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Sequestrin, a CD36 recognition protein on *Plasmodium falciparum* malaria-infected erythrocytes identified by anti-idiotype antibodies

(erythrocyte membrane/sequestration/molecular mimicry/cytoadherence)

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ABSTRACT **The CD36 molecule expressed by human endothelial cells is a receptor for the adhesion of erythrocytes infected with the human malaria parasite *Plasmodium falciparum*. A CD36-specific monoclonal antibody, OKM8, inhibits the adhesion of malaria-infected erythrocytes (IRBC) to purified CD36 and cells expressing CD36. Monospecific polyclonal anti-idiotype (anti-Id) antibodies, raised against monoclonal antibody OKM8, expressed determinants molecularly mimicking the CD36 binding domain for the adhesion of IRBC. Purified rabbit anti-Id antibodies reacted with the surface of IRBC by immunofluorescence, directly supported the adhesion of wild-type *P. falciparum* malaria isolates, and inhibited IRBC cytoadherence to melanoma cells. An ≈270-kDa protein was immunoprecipitated by the anti-Id antibodies from surface-labeled and metabolically labeled IRBC and was competitively inhibited by soluble CD36. These results support the hypothesis that CD36 is a receptor and the ≈270-kDa protein, sequestrin, is a complementary ligand involved in the adhesion of IRBC to host-cell endothelium. Sequestrin is a candidate malaria vaccine antigen, and anti-Id antibodies that recognize this molecule may be useful for passive immunotherapy of cerebral and severe *P. falciparum* malaria.**

Erythrocytes infected with the human malaria parasite *Plasmodium falciparum* adhere to postcapillary venular endothelium of specific organs and tissues, and the sequestration of infected erythrocytes (IRBC) contributes to the morbidity and mortality of severe and cerebral malaria (1). Cytoadherence of IRBC has been studied *in vitro* and involves specific receptor/ligand-mediated interactions between one or more IRBC binding ligands and host endothelial receptors CD36 (2–4), thrombospondin (5), and intercellular adhesion molecule 1 (ICAM-1) (6). CD36 appears to be a major surface receptor for the adhesion of IRBC. Purified CD36 binds culture-adapted (3) and naturally acquired wild-type *P. falciparum*-IRBC (unpublished data). Soluble CD36 inhibits IRBC binding to endothelial cells and melanoma cells bearing surface CD36.

The distribution of CD36 *in vivo* correlates with sequestration of IRBC, and CD36 is expressed by the capillary endothelium of postmortem brain tissue from patients with cerebral malaria (4). The cytoadherence of IRBC to purified CD36 or cells expressing CD36 is inhibited by monoclonal antibodies (mAbs) OKM8 and OKM5 (2, 7). Based on the above observations, we sought to develop an immunologic monospecific probe to define the malaria-IRBC surface molecule mediating cytoadherence. Such an antibody would focus on the ligand specificity of the CD36 receptor as expressed by an anti-idiotype (anti-Id).

Anti-Id antibodies raised against the antigen binding sites of antibodies specific for receptors have been used as cell-surface probes to define the molecular interactions between ligands and receptors (8). Some anti-Id antibodies contain the internal image of the receptor (Ab2) and may recognize a complementary receptor-binding site. In this report we describe a *P. falciparum* malaria sequestration ligand on the surface of IRBC that we identified by anti-Id antibodies (Ab2) against mAb OKM8 (Ab1). These anti-Id antibodies reacted with the combining site of Ab1 and with the surface of IRBC and inhibited their adhesion to purified CD36 and melanoma cells *in vitro*. An ≈270-kDa parasite-derived erythrocyte membrane protein, which we have termed sequestrin, was recognized by both anti-Id antibodies and soluble CD36 and may provide the basis for a component of a multivalent malaria vaccine.

MATERIALS AND METHODS

Receptor Proteins. CD36 (platelet glycoprotein gpIV) was purified from platelets as described (9). CD36 was coupled to CN-Sepharose (Pharmacia) according to the manufacturer's recommendations. The coupling efficiency was determined to be ≈98%, corresponding to 1 mg of CD36 per ml of beads.

