (c) Peptide:N-glycosidase F (N-glycanase), 0.5 U in 50 μl 0.1% SDS, 1% Nonidet P-40, 150 mM sodium phosphate (pH 7.4), 30 mM β -mercaptoethanol, 10 mM 1,10 phenanthroline. Control tubes were incubated identically without glycosidase. The digests were analyzed by Superose 12 chromatography.

Monosaccharide compositional analysis. CHIP protein was purified from 100 ml of human group AB red cells by POROS Q/H chromatography (15). SDS was added to a final concentration of 1%, and 900 μg CHIP protein was electrophoresed through a 5-cm SDS-PAGE acrylamide column using a model 491 Prep Cell (Bio-Rad). The glyCHIP was eluted into electrophoresis buffer (28), concentrated over a Centricon-10 (Amicon), and washed with 0.01% SDS. Duplicate microtubes with 0.28 nmol of glyCHIP protein or tubes with an equivalent volume of buffer control were precipitated with ethanol at -20°C . As a control, bovine fetuin (0.82 nmol) was analyzed in parallel without alcohol precipitation. The samples were hydrolyzed by addition of 2 N trifluoroacetic acid or 6 N HCl, incubated for 4 h at 100°C , and dried under vacuum. The hydrolysates were resuspended in 200 μl deionized water. Samples equivalent to 0.122 nmol of fetuin (known) or not more than 0.20 nmol of glyCHIP (based on original starting material) were injected onto the Dionex Glyco Station (Dionex Corp., Sunnyvale, CA) and analyzed using manufacturer's instructions.

Colton immunoprecipitations, intact red cells. 1 ml of packed red cells was incubated in 4 ml of 5 mM sodium phosphate (pH 8), 150 mM NaCl (PBS) with 10 mg of trypsin (bovine pancreas, 10,200 U/

mg) for 45 min at 37°C and then was washed four times with PBS (pH 7.3) as described (29). 5 ml of anti-Co^a, anti-Co^b, or anti-Co³ serum was added and incubated for 1 h at 37°C and then incubated overnight at 4°C . The red cells were pelleted and washed three times in 30 ml of PBS, and membranes were prepared by lysis in 30 vol of 5 mM sodium phosphate (pH 7.4) as described (30). The membranes were suspended in 6 vol 2% (wt/vol) Triton X-100 in PBS (pH 7.3) containing 2 mM phenylmethylsulfonyl fluoride and 5 mM K_2EDTA (TX-PBS) for 15 min at 20°C before centrifugation (40,000 g for 30 min at 4°C). 60 μl of a 50% (vol/vol) suspension of protein G Sepharose in TX-PBS was added to the supernatant for 1 h. The resin was washed five times in the same buffer, and the immune complexes were eluted with 5% SDS and analyzed by SDS-PAGE immunoblot.

Colton immunoprecipitations, Triton X-100-solubilized membranes. Group O red cell membranes at 2 mg/ml were solubilized in 3% (vol/vol) Triton X-100, 20 mM Tris-HCl (pH 7.5), 1 mM Na_2EDTA , 1 mM NaN_3 by shaking for 1 h at 20°C . After pelleting at 44,000 g for 45 min at 4°C , the supernatant was diluted to 1% (vol/vol) Triton X-100 and divided into three aliquots and mixed with an equal volume containing: (a) group O pooled plasma (negative control); (b) anti-Co^a plasma from a group O donor (titer 64); or (c) 0.16 μg of affinity-purified anti-CHIP (positive control). The tubes were incubated for 30 min at 37°C and then for 18 h at 4°C before addition of 0.5 vol of protein A Sepharose CL-4B for 2 h at 4°C . The resin was pelleted, washed three times with 0.1% Triton X-100 in the same buffer, eluted in 2% (wt/vol) SDS, and analyzed by SDS-PAGE immunoblot with anti-CHIP.

