A dodecasaccharide motif of the low-sulfated chondroitin 4-sulfate (C4S) mediates the binding of *Plasmodium falciparum*-infected red blood cells (IRBCs) in human placenta. Here we studied the detailed C4S structural requirements by assessing the ability of chemically modified C4S to inhibit IRBC binding to the placental chondroitin sulfate proteoglycan. Replacement of the N-acetyl groups with bulky N-acetyl or N-benzoyl substituents had no effect on the inhibitory activity of C4S, whereas reduction of the carboxyl groups abrogated the activity. Dermatan sulfates showed ~50% inhibitory activity when compared with C4S with similar sulfate contents. These data demonstrate that the C4S carboxyl groups and their equatorial orientation but not the N-acetyl groups are critical for IRBC binding. Conjugation of bulky substituents to the reducing end N-acetyl-galactosamine residues of C4S dodecasaccharide had no effect on its inhibitory activity. Based on these results, we prepared photoaffinity reagents for the identification of the parasite proteins involved in C4S binding. Cross-linking of the IRBCs with a radioiodinated photoactivable C4S dodecasaccharide labeled a ~22-kDa novel parasite protein, suggesting strongly for the first time that a low molecular weight IRBC surface protein rather than a 200–400-kDa PfEMP1 is involved in C4S binding. Conjugation of biotin to the C4S dodecasaccharide photoaffinity probe afforded a strategy for the isolation of the labeled protein by avidin affinity precipitation, facilitating efforts to identify the C4S-adherent IRBC protein(s). Our results also have broader implications for designing oligosaccharide-based photoaffinity probes for the identification of proteins involved in glycosaminoglycan-dependent attachment of microbes to hosts.

An unusual feature of *Plasmodium falciparum* infection compared with the three other human malarial parasites, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae*, is that only the former parasite is able to sequester in the microvascular capillaries of various organs (1, 2). In *P. falciparum*-infected individuals, the infected red blood cells (IRBCs) adhesion molecules. This adherence property is believed to play an important role in the development of cerebral and other severe malaria syndromes (2–4). Several host molecules have been implicated in the IRBC adherence, including thrombospondin, CD36, intercellular adhesion molecule 1, vascular cell adhesion molecule 1, E-selectin, P-selectin, platelet endothelial cell adhesion molecule/CD31, and chondroitin 4-sulfate (1–6). In response to the development of adherent phenotype-specific immunity by hosts, parasites with different adherent receptor specificity evolve to efficiently survive in the host. However, eventually, when immunity to various adhesive parasite phenotypes is developed, the host effectively controls infection and avoids pathogenesis. This is generally true for malaria-immune people regardless of gender. However, in the case of pregnant women, placenta presents a new receptor for IRBC adherence allowing selection and propagation of parasites with different adherent specificities to which the host was not previously exposed. Accumulation of red blood cells infected with these phenotypically distinct parasites and subsequent monocyte/macrophage infiltration in the placenta lead to pregnancy-associated malaria characterized by poor pregnancy outcomes and maternal fatalities (7–9).

The discovery by Fried and Duffy (10) in 1996 that placental adherent IRBCs bind specifically to C4S but not to other glycosaminoglycans was confirmed by us and others (11–13). Particularly we showed that an unusually low sulfated CSPG local-
ized predominantly in the intercellular space is the natural receptor for the placental IRBC adherence (14). Further we and others demonstrated that optimal IRBC binding requires the participation of both 4-sulfated and 4-nonsulfated disaccharide moieties (15–18). We have also shown that a dodecasaccharide motif of the C4S with at least two 4-sulfated disaccharides is the minimum requirement for the efficient IRBC binding (15).

Whereas C4S has been conclusively shown to be the receptor for placental IRBC adherence, the identity of the parasite adhesive ligand expressed on the surface of the IRBCs remains unclear (19). It is widely thought that a subset of PfEMP1s containing domains analogous to the erythrocyte Duffy-binding ligands of P. vivax, which are known to be expressed on the IRBC surface, mediate the IRBC adherence to various host receptors, including CD36, intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and C4S (20–22). PfEMP1s comprise a family of 200–400-kDa, antigenically variant proteins encoded by ~60 var genes. It appears that the primary function of PfEMP1 is to prevent IRBCs from being readily recognized by the host immune system (23, 24). In 1999, two groups reported that two distinct PfEMP1 variants, one termed FCR3 var 1CSA (from FCR-3 parasite strain) and the other termed CS2 var 1CSA (from CS2 parasite strain), mediate IRBC binding to C4S in a strain-dependent manner (25, 26). In both cases, a specific domain called DBL-3y was reported to be involved in the IRBC binding. However, it remains to be authenticated whether PfEMP1 is the ligand for placental IRBC adherence because although some subsequent studies support the above observations others do not (19). Recently a well conserved var gene subfamily PfEMP1 named var 2CSA has been proposed to be the parasite ligand involved in C4S binding (27–29). The var 2CSA var gene has been shown to transcribe at high levels in several C4S-binding parasite strains, and antibodies raised against a recombinant var 2CSA can bind to IRBC surface in a gender-specific and parity-dependent manner (27, 28, 30, 31). However, the ability of the recombinant var 2CSA or anti-var 2CSA antibodies to inhibit IRBC binding to C4S has not been demonstrated. Finally direct biochemical evidence in support of either DBL-3y domain of var 1 or var 2 PfEMP1 binding to C4S is lacking, and the possible involvement of other proteins cannot be ruled out.

A major reason hindering the efforts to definitely identify the P. falciparum C4S-adhesive protein(s) is that the very low affinity of C4S-IRBC interaction precludes affinity purification. One approach to circumvent the problem will be to photoaffinity label the protein prior to affinity purification. A prerequisite for this approach is the elucidation of the role of functional groups in the C4S motif involved in binding to IRBC. Accordingly in this study, we investigated the potential of chemically modified C4S to inhibit the binding of IRBC to the placental CSPG. Based on the results, we prepared 125I-labeled photoactivatable derivatives of C4S dodecasaccharide by partially replacing the acetyl groups of GalNAc by 4-azidosalicylyl groups. The C4S-IRBC adhesion-inhibitory activities of these derivatives were almost comparable to that of the unmodified oligosaccharide. Cross-linking of the photoaffinity probes to the C4S-adherent IRBC surface specifically labeled a ~22-kDa parasite protein. In another approach, photoaffinity cross-linking of a probe containing a bioin residue at the C-1 of the reducing end of C4S dodecasaccharide to IRBC surface proteins enabled the isolation of the proteins by avidin affinity precipitation. Thus, our approaches offer, for the first time, strategies for the purification and biochemical characterization of the parasite adhesive protein(s). Moreover these procedures have important implications for designing photoactivatable probes for the identification of adhesive protein(s) involved in the glycosaminoglycan-dependent adherence of various pathogens to host cells.