Preparation of Anti-Id Antibodies. mAb OKM8 was used as an immunogen for the preparation of polyclonal anti-Id. New Zealand White rabbits were immunized by subcutaneous injection at multiple sites with 50 μg of mAb OKM8 coupled to keyhole limpet hemocyanin (KLH) and administered in Freund's complete adjuvant. Rabbits received boosters twice at 3-week intervals with 25 μg of KLH-conjugated mAb OKM8 emulsified in Freund's incomplete adjuvant, followed by three weekly i.v. injections of unconjugated mAb OKM8 (10 μg). Serum was collected for testing 10 days after the last immunization. Purified monospecific anti-Id antibodies were obtained by passing the rabbit antiserum sequentially through an affinity column of mouse Ig coupled to Sepharose (Cappel Laboratories), followed by affinity chromatography with mAb OKM8 coupled to Affi-Gel 10 (Bio-Rad). Bound antibodies were eluted with 0.1 M glycine·HCl (pH 2.5) and dialyzed against phosphate-buffered saline to prepare rabbit anti-mouse immunoglobulin antibodies and anti-Id antibodies. This purification scheme was designed to obtain antibodies against the antibody-combining site ("paratope") of mAb OKM8 by removal of anti-isotypic and anti-allotypic rabbit antibodies prior to the adsorption and elution from the CD36-specific mAb column. Preimmune rabbit IgG was obtained by adsorption of whole rabbit serum to protein A-Sepharose CL-4B (Pharmacia) and elution of IgG from the column as described above.

Parasites. *P. falciparum* culture-adapted clones CAMP (Malayan) and ItG2F6 (Brazilian) were cultured as described (10). The CAMP strain parasites maintained in continuous long-term culture were deficient in surface knobs and did not cytoadhere to C32 melanoma cells (K^- C^- phenotype). The

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Abbreviations: IRBC, infected erythrocytes; anti-Id, anti-idiotype; mAb, monoclonal antibody.

parental clones of CAMP ($K^- C^-$) and Itg2F6 ($K^+ C^+$) IRBC were selected for different adherence phenotypes by panning the parasitized erythrocytes on purified CD36 adsorbed to plastic Petri dishes (unpublished data). CAMP IRBC selected for increased adhesion to CD36 acquired a CD36-specific binding phenotype while remaining knob-negative (designated $K^- C^+$). ItG2F6 IRBC selected on purified CD36 were designated ItG-CD36 ($K^+ C^+$). Malayan CAMP IRBC with concomitant knobby and cytoadherent phenotypes ($K^+ C^+$) adapted to continuous culture in human erythrocytes were obtained from malaria-IRBC from an *Aotus* monkey.

Primary *P. falciparum* isolates from naturally infected Thai patients with uncomplicated (CTD-9282) or cerebral (GL5; TNF28; CY49) malaria were adapted to continuous culture and used in adhesion assays within five cycles of multiplication.

Immunofluorescence and Flow Cytometry. Infected erythrocytes of cytoadherent and nonadherent CAMP and ItG2F6 parasites were examined for cell surface and cytoplasmic immunofluorescence staining with anti-Id sera as follows. Rabbit antiserum or protein A-purified rabbit IgG were diluted (1:10–1:50) into RPMI 1640 medium with 10% (vol/vol) fetal calf serum (FCS) containing the parasitized cells. Approximately 2×10^7 to 1×10^8 cells (3–5% parasitemia) were allowed to react in 200 μl medium containing antiserum. IRBC were incubated with antibodies for 60 min at 25°C on a rocking platform, washed twice in RPMI 1640 medium with 10% FCS, and cell-bound rabbit antibodies were detected with fluorescein-conjugated goat anti-rabbit IgG (1:10 dilution). Cells were washed twice with 100% FCS, suspended in 100% FCS (50% hematocrit), and mounted onto slides as thin blood films. The erythrocytes were air-dried, methanol-fixed, and stained with monoclonal antibody 3B10.1 against *P. falciparum* gp195. Bound mouse antibodies were detected with rhodamine-conjugated goat anti-mouse κ light chain-specific antibodies.