Molecular genetic studies. Genomic DNA was isolated from peripheral blood lymphocytes and from EBV transformed lymphoblastoid cell lines as described (31). The following oligonucleotide primers corresponding to the four exons were used to amplify DNA templates (sequences complementary to those of the CHIP gene are in capital letters):

1.1S: 5'-gcgaaTTCAAGAAGAAGCTCTTCTGG-3'

1.1A: 5'-cgggtACCAGTGCCTTTCAGCATCA-3'

2.1S: 5'-cgggtACCAGTCTCACCACCTCTC-3'

2.1A: 5'-cggaaTTCCCCACCTCCATCTGG-3'

3.1S: 5'-CTATGACTCTCTGCCTTCGC-3'

3.1A: 5'-GGGACACCAAAGCTTCCCAC-3'

4.1S: 5'-GAGTGGAGCCCTCTGAACAC-3'

4.1A: 5'-CAGACCCCTTCTATTGGGC-3'.

Restriction endonuclease sites (underlined) facilitated directional subcloning of the PCR products. Sense oligonucleotide 1.1S is complementary to bases 13–33 of the human CHIP cDNA (18). Antisense oligonucleotide 4.1A is complementary to sequences near exon IV translational termination sequences. Other oligonucleotides are complementary to sequences from splice donor (S) and acceptor (A) CHIP intron sequences (GenBank accession numbers L11321–L11326; reference 26).

Polymerase chain reactions contained 100 ng genomic DNA, 25 pmol of each primer, 0.2 mM of each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 0.7–1.0 mM MgCl_2 in a 50- μl volume. After denaturation for 5 min at 95°C , AmpliTaq DNA polymerase (2 U; Perkin-Elmer Corp., Norwalk, CT) was added, and 35 cycles of amplification were performed: denaturation for 1 min at 94°C , annealing for 1 min at 50 – 60°C , and extension for 1 min at 72°C . PCR products were separated on 3–4% agarose gels and stained with ethidium bromide. Exon I PCR samples of genomic DNA from two unrelated individuals with each of the phenotypes Co(a+b-), Co(a+b+), and Co(a-b+) were also digested with PflMI (United States Biochemical Corp., Cleveland, OH), run on a 6% polyacrylamide gel in TBE (90 mM Tris, pH 8.0, 90 mM boric acid, 2 mM Na_2EDTA), and stained with ethidium bromide. The PCR products were gel purified and subjected to double-

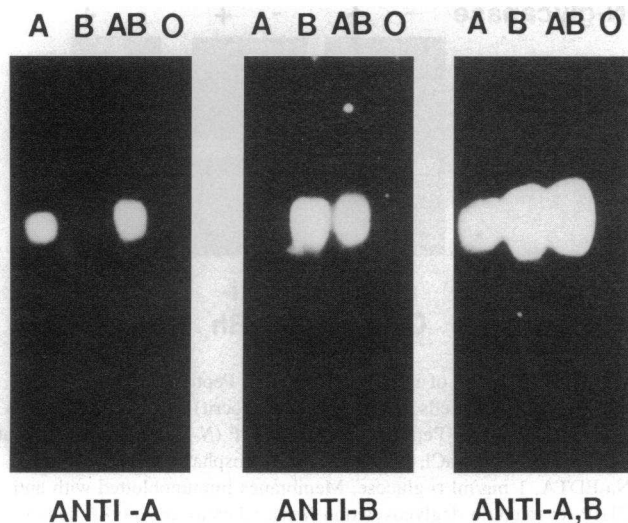


Figure 1. Immunoblot analysis of Aquaporin glyCHIP subunits isolated from red cells of defined ABO phenotypes. Red cell CHIP protein from group A, group B, group AB, and group O donors was purified to homogeneity, and 40–60-kD glyCHIP was isolated. Approximately 7 μ g of glyCHIP protein was electrophoresed into 12% SDS-PAGE gels, transferred to nitrocellulose membrane, incubated with antisera specific for A and B antigenic structures, and visualized by autoradiography (see Methods).

stranded DNA sequencing (GIBCO BRL) with [γ - 32 P]ATP (5,000 Ci/mmol; Amersham Corp.). Exon I PCR products from Co(a+b $-$) and Co(a-b+) samples were also digested with EcoRI and KpnI, ligated into pBluescript II (Stratagene, La Jolla, CA), and sequenced by the dideoxynucleotide chain termination with Sequenase 2.0 (United States Biochemical Corp.).