### EXPERIMENTAL PROCEDURES

**Materials**—Proteus vulgaris chondroitinase ABC (110 units/mg), sturgeon notochord C4S (98% 4-sulfated, 1.5% 6-sulfated, and 0.5% nonsulfated disaccharides), and chondroitin sulfate D (28% 4-sulfated, 46% 6-sulfated, 23% 4,6-disulfated, and 3% nonsulfated disaccharides) were from Seikagaku America (Falmouth, MA). Ovine testicular hyaluronidase (2160 units/mg) was from ICN Biomedicals (Costa Mesa, CA), N-O-Bis(trimethylsilyl)acetamide; bCSA (52% 4-sulfated, 39% 6-sulfated, and 9% nonsulfated disaccharides); EDAC; DS (pig intestinal mucosa); sodium cyanoborohydride; anhydrous hydrazine; hydrazine sulfate; and acetic, propionic, butyric, hexanoic, and benzoic anhydrides were from Sigma. Percoll, Na125I (100 mCi/ml) Sephadex G-15, Sepharose CL-4B, Sepharose CL-6B, DEAE-Sepharose, and blue dextran were from Amersham Biosciences. Bio-Gel P-6, Bio-Gel P-10, 4–15% polyacrylamide gradient minigels, and protein molecular mass markers (10–250 kDa) were from Bio-Rad. Protease Inhibitor Mixture set I was from Calbiochem. ASBA, NHS-ASA, biotin hydrazide, chloramine T-immobilized polystyrene beads (lodobeads), biotin-immobilized avidin-agarose beads (NeutrAvidin beads), HPLC grade 6 M HCl, and micro-BCA protein assay kit were from Pierce. SYPRO Ruby protein gel stain was from Molecular Probes (Eugene, OR). Glyko® FACE ANTS labeling reagent kit was from Prozyme (San Leandro, CA).

**Placental CSPG**—The low sulfated, P. falciparum-IRBC-binding CSPG of the human placenta was isolated and purified as reported previously (13, 16).

**Parasites**—C4S-adherent F. falciparum were selected from a 3D7 parasite clone (derived from NF-54 strain) on plastic plates coated with the low sulfated CSPG purified from human placenta (13). These parasites are the same as those used in our previous studies (13, 15, 16). They bind efficiently to the placental CSPG and show exclusive specificity to C4S. The parasites were cultured in RPMI 1640 medium using human O-positive erythrocytes and O-positive serum as described previously (15).

**IRBC Adhesion Inhibition Assay**—Adhesion/inhibition assays were carried out according to Alkhalil et al. (15). Briefly a solution of the placental CSPG (200 ng/ml) in PBS, pH 7.2, was coated onto polystyrene Petri dishes (Falcon 1058) as circular spots (~4-mm diameter) for 4 h at room temperature and blocked with 1% BSA in PBS for 1 h. Suspensions (2%) of parasite culture (20–35% parasitemia) cell pellet in PBS, pH 7.2, were preincubated with C4S or the test C4S derivatives at room temperature for 30 min with occasional shaking and then overlaid onto the CSPG-coated spots. After 30 min at room temperature, the unbound cells were removed by washing with PBS,
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pH 7.2. The bound cells were fixed with 2% glutaraldehyde, stained with 1% Giemsa, and counted under a light microscope at 40× magnifications in five to six different fields. The number of cells in one-fourth (one grid) of these fields was counted. The average number of cells per grid was typically 65–110. These values were used to calculate the number of cells (4000–7000) bound per 1 mm² of CSPG-coated plates. In inhibition studies, the number of cells bound per 1 mm² in the presence of the inhibitor was calculated as a percentage of that bound in the absence of inhibitors. All assays were performed in duplicates and repeated two to three times.

Regioselective 6-O-Desulfation of bCSA—A partially 4-sulfated C4S that efficiently inhibits IRBC binding to the placental CSPG was prepared as reported previously (15). bCSA (0.5 g) was converted into its pyridinium salt by chromatography on Dowex 50W-X 8 (H⁺) followed by neutralization with pyridine. The lyophilized material was dissolved in anhydrous pyridine (100 ml), and N,O-bis(trimethylsilyl)acetamide (20 ml) was added and heated at 80 °C for 4 h under anhydrous condition. After cooling in an ice bath and dilution to 250 ml with ice-cold water to decompose excess sialylating reagent, the reaction mixture was neutralized with 0.2M NaOH, dialyzed against water, and lyophilized. The 6-O-desulfated chondroitin sulfate was applied onto a DEAE-Sephrose column (1.5 cm × 10 cm) in 50 mM NaOAc, pH 5.5, containing 100 mM NaCl, washed with the above buffer containing 0.2 M NaCl, and eluted with a gradient of 0.2–1 M NaCl. Fractions (3 ml) were collected, and aliquots were assayed for uronic acid content of the partially desulfated C4S and DS ranged from 28 to 78% (see Table 2).

Preparation of C4S Oligosaccharides—Oligosaccharides of varying sizes were prepared by the testicular hyaluronidase digestion of C4S (10 mg) followed by chromatography on a Bio-Gel P-6 column as described previously (15).

N-Decaetylation of C4S—The C4S was N-deacetylated by hydrazinolysis according to Shaklee and Conrad (33). C4S (70 mg) was dissolved in anhydrous hydrazine (1.2 ml) containing 1% hydrazine sulfate in a reaction ampoule and flushed with nitrogen. The ampoule was sealed and allowed to stand at room temperature for 24 h. The reaction mixture was neutralized with 0.1 M NaOH, dialyzed, and lyophilized. The extent of N-deacetylation was determined by nitrous acid treatment followed by analysis of the products on Bio-Gel P-10 columns (35). With hexanoic and benzoic anhydrides, the N-acetylation procedure was repeated three times to achieve near complete acylation of free -NH₂ groups, and any remaining -NH₂ groups were acetylated with acetic anhydride.

Reduction of Carboxyl Groups—To a solution of C4S (100 mg) in 15 ml of water solid EDAC (380 mg) was added, and the pH was maintained at 4.75 with 0.1 M HCl. After 2 h at room temperature, solid sodium borohydride (0.5–150 mg) was added over a period of 1 h, and the pH of the reaction mixture was maintained at 7.0 with the addition of 1 M HCl (36). The solution was then acidified to pH 5 with 0.1 M HCl to decompose the excess of borohydride. The carboxyl-reduced C4Ss were treated with 50 mM NaOH at 37 °C for 10–12 h to hydrolyze any EDAC ester of unreduced carboxylic acid groups, neutralized with 2 M acetic acid, dialyzed, and lyophilized.