Uninfected erythrocytes or CAMP ($K^+ C^+$) IRBC were suspended in 200 μl of phosphate-buffered saline (PBS) containing 10 μl of ethidium bromide (200 μg/ml) for 30 min at room temperature to detect intraerythrocytic parasite DNA. The cells were washed and resuspended in 50 μl of preimmune or anti-Id serum (1:50 dilution) for 60 min at room temperature. The erythrocytes were washed twice with PBS and resuspended in fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (1:20 dilution) for 60 min at 4°C to detect surface bound anti-Id. The erythrocytes were washed, and fluorescence analysis (50,000 events) was performed on a Becton Dickinson FACS analyzer.

Adhesion Assay. IRBC from laboratory-adapted and wild isolates were assayed for adhesion to CD36-coated plastic (1 μg/ml), purified anti-Id antibodies (140 μg/ml), and rabbit anti-mouse immunoglobulin (140 μg/ml) as described (3). In some experiments, inhibition of binding of IRBC to purified CD36 or C32 melanoma cells was performed.

Surface and Metabolic Labeling. Trophozoite and schizont stage IRBC were purified to >95% parasitemia by centrifugation through a Percoll/sorbitol gradient prior to surface radioiodination (11). CAMP IRBC ($K^- C^+$) were surface-labeled with $Na^{125}I$ by the lactoperoxidase technique. The IRBC were washed in PBS and extracted with 1% Triton X-100 in the presence of protease inhibitors, chymostatin (10 μg/ml), and leupeptin (10 μg/ml) to a final concentration of 5×10^8 IRBC per ml. The Triton X-100 soluble lysate was used for subsequent immunoprecipitation and gel electrophoresis analysis. In some experiments, intact radioiodinated IRBC were incubated with trypsin (10 μg/ml) for 10 min at room temperature followed by the addition of soybean trypsin inhibitor (100 μg/ml). The trypsinized IRBC were extracted with Triton X-100 as described above.

Synchronous ring-stage IRBC were cultured in isoleucine-free medium containing 100 μCi (3700 kBq) of [^{3}H]isoleucine (Amersham) per ml for 30 hr. The IRBC were washed twice and then concentrated by Percoll gradient centrifugation (Camp $K^- C^+$) prior to extraction with Triton X-100.

Immunoprecipitation and Gel Electrophoresis. Aliquots of the Triton X-100/PBS-soluble extract were precleared by incubating 50 μl of pooled rabbit sera for 60 min at 4°C, followed by incubation with goat anti-rabbit Sepharose beads for 60 min at room temperature. The precleared lysate was divided into two portions, and each portion was incubated with 50 μl of preimmune or anti-Id serum. After 60 min, the soluble immune complexes were precipitated by incubation with goat anti-rabbit immunoglobulin-conjugated Sepharose beads. The precipitated immunocomplexes were washed and then dissolved in sample buffer, and the proteins (equivalent to 2.5×10^7 IRBC per well) were resolved by 5–10% or 5–15% gradient SDS/PAGE with 5% mercaptoethanol and were processed for autoradiography and fluorography.

RESULTS

Anti-Id Antibodies Binding to Parasitized Erythrocytes. Polyclonal anti-Id antibodies were prepared by immunizing rabbits with mouse mAb OKM8. Serial dilutions of anti-Id antisera but not preimmune antisera contained antibodies (Ab2) that specifically recognized the OKM8 idiotype (Ab1) in a two-site immunoradiometric assay and not an irrelevant mAb of similar isotype (data not shown). The anti-Id antibody inhibited the binding of ^{125}I-labeled CD36 to mAb OKM8 in a dose-dependent manner, suggesting that a specific subset of anti-Id antibodies ($Ab2_\beta$) contained internal-image analogues of CD36 mimicking the OKM8 binding domain (data not shown).