Results

Aquaporin CHIP-glycan. Although a subset of CHIP subunits are known to bear asparagine-linked carbohydrates (11), the molecular composition of these oligosaccharides has not been defined. CHIP was purified to homogeneity from red cells of known ABO phenotype. To separate the 40–60-kD glycosylated subunits (glyCHIP) from the 28-kD nonglycosylated subunits, purified total CHIP was denatured by SDS, loaded onto a preparative SDS-PAGE slab gel, and the glyCHIP fractions were eluted and concentrated. Specific reactions were observed when the glyCHIP fractions from typed blood were analyzed by SDS-PAGE immunoblot with antisera specific for A and B blood group antigens (Fig. 1).

The structures of the CHIP-glycans were characterized further by digestion with specific glycosidases. Purified CHIP was labeled with 125 I, and 28-kD nonglycosylated CHIP subunits and 40–60-kD glycosylated subunits were eluted separately from preparative SDS-PAGE gels and digested with α -chymotrypsin. Whole digests were then analyzed by gel filtration chromatography over a Superose 12 column (Fig. 2, A and B). The profiles were identical except for a small peak with more rapid elution in the glyCHIP preparation (Fig. 2 B, *bracket*). This peak which consistently exhibited the same mobility when rechromatographed over the same column (not shown) represented the 125 I-glycopolypeptides. Aliquots of the glyCHIP peak were digested with specific glycosidases, and the elution pattern of control

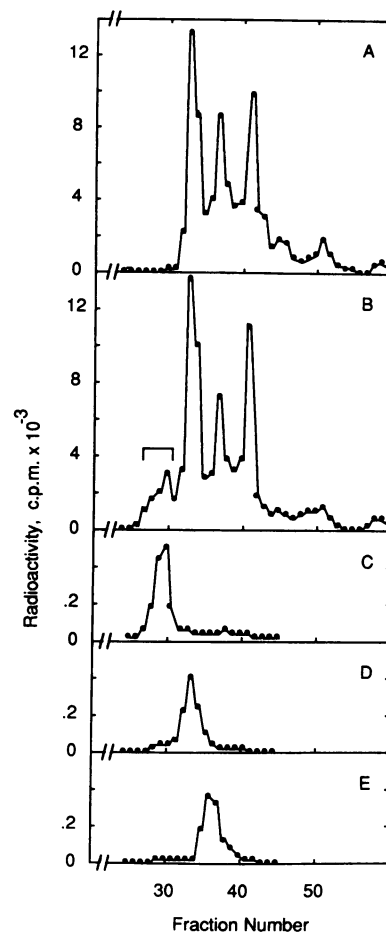


Figure 2. Gel filtration chromatography of 125 I-glyCHIP peptide fragments after digestion with various glycosidases. (A) Isolated 28-kD CHIP or (B) 40–60-kD glyCHIP was labeled previously with 125 I, digested with α -chymotrypsin, and injected onto a Superose 12 column while 0.5-ml fractions were collected (see Methods). The 125 I-glyCHIP peptide fraction (B, *bracket*) was rechromatographed directly (not shown) or (C) after digestion with endoglycosidase H, (D) endo- β -galactosidase, or (E) Peptide:N-glycosidase F.