2-O-Desulfation—Chondroitin sulfate D (5 mg) was dissolved in 10 ml of 0.05 M sodium hydroxide, and pH of the solution was adjusted to 12.5 by the addition of 1 M sodium hydroxide to a final concentration of 0.2 M (37). The solution was lyophilized, dissolved in 10 ml of water, dialyzed, and lyophilized.

Digestion of C4S and Its N-Acylated Derivatives with Chondroitinase ABC—The C4S and its derivatives (100 µg each) were digested with chondroitinase ABC (50 milliunits) in 50 µl of 100 mM Tris-HCl, pH 8.0, containing 30 mM NaOAc and 0.01% BSA at 37 °C for 5 h as described previously (13). The samples were analyzed by gel filtration on Bio-Gel P-10 columns (1.5 × 70 cm).

Analysis of N-Decaetylated C4S and Re-N-acetylated C4S by Nitrous Acid Degradation—The N-deacetylated, re-N-acetylated, or re-N-benzyloxy C4Ss (100 µg) were dissolved in 100 µl of 200 mM sodium acetate buffer, pH 3.8 (35). An equal volume of 1 M sodium nitrite was added, and the reaction mixture was allowed to stand at room temperature for 24 h and then chromatographed on Bio-Gel P-10 columns.

Gel Permeation Chromatography—The analysis of modified C4S and its nitrous acid or chondroitinase ABC degradation products was performed on columns of Sepharose CL-6B (1 × 50 cm), Bio-Gel P-6 (1.5 × 70 cm), or Bio-Gel P-10 (1.5 × 70 cm) using either 0.2 M sodium chloride or 0.1 M pyridine, 0.1 M acetic acid, pH 5.5. Fractions (0.67 or 2 ml) were collected and assayed for the uronic acid content.

Disaccharide Compositional Analysis—The C4S (5–10 µg) in 50 µl of 100 mM Tris-HCl, pH 8.0, containing 30 mM NaOAc and 0.01% BSA was digested with chondroitinase ABC (2.5–5 milliunits) at 37 °C for 6 h (13). The unsaturated disaccharides that are formed were analyzed on an amino-bonded silica PA03
column (YMC Inc., Milford, MA) using a Waters 600E HPLC system with a linear gradient of 16–530 mM NaH₂PO₄ over a period of 70 min at room temperature at a flow rate of 1 ml/min as described by Sugahara et al. (38). The elution of disaccharides was monitored on line by measuring absorption at 232 nm with a Waters 484 variable wavelength UV detector. The data were processed with the Millennium 2010 chromatography manager using an NEC PowerMate 433 data processing system.  

Polycyclamid Gel Electrophoresis of C4S Oligosaccharides—The oligosaccharide fractions (2–4 μg each) obtained after the Bio-Gel P-6 chromatography of the ovine testicular hyaluronidase digest of C4S were electrophoresed on 10% polyacrylamide gel (14 x 23.5 cm, 1.5-mm thick) using 90 mM Tris borate buffer, 2 mM EDTA, pH 8.3, as described previously (15). A total enzyme digest of C4S was used as oligosaccharide size marker. The gels were stained with 0.03% Alcian blue followed by silver nitrate (39).

Coupling of ANTS to the Reducing End of C4S Dodecasaccharide—Derivatization of the oligosaccharide at the reducing end with ANTS was carried out using the Glyko FACE oligosaccharide profiling kit according to the manufacturer's protocol (40, 41). The dried C4S dodecasaccharide (50 μg) was dissolved in 5 μl of ANTS reagent. After 15 min at room temperature, 5 μl of the reagent containing sodium cyanoborohydride was added and incubated at 37 °C for 18 h. In parallel, a mixture of oligosaccharides (50 μg) obtained by treatment of C4S with testicular hyaluronidase was derivatized with ANTS as above and used as size marker. Aliquots of the reaction mixture were analyzed by electrophoresis on 10% polyacrylamide gels using 90 mM Tris borate buffer, 2 mM EDTA, pH 8.3, and oligosaccharide bands were visualized either under UV light or by Alcian blue/silver nitrate staining (15, 39). The derivatized oligosaccharides were purified on Bio-Gel P-6 columns (1.5 x 70 cm).

Preparation of Photoactivable ASBA-C4S Dodecasaccharide—To a solution of C4S dodecasaccharide (150 μg) in 200 μl of 0.2 M sodium borate buffer, pH 7.8, ASBA (125 μg) in 10 μl of Me₂SO was added. After gentle shaking at room temperature for 1 h, sodium cyanoborohydride (50 μg) in 5 μl of 0.2 M sodium borate buffer, pH 7.8, was added three times at 10-min intervals. After 4 h, the ASBA-oligosaccharide conjugate was purified by gel filtration on Sephadex G-15 columns (0.6 x 30 cm) using 0.2 M NaCl. The column effluents were collected in 0.4-ml fractions. The excluded fractions containing ASBA-C4S dodecasaccharide conjugate were pooled, dialyzed against water using 500 molecular weight cutoff membranes, and lyophilized. The UV absorption of the conjugate was recorded (42).

Preparation of Photoactivable N-4-Azidosalicylyl Derivative of C4S Dodecasaccharide—The C4S dodecasaccharide was N-deacetylated by hydrazinolysis with a minor modification to the procedure described above for C4S. Briefly C4S dodecasaccharide (0.5 mg) dissolved in 200 μl of anhydrous hydrazine was heated in a sealed ampoule at 100 °C for 3 h. The solution was processed as outlined above for N-deacetylated C4S, and N-deacetylated oligosaccharide was purified by chromatography on Bio-Gel P-6 using 0.1 M pyridine, 0.1 M acetic acid, pH 5.5.

The N-deacetylated C4S dodecasaccharide (200 μg) was dissolved in 200 μl of PBS, pH 7.2, and 75 μg of NHS-ASA in Me₂SO (20 μl) was added (43, 44). After stirring overnight at room temperature in the dark, solid NaHCO₃ was added to saturation, and then 10 μl of 10% acetic anhydride in acetone was added two times at 10-min intervals to N-acetylate the remainder of the -NH₂ groups. The labeled N-4-azidosalicylicyl derivative of the C4S oligosaccharide was purified by chromatography on Sephadex G-15 columns (0.6 x 30 cm) using 0.2 M NaCl. The effluent was collected in 0.4-ml fractions, and the void volume fractions containing the derivatized oligosaccharide were pooled, dialyzed against water using 500 molecular weight cutoff membranes, and lyophilized.