We hypothesized that anti-Id antibodies ($Ab2_\beta$) would recognize a conserved *P. falciparum*-IRBC surface antigen by its CD36-binding site, and that adhesion of IRBC to anti-Id antibodies would be neither strain-specific nor knob-specific. Culture-adapted IRBC and IRBC from patients with uncomplicated and cerebral malaria bound to purified anti-Id antibodies but not to rabbit anti-mouse allotypic and isotypic antibodies (Fig. 1).

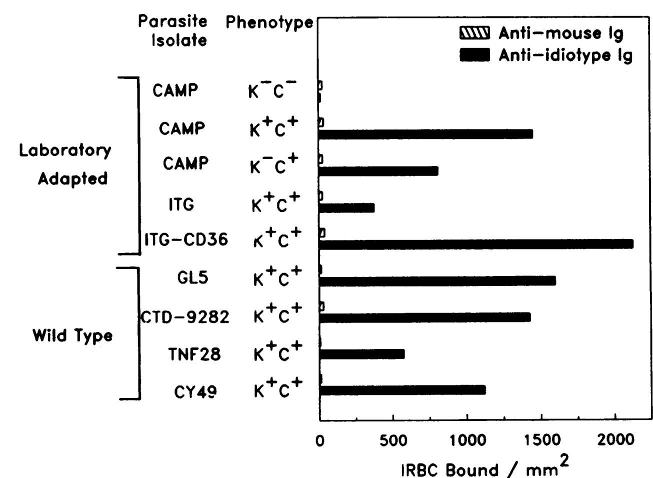

FIG. 1. Binding of *P. falciparum*-IRBC to purified anti-Id antibody. Human erythrocytes infected with culture-adapted or wild-type isolates were cultured to the mature trophozoite stage of parasite development. IRBC (4% suspension, 10–12% parasitemia) were added to plastic tissue-culture plates containing immobilized anti-Id or anti-mouse immunoglobulin (140 μg/ml). After 60 min at room temperature, unbound erythrocytes were removed by rinsing the plates, and the bound IRBC were fixed, stained, and counted. Results (duplicate samples) represent the mean number of IRBC bound per mm^2 of surface area.