aliquots incubated without glycosidase was not altered (not shown). After digestion with endoglycosidase H, the elution profile of 125 I-glyCHIP was identical to the undigested control (Fig. 2 C), indicating the lack of high mannose structures within the glycan. The elution of 125 I-glyCHIP was significantly retarded after digestion with endo- β -galactosidase (Fig. 2 D), indicating the existence of internal galactose-*N*-acetylglucosamine repeating units. A single peak with still slower chromatographic elution was found after digestion with Peptide:N-glycosidase F, an enzyme known to strip the oligosaccharides from their asparagine linkage yielding free 125 I-peptides (Fig. 2 E). Compositional analysis of oligosaccharides isolated from purified glyCHIP was performed with the Dionex Glyco Station (Dionex Corp.) which confirmed the existence of sugars known to comprise poly-lactosaminoglycans similar to that of band 3:*N*-acetylglucosamine, mannose, galactose, and fucose (Table I). In addition, the presence of ABH antigens within the CHIP glycan was also confirmed by the identification of the sugars which define ABH antigenic sites: *N*-acetylgalactosamine, galactose, and fucose.

Table 1. Monosaccharide Composition of CHIP-Glycan from a Group AB Blood Donor

| | Fetuin* | | | CHIP [‡] | |
|-----------|-------------|---------|--------------------|-------------------|----------------------------------|
| | Composition | | Ratio* expected | Composition | Ratio [‡] calculated |
| | nmol | mol/mol | | nmol | |
| Fucose | 0 | 0 | 0 | 0.15 | 3.3 |
| GalNAc | 0.49 | 4.0 | 3 | 0.17 | 3.6 |
| GlcNAc | 2.27 | 18.6 | 15 | 0.67 | 14.4 |
| Galactose | 1.65 | 13.5 | 12 | 0.42 | 9.0 |
| Mannose | 1.17 | 9.6 | 9 | 0.14 | 3.0 |

* Composition corresponds to monosaccharides recovered from 0.122 nmol of fetuin. The expected ratios of monosaccharides were obtained from published analyses (44). [‡] Composition corresponds to monosaccharides recovered from 0.2 nmol of CHIP. An efficiency of ~25% resulted from the alcohol precipitation step before hydrolysis (see Methods). [‡] To estimate the monosaccharide composition of an individual CHIP-glycan, the quantity of each monosaccharide was normalized to the quantity of mannose which exists as a tri-mannose core in the polylectosaminoglycan on band 3 (25).

The accessibility of the glycan at the red cell surface was evaluated by incubating intact red cells with Peptide:*N*-glycosidase F. When assessed by anti-CHIP immunoblot, the electrophoretic mobility of glyCHIP was not altered by this digestion (Fig. 3). The same blots were stripped and incubated with anti-glyRh, an antibody specific for the 50-kD glycosylated subunits of the Rh antigens (Rh50A) (32), and complete digestion to the 30-kD core peptide was confirmed. Inspection of silver-stained SDS-PAGE slabs also revealed increased mobility of glycophorin A after digestion. The inaccessibility of CHIP to Peptide:*N*-glycosidase F persisted even when intact red cells were previously digested with chymotrypsin (data not shown), suggesting that the glycosylation site was not inaccessible simply because of steric obstruction by other surface components. This inaccessibility was only observed while CHIP was in the membrane. SDS-denatured, purified CHIP is highly sensitive to Peptide:*N*-glycosidase F digestion (10, 11), and octylglucoside solubilized, purified CHIP was also digested by the enzyme at high concentrations (data not shown).

Colton blood group antigens on Aquaporin CHIP. Membranes prepared from red cells of known Colton phenotypes were immunoblotted with anti-CHIP. Membranes from Co(a+b-) and Co(a-b+) individuals exhibited equivalently strong reactions over CHIP28 and glyCHIP subunits, whereas membranes from a Co(a-b-) sample (donor G.S.) gave no reaction (Fig. 4 A). Intact Co(a+b-), Co(a-b+), and pooled red cells were reacted with anti-Co^a, anti-Co^b, or anti-Co³, and immunoprecipitates were analyzed on immunoblots with anti-CHIP (Fig. 4 B). Anti-Co^a precipitated CHIP from Co(a+b-) red cells but not from Co(a-b+) red cells. Conversely, anti-Co^b precipitated CHIP from Co(a-b+) red cells but not from Co(a+b-) red cells. Anti-Co³ is an antibody from a patient with the Colton null phenotype and reacts with red cells of all Colton phenotypes (33); anti-Co³ precipitated CHIP from pooled red cells (Fig. 4 B). To assess the possibility that the Colton antigens are simple peptide epitopes, immunoblotting