Biotinylination of the Photoaffinity Probe—N-4-Azidosalicylicyl derivative of C4S dodecasaccharide (150 μg) was dissolved in 200 μl of 0.1 M sodium acetate, pH 5.5, and biotin hydrazide (25 μg) in Me₂SO (20 μl) was added (45). After stirring at room temperature in the dark for 2 h, sodium cyanoborohydride (50 μg) in 1 mM sodium hydroxide (5 μl) was added three times at 10-min intervals and stirred at room temperature for 16 h. The biotinylated oligosaccharide was chromatographed on Sephadex G-15 column (0.6 x 30 cm) using 0.2 M NaCl. The oligosaccharide derivative eluting at the void volume was collected, dialyzed as above, and lyophilized.

Radioiodination of the Photoactivable Probe—Na¹²⁵I solution (10 μl, 1 mCi) and two iodobeads were added to a solution of N-4-azidosalicylicyl derivative of C4S dodecasaccharide (40 μg) in 100 μl of PBS, pH 7.2 (42, 43). The solution was allowed to stand at room temperature for 1 h in the dark and then chromatographed on Sephadex G-15 columns (0.6 x 30 cm) using 0.2 M NaCl. The effluent was collected in 400-μl fractions, and the void volume fractions containing the radiolabeled (assessed by measuring ¹²⁵I activity in a γ-counter) photoactivable oligosaccharide derivative were used for cross-linking to the IRBC surface proteins.

Photoaffinity Labeling of the IRBC Surface Proteins—Suspensions of C4S-adherent or non-adherent parasite cultures (400-μl packed cell pellet in 2 ml of incomplete medium) with ~30% parasitemia were layered on sequential cushions of 40, 60, 70, 80, and 90% Percoll (2 ml each in PBS containing 6% sorbitol) and centrifuged at 3000 rpm for 10 min at room temperature in a Jouan CR 412 centrifuge (46, 47). The IRBC layers on 80 and 90% Percoll cushions that contained predominantly the late ring and early trophozoite stage parasites were collected, pooled, and washed five times with PBS, pH 7.2. To a suspension of enriched IRBCs (50 – 60-μl packed cell pellet) in 400 μl of PBS, pH 7.2, ¹²⁵I-labeled C4S dodecasaccharide derivative (1 μg, 10–12 x 10⁶ cpm) was added, incubated at 37 °C for 30 min in the dark, and irradiated with 254 nm UV light at room temperature for 30 min. The IRBCs were then washed four times each with 1 ml of PBS, pH 7.2.

To isolate the photolabeled C4S-adherent proteins of the IRBC surface by biotin-avidin affinity precipitation, IRBCs were cross-linked with biotin-conjugated, nonradiolabeled photoactivable oligosaccharide probe using the procedure outlined above. To test the specificity of the photoaffinity labeling, the IRBCs were first incubated with underivatized C4S oligosaccharide, the nonradiolabeled photoactive derivative (40 μg),
or heparin (20 μg) at 37 °C for 30 min (43). The IRBCs were washed with PBS, pH 7.2, and then labeled with 125I-labeled N-4-azidosalicyl derivative of C4S dodecasaccharide as described above.

**Extraction of Photoaffinity-labeled Parasite Proteins and Isolation of C4S-adherent Proteins by Avidin Affinity Binding**—The IRBC pellets, after labeling with 125I-labeled photoaffinity C4S oligosaccharide probe, were extracted sequentially with buffers containing 0.05% saponin, 0.5% Triton X-100, and 2% SDS. A mixture of protease inhibitors was added to the extraction buffers (48, 49). Thus, IRBCs were first extracted with 100 μl of 50 mM Tris-HCl, 150 mM NaCl, pH 6.8 (TBS) containing 0.05% saponin at 4 °C for 5 min followed by 2 × 50 μl of the above buffer containing 0.5% Triton X-100 at 4 °C for 30 min and finally with 2 × 50 μl of the buffer containing 2% SDS for 40–50 min at room temperature.

To test the feasibility of isolating C4S-adherent proteins by avidin affinity binding, the 2% SDS extracts of IRBCs labeled with biotin-conjugated photoactivable oligosaccharide were diluted to 0.2% SDS with PBS, pH 7.2, containing 0.5% Triton X-100. Avidin-immobilized agarose beads (25 μl), pretreated with 1% BSA and washed, were added and incubated overnight at 4 °C with gentle rocking. The beads were washed five times with 50 mM Tris-HCl, 150 mM NaCl, pH 7.2, and the bound proteins were released by heating the beads with 25 μl of 2× SDS-PAGE reducing sample buffer containing 100 mM dithiothreitol in a water bath at 90 °C for 6–8 min. The suspension was centrifuged, and the supernatant was analyzed by SDS-PAGE.

**Analysis of Photoaffinity-labeled Parasite Proteins by SDS-PAGE**—The IRBC extracts were electrophoresed under reducing conditions on 4–15% gradient gels according to Laemmli (50). The gels with radiolabeled proteins were fixed, dried, and exposed to x-ray films for autoradiography. The gels with non-radiolabeled proteins were fixed with 50% MeOH, 10% AcOH, exposed to x-ray films for autoradiography. The gels with radiolabeled proteins were fixed, dried, and exposed to x-ray films for autoradiography. The gels with non-radiolabeled proteins were fixed with 50% MeOH, 10% AcOH, 40% water and stained with SYPRO Ruby, and the protein bands were photographed using Eagle Eye™II with a dual intensity UV transilluminator (Stratagene).

**RESULTS**

**Assessment of the Functional Groups of C4S in the Binding of P. falciparum-IRBCs to Human Placental CSPG Receptor**

We have shown previously that chondroitin sulfate containing 30–50% of the disaccharide moieties 4-sulfated and the remainder nonsulfated can maximally inhibit the binding of *P. falciparum*-IRBC to human placental CSPG receptor (15). Accordingly a partially sulfated C4S (with 40% 4-sulfated, 59% nonsulfated, and 1% 6-sulfated disaccharides) was prepared by the regioselective 6-desulfation of bCSA (15, 32), referred to as unmodified C4S or C4S, and used for all experiments in this study.

**The Acetamido Group**—To determine whether the acetamido groups of GaINAc in C4S interact with IRBCs, the acetamid groups were removed by hydrazinolysis. Nitrous acid degradation followed by Bio-Gel P-10 chromatography indicated that the majority of the N-acetyl groups were removed because less than 90% of N-deacetylated C4S was converted into di- and tetrasaccharides (Fig. 1A). The N-deacetylated product was re-

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other structural alteration. When treated with chondroitinase ABC, N-hexanoyl and N-benzoyl derivatives of C4S were completely resistant to the action of the enzyme, whereas N-propionyl and N-butyryl derivatives of C4S were degraded into disaccharides at slower rates compared with N-acetylated C4S (Fig. 1B and data not shown). Under the conditions required for the quantitative conversion of N-acetylated C4S into disaccharides, ~70 and 20% of N-propionylated and N-butyrylated C4S, respectively, were degraded into disaccharides (Fig. 1B).