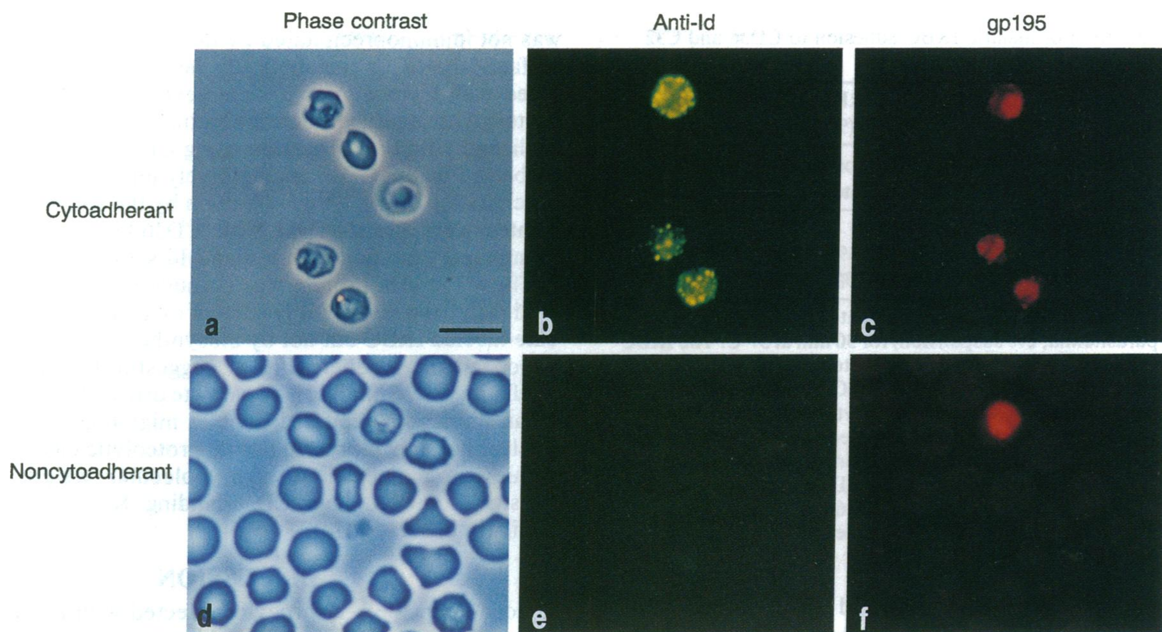

FIG. 2. Immunofluorescence reactivity of CAMP $K^- C^+$ (*a–c*) and $K^- C^-$ (*d–f*) IRBC. IRBC thin blood films were photographed with phase-contrast micrography (*a* and *d*) and UV fluorescence after sequential reactions with anti-Id antisera and fluoroscein isothiocyanate-conjugated anti-rabbit antibody (*b* and *e*) followed by anti-gp195 mAb and rhodamine-conjugated goat anti-mouse κ light chain antibody (*c* and *f*) as described.

Culture-adapted knob-negative (K^-) IRBC bind to C32 melanoma cells and purified CD36 (12–14). These observations led us to select a CD36-binding parasite population from a parental knobless noncytoadherent clone, CAMP $K^- C^-$, by repeated panning of the knobless parasitized erythrocytes on purified CD36. IRBC adhesion to plastic-adsorbed anti-Id antibodies correlated with binding to CD36. A greater number of CAMP $K^- C^+$ IRBC and ItG-CD36 IRBC, selected by panning on CD36, bound to anti-Id antibodies compared with the adhesion of parental clones CAMP $K^- C^-$ and ItG2F6, respectively.

Anti-Id antibody reacted in an immunofluorescence assay with viable IRBC containing mature cytoadherent *P. falciparum* parasites (Fig. 2). Uninfected erythrocytes and erythrocytes infected with a clone of CAMP strain parasites that fail to cytoadhere did not react with the anti-Id antibody. Two-color immunofluorescence permitted the simultaneous detection of surface-bound anti-Id (green fluorescence) antibody with an internal parasite antigen defined by a GP195-specific mAb (red fluorescence). A punctate pattern of surface fluorescence on $K^- C^+$ IRBC was observed similar to that of K^+ IRBC.

Flow cytometry quantitatively confirmed that anti-Id antibody reacts with cytoadherent-positive (C^+) IRBC but not uninfected erythrocytes (Fig. 3). Approximately 85% of IRBC displayed positive fluorescence when treated with the anti-idiotype sera. Soluble CD36 inhibited the binding of anti-Id antibody to CAMP $K^+ C^+$ IRBC, confirming that CD36 competes with anti-Id antibody for the IRBC-binding ligand.

Inhibition of IRBC Adhesion to CD36 and Melanoma Cells by Anti-Id Antibodies. Since IRBC bind to melanoma cells by various receptors [e.g., CD36 and intercellular adhesion molecule 1 (unpublished data)], we tested whether anti-idiotype IgG would inhibit IRBC adhesion to C32 melanoma cells. Polyclonal anti-Id IgG but not preimmune rabbit IgG inhibited the adhesion of CAMP $K^+ C^+$ IRBC to C32 melanoma cells (Table 1). Anti-Id IgG also inhibited IRBC binding to purified CD36, suggesting that anti-idiotypic paratopes manifest internal image analogues of the IRBC-binding domain of the CD36 receptor.