Figure 3. Digestion of intact red cells with Peptide:*N*-glycosidase F. Washed red blood cells (25 μ l, 33% hematocrit) were incubated with 1 U of glycerol-free Peptide:*N*-glycosidase F (*N*-glycanase) for 20 h at 37°C in 150 mM NaCl, 50 mM sodium phosphate (pH 7.4), 1 mM Na₂EDTA, 1 mg/ml D-glucose. Membranes immunoblotted with anti-CHIP revealed no deglycosylation (left), whereas anti-glyRh demonstrated complete deglycosylation of the Rh glycopeptide (center) and silver staining of the SDS-PAGE slab revealed shift in the mobility of glycophorin A (right, arrow).

with anti-Co^a antisera was attempted on nitrocellulose filters containing purified CHIP, however, high background binding obscured interpretation. Attempts to immunoprecipitate SDS-denatured, nonglycosylated CHIP subunits were not successful, suggesting that linear peptide sequence alone may not comprise the Co^a antigen. Extraction of red cell membranes with Triton X-100 is known to solubilize CHIP in its tetrameric form (11). Triton X-100-solubilized CHIP protein was immunoprecipitated with anti-Co^a but not control plasma (Fig. 4 C), suggesting that the surface conformation of the protein may be required for antigenicity. Since the CHIP-associated glycan could not be removed by digestion of intact red cells with Peptide:*N*-glycosidase F (Fig. 3), hemagglutination studies using Colton antisera could not be used to determine the possible role of the oligosaccharide within Colton antigenic structures.

Molecular genetic analysis of Colton polymorphism. CHIP is encoded by *Aquaporin-1*, a gene which contains four exons (26). DNA was obtained from two unrelated individuals with each of the known Colton phenotypes: Co(a+b-), Co(a-b+), and Co(a+b+). Each exon was amplified by polymerase chain reaction using primers corresponding to untranslated and intron sequences, and the nucleotide sequences of each coding region were determined by dideoxynucleotide sequencing. A single difference was noted at nucleotide 134 which was C in DNA from the Co(a+b-) samples and T in the Co(a-b+) samples (Fig. 5 A). This substitution will change the coding sequence at residue 45 from alanine in Co(a+b-) individuals to valine in Co(a-b+). DNA from Co(a+b+) individuals contained both nucleotides (not shown).

The first exon of CHIP contains a site for the restriction endonuclease PflMI which would cleave the 444 bp exon I PCR product into two fragments of 300 bp and 144 bp (18). The C to T substitution at the codon for residue 45 was predicted to create a second PflMI restriction site in CHIP exon I DNA from Co(a-b+) individuals. PflMI digestion would cut the 300 bp fragment of Co^b alleles into 174 and 126 bp pieces (Fig. 5 B). CHIP exon I DNA amplified from each of the Colton phenotypes was digested with PflMI and analyzed by polyacrylamide gel electrophoresis. Samples from two unrelated Co(a+b-) in-