The N-deacetylated and N-acylated or N-benzoylated derivatives of C4S were evaluated for their ability to inhibit *P. falciparum*-IRBC binding to the placental CSPG receptor as reported previously (15). Although the N-deacetylated C4S was completely noninhibitory, all N-acylated C4S and the N-benzoylated C4S could efficiently inhibit the IRBC binding in a dose-dependent manner (Fig. 2). Of the latter, in each case, the inhibitory capacity was comparable to that of the unmodified C4S, demonstrating that N-acetyl group has no role in binding of IRBCs to C4S.

The Carboxyl Group—The carboxyl groups of C4S were converted to primary hydroxyl groups by esterification with EDAC followed by sodium borohydride reduction. Reduction with different amounts of sodium borohydride afforded C4S in which 14–80% carboxyl groups were reduced as determined by measuring the uronic acid content and analyzing products of chondroitinase ABC treatment on Bio-Gel P-10 columns (Fig. 3 and Table 1). The C4S in which ~14% of the carboxyl groups were reduced could inhibit IRBC adhesion in a dose-dependent manner comparable to that of the unmodified C4S (Fig. 3 and Table 1). However, the inhibitory activity of C4S in which ~25 and ~33% carboxyl groups were reduced showed, respectively, an ~25 and ~40% decrease, and reduction of ~50% of the carboxyl groups resulted in complete loss of activity (Fig. 4 and Table 1). These data indicate that the carboxyl groups of C4S are crucial for IRBC binding. The carboxyl groups in chondroitin sulfate are equatorial in the stable chair conformation of D-glucuronic acid, whereas carboxyl groups in DS are axially oriented in the stable chair conformation of L-iduronic acid. To determine whether the orientation of the carboxyl groups is important for the efficient interaction of C4S with IRBCs, we compared the inhibitory activity of partially sulfated DS to that of partially sulfated C4S with similar sulfate contents (Ref. 15; Table 2). The CSPG-IRBC binding-inhibitory activities of all the partially sulfated DSs studied were 40–50% lower compared with those of the partially sulfated C4Ss with similar sulfate content (Fig. 5). The most likely reason for the observed
we assessed the role of this hydroxyl group by studying the inhibition of IRBC binding to CSPG using CSD, which contains significant levels of sulfate at C-2, before and after selective 2-O-desulfation (37). The sulfate groups of CSD were regios-electively removed by treatment with alkali and by freeze-drying as described previously (37). Disaccharide composition analysis showed that ~70% of the sulfate groups at C-2 were removed by the above procedure (Table 3). The inhibitory activity of 2-O-desulfated CSD was similar to that of the unmodified CSD (data not shown), suggesting that hydroxyl groups at C-2 of C4S are not involved in IRBC binding.

The Requirement of Reducing End GalNAc of the C4S Dodecasaccharide for IRBC Binding—We have shown previously that the inhibitory activity of the C4S dodecasaccharide is comparable to that of the C4S polysaccharide (13). The reducing end sugar residue of the dodecasaccharide is readily amenable

### Table 1: Composition and P. falciparum IRBC adhesion-inhibitory activity of carboxyl-reduced C4S

| GAG type      | Uronic acid content (%) | Carboxyl group reduction (%) | IRBC adhesion-inhibitory activity (%) |
|---------------|-------------------------|------------------------------|---------------------------------------|
| C4S           | 36                      | 0                            | 100                                   |
| C4S-R1        | 31                      | 14                           | 100                                   |
| C4S-R2        | 27                      | 25                           | 100                                   |
| C4S-R3        | 24                      | 33                           | 60                                    |
| C4S-R4        | 16                      | 56                           | 0                                     |
| C4S-R5        | 12                      | 67                           | 0                                     |
| C4S-R6        | 7                       | 80                           | 0                                     |

* Determined by the carbazole colorimetric assay (13), and these values are in agreement with the extent of resistance of the partially carboxyl-reduced C4Ss to chondroitinase ABC digestion.

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**FIGURE 3.** Analysis of the chondroitinase ABC degradation products of the carboxyl-reduced C4S. The carboxyl groups of C4S were activated with EDAC at pH 4.75 for 2 h and reduced with varying amounts NaBH4 at pH 7.0 for 1 h. The partially reduced C4Ss were treated with chondroitinase ABC and analyzed on Bio-Gel P-10 (1.5 × 70 cm) columns using 200 mM NaCl. Fractions of 2 ml were collected, and aliquots were analyzed for uronic acid content (330 nm). V0 and Glc, respectively, represent void volume of the column and the elution position of glucose, C4S, C4S-R1, C4S-R2, C4S-R3, C4S-R4, C4S-R5, and C4S-R6 in which, respectively, 14, 25, 33, 56, 67, and 80% carboxyl groups were reduced.

**FIGURE 4.** Inhibition of IRBC adherence to the placental CSPG by carboxyl-reduced C4S. The carboxyl group-reduced C4Ss, prepared as outlined in Fig. 3, were assessed for their abilities to inhibit the adhesion of IRBCs to the placental CSPG by adhesion inhibition assay as described in Fig. 2. ○, unmodified C4S; ●, ▲, ■, □, △, and ▼, C4Ss in which, respectively, 14, 25, 33, 56, 67, and 80% carboxyl groups were reduced.

The C-2 Hydroxyl Groups of Glucuronic Acid Residues—A procedure for the specific modification of the C-2 hydroxyl groups of the glucuronic acid in C4S is not available. Therefore, the difference is the altered orientation of the carboxyl groups in C4S versus DS. That is, the equatorial carboxyl group in C4S is critical for the efficient binding of IRBCs. An alternate explanation for the noted differences could be dissimilarities in the pattern of 4-sulfation in these polysaccharides.

**FIGURE 2.** Inhibition of IRBC adherence to the placental CSPG by N-acyl and N-benzyol C4S. The amino groups of N-deacetylated C4S were acylated or benzoylated as outlined in Fig. 1 and assessed for inhibition of IRBC adhesion to the purified placental CSPG. The plastic Petri plates were coated with a 200 ng/ml solution of the placental CSPG, and the spots were blocked with 2% IRBC suspension for 30 min, and the cell suspensions were overlaid onto the CSPG-coated spots. After 30 min at room temperature, the plates were washed with PBS, and the bound cells were fixed with 2% glutaraldehyde, stained with Giemsa, and measured by light microscopy. ●, C4S; ○, N-deacetylated C4S; ▲, re-N-acetylated C4S; △, N-propionyl C4S; ■, N-butyryl C4S; □, N-hexanoyl C4S; ×, N-benzoyl C4S.