Immunoprecipitation of Surface- and Metabolic-Labeled CD36 Binding Protein. To identify the complementary CD36-binding ligand on the IRBC surface, Triton X-100-soluble detergent lysates from radioiodinated CAMP $K^- C^+$ IRBC

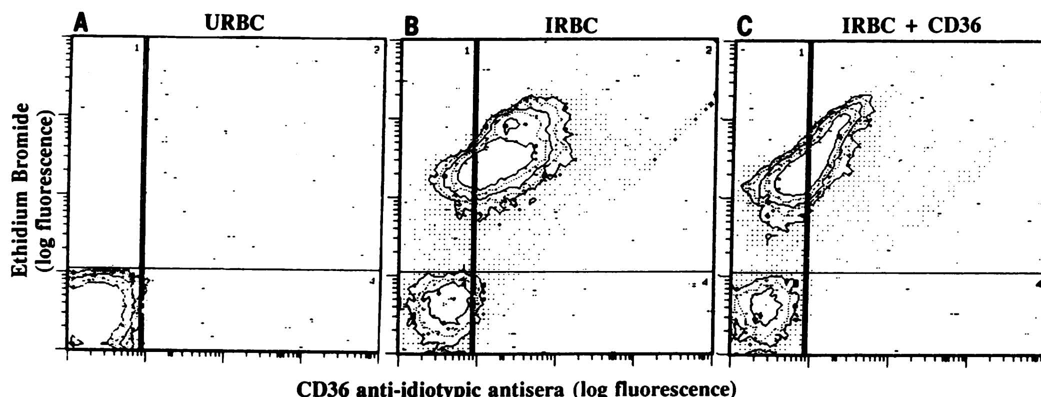

FIG. 3. (*a*) Cytofluorographic profiles of uninfected erythrocytes (URBC). (*b*) IRBC CAMP ($K^+ C^+$) stained with anti-Id antibody (green fluorescence) and ethidium bromide (red fluorescence). (*c*) Inhibition of binding of anti-Id antibody to IRBC by soluble CD36 (5 μg/ml). IRBC were incubated with CD36 for 30 min at room temperature prior to addition of anti-Id antiserum.

Table 1. Inhibition of malaria-IRBC adhesion to CD36 and C32 melanoma cells by anti-Id antibodies

		CAMP K^+ C^+ IRBC adhesion, mean no. ± SD (% inhibition)	
IgG serum	Amount, μg/ml	IRBC per mm^2 of CD36-coated plate	IRBC per 100 C32 melanoma cells
None	—	8200 ± 546	873 ± 48
Preimmune	200	8012 ± 328 (2.3%)	794 ± 28 (9.0%)
Anti-Id	200	3116 ± 783 (62%)	195 ± 41 (78%)

Preimmune and anti-Id IgG were incubated with CAMP K^+ C^+ IRBC (8% parasitemia, 2% suspension) for 30 min at 37°C. The IRBC suspension was added to tissue-culture plates coated with CD36 or to tissue culture plates plated with 4×10^4 C32 melanoma cells and incubated for 60 min at 37°C. Unattached erythrocytes were removed by washing three times with RPMI 1640 medium, and bound IRBC were fixed wtih 2% glutaraldehyde, stained with Giemsa, and counted. Results represent the mean ± SD of triplicate samples. Inhibition of IRBC binding to purified CD36 and to melanoma cells was calculated by comparing control samples incubated with RPMI 1640 medium alone.

were immunoprecipitated with anti-Id sera and coprecipitated with CD36-Sepharose (Fig. 4). Anti-Id but not preimmune sera immunoprecipitated an ≈270-kDa surface protein from detergent-soluble lysates of K^- C^+ IRBC. This protein was not immunoprecipitated by the anti-Id sera when intact surface-labeled or metabolically labeled IRBC were incubated with trypsin prior to detergent extraction. A surface protein with identical electrophoretic mobility was identified by ligand-affinity purification using CD36-Sepharose as absorbant. Finally, CD36 competitively inhibited the immunoprecipitation of the 270-kDa protein by the anti-Id sera when lysates were preadsorbed with CD36-Sepharose prior to immunoprecipitation with the anti-Id sera.

The ≈270-kDa protein was immunoprecipitated with anti-Id sera from detergent lysates of cytoadherent [^{3}H]isoleucine-labeled IRBC but not by biosynthetically labeled IRBC which fail to cytoadhere (Fig. 4), suggesting that the molecule mediating CD36 binding is of parasite origin. The significance of an immunoprecipitated doublet migrating at 270 kDa is unclear but may represent partial proteolytic cleavage of the sequestrin molecule. A high molecular weight trypsin-sensitive doublet from CD36-binding K^- C^+ IRBC was similarly observed (14).