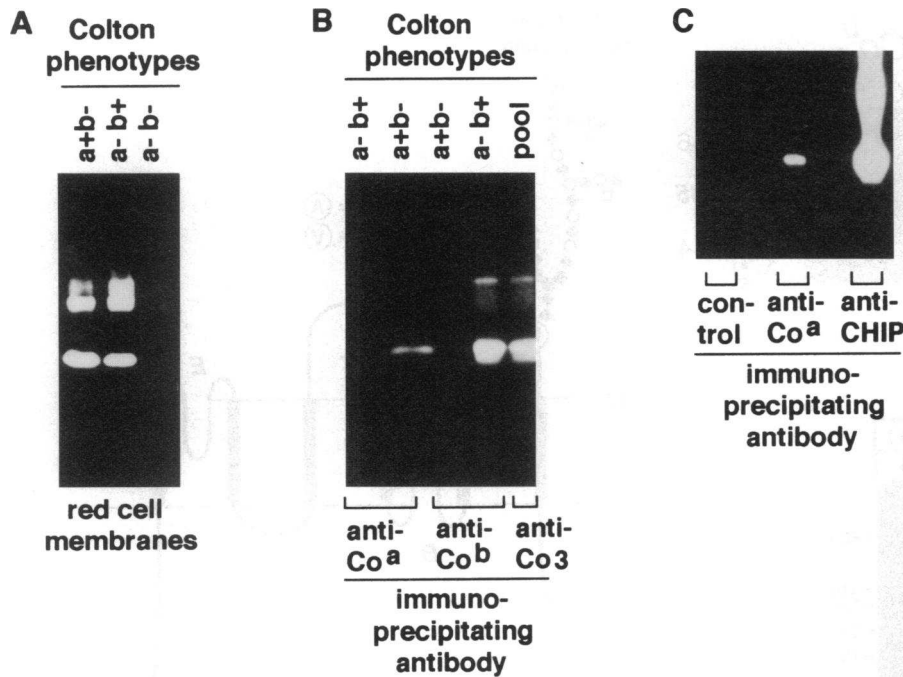


Figure 4. Anti-CHIP immunoblots of red cell membranes and Colton immunoprecipitates. (A) Membranes were prepared from red cells of defined Colton phenotypes and analyzed by immunoblot with anti-CHIP specific for the carboxy terminus or antibody specific for amino terminus (not shown). (B) Intact red cells of defined Colton phenotypes were reacted with the Colton-specific antisera (see Methods), and immunoprecipitates were analyzed by immunoblot. (C) Membranes prepared from group O, Co(a+b-) red cells were solubilized in Triton X-100 and then reacted with group O plasma (control), anti-Co^a, or anti-CHIP, and immunoprecipitates were analyzed by immunoblot.

dividuals contained bands of ~ 300 and 144 bp, while samples from two unrelated Co(a-b+) individuals contained bands of ~ 174, 144, and 126 bp. As expected, the samples from the unrelated Co(a+b+) individuals contained both patterns (Fig. 5 B).

Discussion

These studies have identified and characterized antigens from two different blood group systems on Aquaporin CHIP (Fig. 6). The oligosaccharide attached to asparagine-42 resembles a band 3 polylactosaminoglycan with ABH determinants, and the Colton polymorphism was defined at residue 45. These findings support the generally held view that blood group antigens are polymorphisms in red cell membrane surface structures. More importantly, evaluation of these CHIP-associated antigens has also provided new insight into the structure of the molecule and promises to provide further explanations for the importance of CHIP in transmembrane water movements.

Previous studies revealed that a subpopulation of 40–60-kD CHIP subunits (glyCHIP) contained a large oligosaccharide which could be cleaved with Peptide:N-glycosidase F after the protein is denatured in SDS (10). Curiously, digestion of intact red cells with Peptide:N-glycosidase F failed to cleave the glycan (Fig. 3). Triton X-100-solubilized CHIP oligomers were quantitatively adsorbed onto wheat germ agglutinin-Sephadex, although free 28-kD subunits were not, providing evidence that the protein is an oligomer (11). Additionally, the glycan was apparently attached to only a subpopulation of CHIP subunits in young and old red cells, indicating that deglycosylation was not ongoing (11). Substoichiometric glycosylation of CHIP was also found when the single CHIP cRNA was injected into *Xenopus* oocytes (14). Other integral membrane proteins are entirely glycosylated (e.g., band 3, glycophorins, and glucose transporters), although a few are entirely nonglycosylated and exist in the membrane in a complex with glycosylated polypeptides produced by different genes (e.g., the 30-kD Rh polypeptides) (32).