**TABLE 1** Composition and P. falciparum IRBC adhesion-inhibitory activity of carboxyl-reduced C4S

The carboxyl groups of C4S were partially reduced by EDAC activation followed by treatment with limiting amounts of NaBH4. The extent of carboxyl group reduction was determined by measuring the uronic acid contents. The carboxyl-reduced C4Ss were evaluated for inhibition of IRBC binding to human placental CSPG. GAG, glycosaminoglycan.
TABLE 2  
Disaccharide composition of partially sulfated C4S and DS used for evaluating IRBC adhesion-inhibitory activity shown in Fig. 5  
The partially sulfated C4Ss and DSs were prepared by solvolytic desulfation of fully sulfated sturgeon notochord C4S and pig intestinal mucosa DS, and their disaccharide compositions were determined. GAGs, glycosaminoglycans; ΔDi-0S, non-sulfated disaccharide containing Δ^{4-5}-unsaturated glucuronic acid; ΔDi-4S, 4-sulfated disaccharide containing Δ^{4-5}-glucuronic acid; ΔDi-6S, 6-sulfated disaccharide containing Δ^{4-5}-glucuronic acid.

| GAGs | ΔDi-0S | ΔDi-4S | ΔDi-6S |
|------|--------|--------|--------|
| C4S-1 | 21     | 78     | 1.0    |
| C4S-2 | 48     | 52     | 0      |
| C4S-3 | 62     | 38     | 0      |
| C4S-4 | 70     | 30     | 0      |
| DS-1  | 25     | 75     | 0      |
| DS-2  | 45     | 55     | 0      |
| DS-3  | 58     | 42     | 0      |
| DS-4  | 72     | 28     | 0      |

* Determined by HPLC analysis of disaccharide released by digestion with chondroitinase ABC (38).

FIGURE 5. Inhibition of IRBC adherence to placental CSPG by partially sulfated DS and C4S. The DS from pig intestinal mucosa with 94% 4-sulfated disaccharides and chondroitin 4-sulfate from sturgeon notochord with 98% 4-sulfated disaccharides were solvolytically 4-O-desulfated as described previously (15). The partially desulfated DS and C4S with comparable levels of sulfate contents were analyzed for inhibition of IRBC adhesion to placental CSPG. C4S with, respectively, 30, 38, 52, and 75% 4-sulfate; DS with, respectively, 28, 42, 55, and 75% 4-sulfate. Glycosaminoglycan (42).

TABLE 3  
Disaccharide composition of CSD and 2-O-desulfated CSD  
The sulfate groups at C2 of the GlcA residues in shark cartilage CSD were selectively removed by freeze-drying of an alkaline solution of CSD followed by lyophilization as reported (37). The polysaccharides were digested with chondroitinase ABC, and the released disaccharides were analyzed by HPLC (15). GAG, glycosaminoglycan; ΔDi-0S, non-sulfated disaccharide repeat unit containing Δ^{4-5}-unsaturated glucuronic acid; ΔDi-4S, 4-sulfated disaccharide containing Δ^{4-5}-glucuronic acid; ΔDi-6S, 6-sulfated disaccharide containing Δ^{4-5}-glucuronic acid; ΔDi-2,6dis, 2,6-di sulfated disaccharide containing Δ^{4-5}-glucuronic acid.

| GAGs | ΔDi-0S | ΔDi-4S | ΔDi-6S | ΔDi-2,6dis |
|------|--------|--------|--------|-----------|
| CSD  | 3      | 28     | 46     | 23        |
| 2-O-Desulfated CSD | 3 | 28 | 62 | 7 |

FIGURE 6. Preparation of the ANTS derivative of C4S dodecasaccharide and analysis of IRBC adhesion inhibitory activity of the derivative. The C4S dodecasaccharide was isolated by the Bio-Gel P-6 chromatography of the testicular hyaluronidase digest of C4S as reported previously (15). The enzyme digestion products and oligosaccharide fraction containing predominantly dodecasaccharide were conjugated with ANTS using the Glyko FACE oligosaccharide profiling kit according to the manufacturer’s instructions. Aliquots of the oligosaccharides before and after ANTS derivatization were analyzed by polyacrylamide gel electrophoresis (see the inset). The gels with the derivatized oligosaccharides were stained with Alcian blue followed by silver nitrate (A), and those with derivatized oligosaccharides were directly viewed under UV light and photographed (B). In both panels, lane 1 represents the total hyaluronidase digestion products, and lane 2 represents C4S dodecasaccharide fraction isolated by Bio-Gel P-6 chromatography. The size and electrophoretic mobility of the oligosaccharides are indicated on either side of the inset. The dodecasaccharide-ANTS derivative was purified on Bio-Gel P-6 and assessed its ability to inhibit the binding of IRBC to the placental CSPG as outlined in Fig. 2. ○, C4S dodecasaccharide; O, Oligo-ANTS.

Preparation of Photoaffinity Labeling Derivatives of C4S Dodecasaccharide and Their Application for the Identification of P. falciparum-IRBC-binding Protein(s)  
The results of our structure-activity studies indicated that the acetamido groups of C4S and the reducing end GalNAc of the C4S dodecasaccharide are not required for IRBC binding to C4S and that coupling of bulky substituents at these sites does not significantly affect the IRBC-C4S binding inhibitory activity. Based on this information, we prepared radioiodinatable and photoactivable C4S dodecasaccharide derivatives for affinity labeling of parasite adhesive proteins and studied the practical utility of these derivatives.