DISCUSSION

Cytoadherence of erythrocytes infected with *P. falciparum* involves specific receptor–ligand interactions. CD36, thrombospondin, and intercellular adhesion molecule 1 are putative receptor molecules, but no direct evidence has linked an

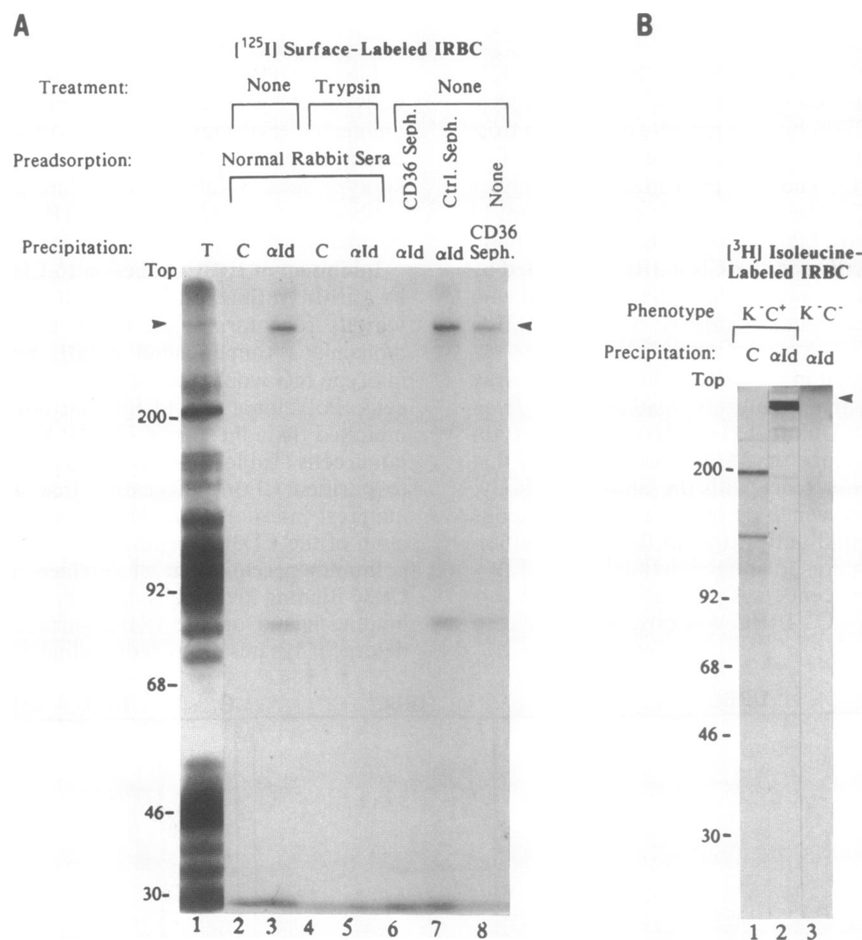

FIG. 4. Immunoprecipitation and SDS/PAGE of sequestrin. (*A*) Autoradiograph of radioiodinated 270-kDa protein of CAMP K^- C^+ IRBC precipitated by anti-Id antibody (αId) and soluble CD36 and electrophoresed on 5–10% gradient SDS/PAGE. Intact IRBC were radioiodinated and solubilized in 1% Triton X-100 to obtain a soluble detergent lysate (lane 1) prior to immunoprecipitation. Some intact IRBC samples (lanes 4 and 5) were incubated with trypsin prior to detergent solubilization. Soluble lysates were immunoprecipitated with preimmune rabbit sera (lanes 2 and 4) or anti-Id antisera (lanes 3 and 5). Soluble lysates were adsorbed with CD36-Sepharose (lane 6) or bovine serum albumin-Sepharose (lane 7) prior to immunoprecipitation of the lysate with anti-Id antisera. Direct ligand affinity purification with CD36-Sepharose is shown in lane 8. (*B*) Fluorograph of [^{3}H]isoleucine-labeled K^- C^+ (lanes 1 and 2) and K^- C^- (lane 3) IRBC detergent lysates immunoprecipitated with preimmune (lane 1) or anti-Id (lanes 2 and 3) rabbit sera and electrophoresed on a 5–15% gradient SDS/PAGE. Molecular mass standards (kDa) are shown to the left of both gels. The 270-kDa protein, sequestrin, is marked with an arrowhead.