Substoichiometric glycosylation of CHIP may be explained by physical constraints at the surface glycosylation site which confer relative inaccessibility to glycosyltransferases during biogenesis or glycosidases during *in vitro* studies. Asparagine-42 was identified as the only surface N-glycosylation site (19). If asparagine-42 residues on individual subunits are located near each other while within the CHIP tetramer, the glycosyltransferases may gain access to only one potential site. Alternatively, glycosylation of asparagine-42 may be hindered if it is located very close to the outer leaflet of the lipid bilayer. If CHIP subunits are oligomerized cotranslationally, three nonglycosylated CHIP28 subunits existing in a tetramer with one glyCHIP subunit will be rapidly targeted together to the Golgi apparatus and plasma membrane before unoccupied potential N-glycosylation sites are modified by the transferase. Consistent with this, studies of mutant CHIP molecules expressed in *Xenopus* oocytes revealed that dysfunctional mutants which were apparently retained in the endoplasmic reticulum were disproportionately glycosylated with high mannose glycans, all four subunits of certain CHIP mutants being glycosylated (34, 35). These studies endorse the hypothesis that substoichiometric glycosylation is a kinetic feature resulting from relative inaccessibility of asparagine-42.

Identification of the Colton polymorphism at residue 45 of CHIP resulted from the observation that the chromosomal loci both exist on the short arm of human chromosome 7 (26, 27). The presence of this protein blood group antigen at loop A of the CHIP polypeptide further supports the proposed membrane topology (19). It cannot be stated yet whether the glycan at asparagine-42 contributes to the Colton recognition site at residue 45, since it proved impossible to deglycosylate CHIP while the protein was imbedded in the membrane. Also, preliminary attempts to confer Co^a antigenic sites on *Xenopus* oocytes expressing CHIP were unsuccessful, although other oocyte surface structures or altered glycosylations may obscure the antigen.

A potentially important benefit which may emerge from these studies may be the identification of humans with mutations

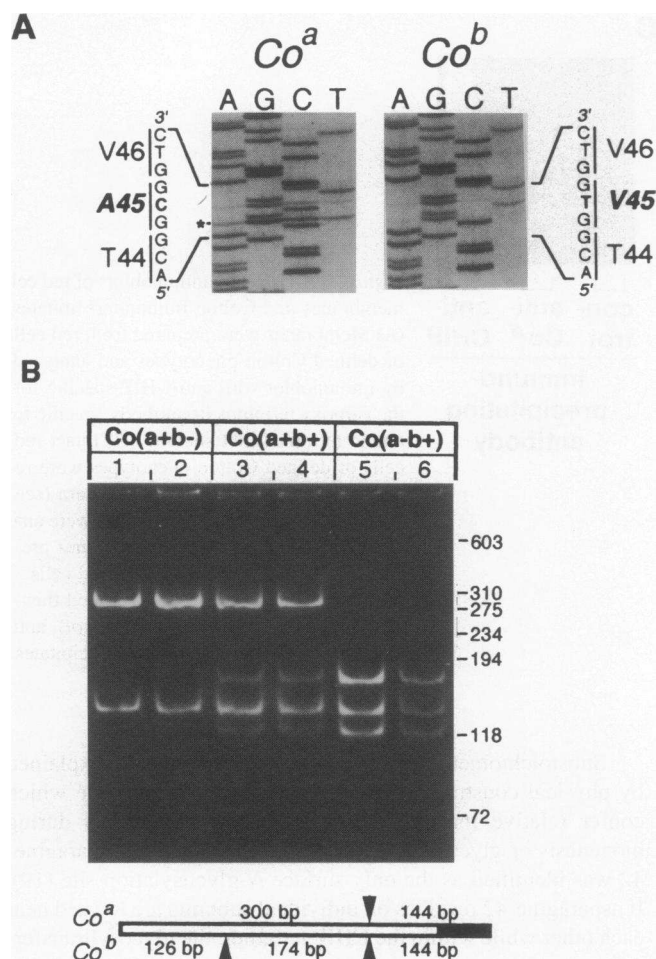


Figure 5. Molecular genetic identification of the Colton polymorphism. (A) Dideoxynucleotide sequencing of exon I polymerase chain reaction product from DNA of a Co(a+b⁻) and a Co(a-b⁺) individual. A single nucleotide difference was identified (C and T) resulting in a coding polymorphism (alanine and valine). Electrophoretic compression artifact is noted with an asterisk. (B) PflMI digestion patterns of the 444 bp exon I polymerase chain reaction product from DNA of individuals with defined Colton phenotypes. The diagram depicts the fragments of 300 and 144 bp in Co^a alleles and fragments of 126, 174, and 144 bp in Co^b alleles.