Preparation of Photoactivable Derivatives of C4S Dodecasaccharide and Evaluation of Their IRBC Adhesion-inhibitory Activity—Two alternative procedures were used for the preparation of photoactivable derivatives of C4S dodecasaccharide that have the potential for photoaffinity labeling of the parasite to derivatization. To determine whether it is involved in IRBC binding, we coupled a fluorescent compound, ANTS, to C4S at the reducing end GalNAc by reductive amination using the Glyko FACE oligosaccharide profiling kit. The coupling efficiency of ANTS to various oligosaccharides has been established to be essentially quantitative (40, 41). The derivatized oligosaccharides were purified by gel filtration and characterized by PAGE (Fig. 6, inset). The inhibitory activity of C4S dodecasaccharide-ANTS conjugate was ~90% of the unmodified dodecasaccharide. These marginally lower values suggest that the reducing end GalNAc residue does not significantly influence the binding of the dodecasaccharide to IRBC (Fig. 6).
adhesive proteins. (i) ASBA was conjugated to the reducing end of C4S dodecasaccharide by the reductive amination, and the conjugate (Oligo-ASBA in Scheme 2) was purified by Sephadex G-15. The UV spectrum of the conjugate showed the presence of characteristic absorption maxima at 270 nm with a small shoulder at 320 nm (data not shown), indicating the derivatization of C4S dodecasaccharide (42). (ii) The acetamido groups of C4S dodecasaccharide were partially replaced with N-4-azidosalicyl groups (Scheme 3A). C4S dodecasaccharide was N-deacetylated, the free amino groups were partially derivatized with N-hydroxysuccinimide esters of photoactivable 4-azidosalicylic acid, and the remainders of the amino groups were re-N-acetylated to obtain Oligo-ASA (Scheme 3A). Further, both derivatization procedures (depicted in Schemes 2 and 3A) were combined for the preparation of a photoactivatable oligosaccharide, which enables photolabeling of parasite adhesive proteins as well as isolation of the labeled proteins by avidin affinity precipitation (Scheme 3B). Thus, the reducing end GalNAc residue of Oligo-ASA was coupled to biotin hydrazide by reductive amination to obtain Oligo-ASA-Biotin. These photoactivatable C4S dodecasaccharide derivatives were purified by gel filtration on Sephadex G-15 and their IRBC-binding capacities were evaluated by measuring the inhibition of IRBC binding to placental CSPG. The inhibitory activity of these photoactivatable derivatives was not significantly different from that of the underivatized C4S dodecasaccharide with the exception of Oligo-ASBA, which appears to be 10–15% less inhibitory (Fig. 7). This validates the usefulness of these derivatives for photolabeling of parasite adhesive proteins(s) on the IRBC surface (see below).

Photoaffinity Labeling of IRBC Surface Proteins and Analysis of Photoaffinity-labeled Proteins—Two different approaches were used for the identification of C4S-adherent proteins on the IRBC surface. The photoactivatable derivatives, Oligo-ASBA and Oligo-ASA (Schemes 2 and 3A), were iodinated with 125I, purified by gel filtration on Sephadex G-15, and cross-linked to IRBC surface by UV irradiation. The photolabeled proteins were analyzed by SDS-PAGE and visualized by autoradiography. In an alternative procedure, the C4S oligosaccharide derivative, Oligo-ASA-Biotin (Scheme 3B), was cross-linked to the IRBC surface, and the labeled proteins were isolated by avidin affinity precipitation and analyzed by SDS-PAGE followed by fluorescent staining of the gels. Photoaffinity labeling of IRBCs was performed by incubating the cell suspensions with photoactivable C4S oligosaccharide probes (~10 nmol/50–60 μl of IRBC pellet) followed by UV irradiation at room temperature.

Initially we studied the ability of 125I-Oligo-ASA and 125I-Oligo-ASBA to cross-link to the IRBC surface proteins. The labeled IRBCs were treated with TBS containing 0.05% saponin to extract the bulk of the IRBC cytoplasmic contents. The pellet was then extracted with TBS, with 0.5% Triton X-100, and finally with TBS, 2% SDS (48, 49). Analysis of these extracts by SDS-PAGE under reducing conditions and autoradiography revealed a prominent ~22-kDa photoaffinity-labeled protein band in the 2% SDS extract of C4S-adherent IRBCs cross-linked with 125I-Oligo-ASA (Fig. 8). The Triton X-100 extract also showed low levels of the ~22-kDa photoaffinity-labeled band, but the band was not detectable in the saponin extract. The incorporation of 125I-Oligo-ASA to the C4S-adherent IRBC surface proteins was efficiently inhibited by preincubation with a 40-fold excess of either unmodified C4S or Oligo-ASA. No significant photolabeling of cell surface proteins was observed when 125I-Oligo-ASA was allowed to cross-link to non-adherent IRBCs or uninfected red blood cells (Fig. 8 and data not shown). It appears that this presumed parasite protein located on the surface of the IRBC is only minimally soluble in 0.5% Triton X-100 and requires 2% SDS for solubilization. One explanation for this could be that charge interactions play a role in the association of 125I-Oligo-ASA with the ~22-kDa IRBC protein. However, preincubation of the IRBCs with a 20-fold excess of heparin only marginally inhibited the photolabeling of the ~22-kDa protein (Fig. 8).

Together these results strongly suggest that the ~22-kDa IRBC protein specifically labeled by 125I-Oligo-ASA represents the parasite protein that either directly binds C4S or is a component of a multiprotein adhesive complex.

In contrast to 125I-Oligo-ASA, under similar photoaffinity cross-linking conditions, 125I-Oligo-ASBA was unable to label
C4S-adherent IRBC surface proteins at significant levels. This is surprising because the reducing end GalNAc residue of C4S dodecasaccharide is not involved in IRBC binding and the photoactive derivative, Oligo-ASBA, could efficiently interact with IRBCs (see Fig. 7). A likely explanation for the inability of the 125I-Oligo-ASBA to cross-link to the IRBC surface could be that the photoactivable ASBA moiety present on the reducing end Gal-NAc residue is not in close proximity to the binding proteins and thus not accessible for cross-linking.

We also photoaffinity-labeled the IRBC surface C4S-adherent proteins by Oligo-ASA-Biotin and isolated the photolabeled proteins by avidin affinity procedure. After cross-linking of the C4S-adherent IRBCs with nonradiolabeled Oligo-ASA-Biotin, the IRBCs were extracted consecutively with buffers containing 0.05% saponin, 0.5% Triton X-100, and 2% SDS (50, 51). The photoaffinity-labeled proteins in the SDS extract were affinity-purified with avidin-immobilized agarose beads and subjected to SDS-PAGE analysis. A specific protein band with a molecular mass of ∼26 kDa was present in the SDS extract (Fig. 9). The reason for higher apparent molecular mass compared with the ∼22-kDa protein band in the SDS extract of the C4S-adherent IRBC photolabeled with 125I-labeled Oligo-ASA (compare Fig. 8 with Fig. 9) is unclear. One possible explanation for this discrepancy is that the hydrophobic biotin moiety in the photoaffinity probe lowers the degree of protein-SDS complex formation and thus the mobility of the band. Together the above data strongly suggest that the ∼22-kDa protein expressed on the surface of the IRBCs and labeled with photoactivable oligosaccharide probes represents the parasite C4S-adhesive protein.