IRBC binding ligand with any of these receptors. Adhesion to a specific receptor(s) implies accessibility to the erythrocyte surface, sensitivity to proteolytic cleavage by trypsin, and molecular changes in erythrocyte membranes of IRBC not present in uninfected or immature ring-stage parasitized erythrocytes. The receptor binding domain should be structurally conserved in natural isolates.

Indirect evidence suggests that PfEMP1, a *P. falciparum* trypsin-sensitive high molecular weight IRBC membrane protein, fulfills some of the criteria expected of a binding ligand (15). However, the identity of a strain-invariant binding domain has eluded investigators for several reasons. The lack of monospecific antibodies against an infected erythrocyte membrane molecule from parasite strains of diverse geographical regions, the paucity of such antigens, their potentially poor immunogenicity, and the detergent-insoluble properties attributed to such a molecule have hampered efforts at purification and characterization of a binding ligand.

These difficulties have been surmounted by the preparation of anti-idiotype antibodies. This approach rests on the assumption that a single conserved ligand is responsible for the recognition of the sequestration receptor. Additionally, we have exploited the detergent-insoluble properties of the binding ligand within the erythrocyte membrane of K^+ C^+ parasites by selecting a knobless parasite line that binds to CD36. This approach described recently by Biggs *et al.* (14) enabled the extraction of a trypsin-sensitive high molecular mass protein from erythrocytes of knobless cytoadherent parasites. In our experiments, immunoprecipitation with anti-Id antibodies and CD36 receptor affinity precipitation of soluble detergent lysates of both surface- and biosynthetic-labeled CAMP K^- C^+ IRBC identified an ≈270-kDa membrane protein of parasite origin. Because of its critical role in sequestration, we propose the name "sequestrin," for the parasite molecule defined by its recognition of purified CD36 and CD36 anti-idiotype analogues.

This work has identified a *P. falciparum* CD36-binding protein that we think is a good candidate for a vaccine component. The evidence that sequestrin is a cytoadherence ligand encompasses the following: (*i*) sequestrin is surface accessible and sensitive to proteolytic cleavage; (*ii*) the functional binding domain is structurally conserved in cytoadherent laboratory-adapted parasite strains and in all wild-type isolates examined to date and is absent in uninfected erythrocytes or noncytoadherent IRBC; (*iii*) adhesion of IRBC to anti-Id antibodies is inhibited by soluble CD36, and cytoadherence to melanoma cells is inhibited by anti-Id antibodies; and (*iv*) anti-Id antibodies and CD36 precipitate an identical high molecular mass antigen from cytoadherent IRBC.

Sequestrin may be a member of a family of high molecular mass antigens (240–400 kDa) on the surface of *P. falciparum*-IRBC (15, 16). Immunoprecipitation reactions with human and monkey immune sera indicate that this antigen has serologically defined epitopes (15), and cytoadherence by immune sera is inhibited in a strain-specific manner, implying antigenic diversity among parasite strains. A structurally conserved invariant CD36-binding site, which may be poorly immunogenic, might be involved in the receptor–ligand interaction. The preparation of mAbs based upon the results with polyclonal anti-Id antibodies might be useful for passive immunotherapy of severe and cerebral *P. falciparum* infections. Monospecific anti-Id reagents should further the investigation of the molecular basis of cytoadherence and the identification of epitopes on sequestrin which may be useful for a malaria vaccine.

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