in the coding sequence of CHIP or mutations affecting CHIP expression. Two common antithetical Colton antigens are known, Co^a and Co^b. Approximately 92% of Caucasians are Co(a+b⁻), approximately 8% are Co(a+b⁺), and only 0.2% are Co(a-b⁺) (36). Colton antigens cause clinical difficulties very infrequently, although maternal-fetal incompatibility and transfusion reactions are known. The alanine-valine Colton polymorphism itself is unlikely to be of any functional significance, since residue 45 is leucine in murine CHIP cDNAs (37, 38). The power of world-wide blood group referencing makes the rarest of phenotypes accessible, and the single Co(a-b⁻) red cell membrane sample in the reference collection was found to lack CHIP by immunoblot. Only a few individuals have ever been reported to lack the Colton antigens. An association of Colton deficiency and monosomy 7 has been reported in some cases of leukemia (39, 40) and a Colton-deficient individual

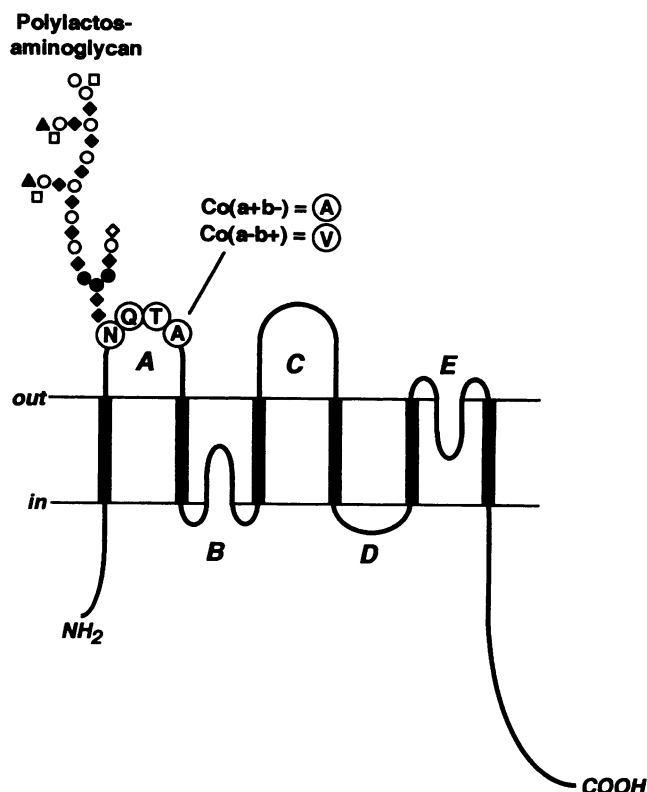


Figure 6. Membrane topology and structure of Aquaporin CHIP showing ABH and Colton antigens. The site of attachment at asparagine-42 and potential structure of the poly-lactosaminoglycan (adapted from reference 45) are illustrated. N-acetylglucosamine, \blacklozenge ; mannose, \bullet ; galactose, \square ; N-acetylgalactosamine, \blacktriangle ; fucose, \square ; or sialic acid, \diamond . The site of the Colton polymorphism at residue 45 is represented: Co^a, alanine (A); and Co^b, valine (V).

with congenital dyserythropoietic anemia has been identified (41, 42). Three other Co(a-b⁻) individuals were recently found with mutations in *Aquaporin-1* and lacked CHIP water channels (43). Future studies of these Co(a-b⁻) individuals are planned to further probe the roles of Aquaporin CHIP.

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