DISCUSSION

The present study gives important insights into the role of the key functional groups of C4S in P. falciparum-IRBC binding to the placental CSPG. First, the data show that replacement of the N-acetyl group by a bulky propionyl, butyryl, hexanoyl, or benzoyl group has no effect on its binding ability. Therefore, it is evident that the acetamido groups of GalNAc residues of C4S are not involved in the P. falciparum-IRBC adherence. Second, the results demonstrate that the equatorial carboxyl groups of the uronic acid residues in C4S are critical for IRBC binding. Third, the results reveal that the GalNAc residues at the reducing end of the C4S dodecasaccharide do not participate in the IRBC binding. This conclusion is supported by the
observation that C4S dodecasaccharide in which the ring structure of the reducing end GalNAc is abolished by conjugating the oligosaccharide to bulky substituents, such as ANTS and ASBA, by reductive amination retains full inhibitory activity. Collectively the above data provided rational strategies for designing probes for the biochemical identification of the parasite adhesive protein.

Based on our observation that the acetamido groups of C4S do not interact with IRBCs and that the substitution of acetyl groups by bulky acyl groups does not affect the IRBC binding activity, we prepared, as shown in Scheme 3, novel photoactivatable N-4-azidosalicylic derivatives of C4S dodecasaccharide with full retention of the IRBC-binding ability. Ongoing studies in our laboratory indicate that IRBCs with parasites at the early developmental stage (late rings and early trophozoites) bind to C4S with maximal affinity, and the binding affinity decreases significantly at the mid- to late trophozoite stages. Cross-linking of the photoactivatable N-4-azidosalicylic oligosaccharide derivatives to the early parasite stage IRBCs could specifically label a novel ~22-kDa parasite protein on the IRBC surface. The results were reproducible in several replicate experiments. Our prediction that the ~22-kDa C4S-adherent protein photolabeled by the above C4S dodecasaccharide is the parasite adhesive protein is supported by the observation that the photolabeling was evident only in the C4S-adherent IRBCs but not in the corresponding non-adherent IRBCs or uninfected red blood cells. Moreover the photolabeling of the ~22-kDa protein on the C4S-adherent IRBC surface could be efficiently inhibited by the unmodified C4S probe or by the non-ioted oligosaccharide probe but not by heparin. Thus, our data strongly suggest that a novel low molecular weight IRBC surface protein but not var1 or var2 PIEMP1 (molecular mass range, 200–400 kDa) is directly involved in C4S binding. However, the data do not exclude the possible involvement of var1 PIEMP1 and/or var2 PIEMP1 in IRBC binding, for example, by forming a functional complex with the ~22-kDa protein identified in this study.

The var2 PIEMP1 has been recently implicated in IRBC binding to C4S (27–29). Thus, we analyzed var2 transcript by reverse transcription-PCR in several batches of C4S-adherent parasites used in this study. It was found that var2 PIEMP1 is consistently expressed at an 8–10-fold higher level in different batches of C4S-selected parasites compared with non-selected parasites; preliminary microarray analysis also gave similar results. In contrast, the var1 PIEMP1 was expressed at a much lower level (1–2-fold), and moreover different genes were expressed in different batches of C4S-selected parasites. Therefore, it appears likely that a multiprotein complex, involving var2 PIEMP1, PIEMP3, knob-associated histidine-rich protein, the 22-kDa novel protein, and possibly other proteins, mediates IRBC adherence to C4S.

Although the C4S dodecasaccharide with photoactivatable

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6 S. V. Madhunapantula, R. N. Achur, and D. C. Gowda, unpublished results.

7 A. Muthusamy, R. N. Achur, and D. C. Gowda, unpublished results.
P. falciparum Adherence to Chondroitin 4-Sulfate

followed by photolabeling of IRBCs and avidin affinity precipitation showed that the ~22-kDa protein that was photolabeled could be specifically precipitated by the immobilized avidin beads. Thus, the photolabeling approach described in this study should facilitate efforts toward full biochemical characterization of the parasite adhesive proteins. Studies in this direction are in progress in our laboratory.

In conclusion, in the present study, we gained understanding on the role of key functional groups of C4S in P. falciparum-IRBC adherence. Notably we found that acetamido groups of GalNAc are not involved in IRBC binding, whereas the equatorial carboxyl groups of the uronic acid are crucial. We also found that the reducing end GalNAc of C4S dodecasaccharide is not required for IRBC binding. Based on these data, we prepared novel C4S dodecasaccharide derivatives by substituting acetyl groups of GalNAc with photoactivable N-4-azidosalicyloyl moieties and by conjugating biotin to the reducing end GalNAc of the oligosaccharide. We also demonstrated that the photoactivable oligosaccharide derivatives are useful for biochemical characterization of parasite adhesive proteins. Finally our studies have broader implications in designing photoaffinity probes for the identification of adhesive proteins of many other microorganisms, such as *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Toxoplasma gondii*, *Leishmania donovani*, and a subset of herpes simplex virus that uses glycosaminoglycans as specific receptors for host cell attachment (51–53). The structure-activity information gained in this study also has important implications for designing C4S derivatives or C4S-based peptide mimetics as possible therapeutics for pregnancy-associated malaria.

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FIGURE 9. Affinity precipitation and analysis of photoaffinity-labeled parasite proteins on the *P. falciparum*-IRBC surface. The C4S-adherent IRBCs were photoaffinity-labeled with Oligo-ASA-Biotin (Scheme 3B) and extracted as outlined in Fig. 8. After extracting the IRBCs with buffer containing 0.5% Triton X-100, the pellet was extracted with TBS, 2% SDS. The SDS extracts were diluted to a final concentration of 50 mM Tris-HCl, 150 mM NaCl, pH 7.2, 0.2% SDS, and 0.5% Triton X-100, and the proteins were affinity-precipitated with avidin-immobilized agarose beads pretreated with 1% BSA. After washing the beads, the bound proteins were released by the addition of 2× SDS-PAGE sample buffer and heating at 90 °C for 6–8 min and electrophoresed on 4–15% SDS-polyacrylamide gradient minigels under reducing conditions according to Laemmli (50). The gels were fixed, stained, and photographed. Lane 1, total 2% SDS extract; lane 2, proteins affinity-precipitated from the 2% SDS extract with avidin-agarose beads. The molecular masses of marker proteins are shown in the left margin, and arrow in the right margin indicates the IRBC surface protein labeled with biotin-containing photoactivatable probe.

ASBA moiety at the reducing end GalNAc was found to be not useful for the photoaffinity labeling of parasite adhesive protein, the results provided us a rationale for designing photoaffinity probe that would allow for the isolation of the labeled protein. The idea was that a C4S dodecasaccharide with photoactivable moiety on the amino groups and biotin-conjugated at the reducing end will photolabel and also allow subsequent isolation of the labeled proteins by affinity precipitation. Thus, conjugation of biotin to the reducing end GalNAc of N-4-azidosalicyloyl derivative of C4S dodecasaccharide (see Scheme 3B)
