Structural, Functional, and Antigenic Differences between Bovine Heart Endothelial CD36 and Human Platelet CD36*

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Endothelial cell CD36 (glycoprotein IV) has been purified from bovine heart tissue by detergent partitioning and immunoaffinity chromatography. Bovine CD36 differs from human CD36 in its apparent mass (85 versus 88 kDa), primary structure, and immunological cross-reactivity. Of the 18 N-terminal residues identified, 17 conformed to the human CD36 sequence. Mouse monoclonal antibodies F-1 and 8A6 defined bovine- and human-specific epitopes, respectively. Because human CD36 has been identified as a receptor for erythrocytes infected with the malaria parasite Plasmodium falciparum, we examined the ability of bovine CD36 to bind infected erythrocytes. Bovine CD36, unlike human CD36, did not bind infected erythrocytes, suggesting that human CD36-specific structural features are responsible for recognition of the infected erythrocyte ligand.

We have purified the bovine counterpart of human CD36, a multifunctional membrane protein which has been shown to mediate binding of thrombospondin, collagen, and malaria-infected erythrocytes. Since CD36 appears to be critical to the pathology of the often lethal human malaria Plasmodium falciparum, we have compared the structural and functional characteristics of the human and bovine proteins as a step in understanding the chemical basis for its role in the progression of malaria infection. Erythrocytes infected with the trophozoite stage of this malaria parasite adhere to venular endothelial cells and thereby escape destruction in the spleen (Luse and Miller, 1971; David et al., 1983; Cranston et al., 1984). The ability of the infected erythrocyte (IRBC) to adhere to endothelial cells has been correlated with the appearance of protrusions or knobs on the surface of the erythrocyte (Trager et al., 1966; Aikawa et al., 1972). The protein component(s) of these structures have yet to be identified. They may be synthesized by the parasite (reviewed by Howard (1988)) or may be modified erythrocyte membrane proteins (Winograd and Sherman, 1989). Recently, much attention has been focused on the endothelial cell receptor for the IRBC ligand. The endothelial cell and platelet membrane glycoprotein CD36 (also referred to as glycoprotein IV (Shaw, 1987)) has been shown to bind IRBC both as a component of cultured cells and as a purified protein (Barnwell et al., 1985, 1989; Ockenhouse et al., 1989). CD36 has also been demonstrated to bind IRBC after transfection and expression in COS cells (Oquendo et al., 1989; Berendt et al., 1989). In addition to its role in the adherence of IRBC, CD36 has also been identified as a receptor for thrombospondin, a large adhesive protein which participates in the secretion-dependent phase of platelet aggregation (Asch et al., 1987; Silverstein et al., 1989; McGregor et al., 1989). Interestingly, thrombospondin itself has been implicated in the adherence of IRBC to endothelial cells (Roberts et al., 1985; Rock et al., 1988). The relationships between the ability of CD36 to bind both thrombospondin and IRBC and the ability of thrombospondin to mediate IRBC binding remain unclear.

CD36 has been purified from both human platelets and the human C32 melanoma cell line (Asch et al., 1987; McGregor et al., 1989; Tandon et al., 1989). In addition, a cDNA for CD36 has been isolated from a human placental cDNA library and sequenced (Oquendo et al., 1989). The polypeptide has a predicted M, of ~63,000 and is heavily glycosylated. Putative transmembrane domains have been identified at both the N and C termini (Tandon et al., 1989; Oquendo et al., 1989). However, nothing is known about the molecular basis of the interactions of CD36 with either IRBC or thrombospondin. In this paper, we report the purification of bovine CD36 and demonstrate that it does not bind IRBC. We demonstrate distinct structural differences between human and bovine CD36 and suggest that study of these differences may help to elucidate the mechanism by which human CD36 recognizes and binds IRBC.

EXPERIMENTAL PROCEDURES

Materials—Aprotinin, 2,2'-azinobis(3-ethylbenzthiazolesulfonic acid), glycine, SDS, Tris, bovine serum albumin, phenylmethylsulfonyl fluoride, Triton X-114, Triton X-100, nitro blue tetrazolium, TEMED, N,N,N',N'-tetramethylethylenediamine; EGTA, [ethylendienetri(oxyethyl]enitrilo)tetraacetic acid; PAS, periodic acid-Schiff; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

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The abbreviations used are: IRBC, infected erythrocyte(s); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TEMED, N,N,N',N'-tetramethylethylenediamine; EGTA, [ethylendienetri(oxyethyl]enitrilo)tetraacetic acid; PAS, periodic acid-Schiff; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; BSA, bovine serum albumin; PBS, phosphate-buffered saline.
Preparation and Extraction of Membranes—Bovine heart tissue was obtained immediately after slaughter from a local abattoir. Thin slices of ventricular tissue were transported to the laboratory on ice. Homogenization was performed in 2 volumes (v/w) homogenization buffer (10 mM Tris, pH 7.4, containing 0.15 M NaCl, 1 mM EGTA, 1% aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM aminocaproic acid). After two 10-s intervals at maximum speed, the homogenate was filtered through two layers of cheesecloth and centrifuged at 10,000 x g for 10 min. The supernatant was further centrifuged for 10 min at 10,000 x g, and the resulting supernatant was again centrifuged for 1 h at 100,000 x g. The microsomal pellet was resuspended in homogenization buffer to a final protein concentration of 5 mg/mL, and Triton X-114 was added to a final concentration of 1%. The suspension was stirred for 1 h at 4 °C and then centrifuged at 100,000 x g for 1 h. Residual lipid was removed from the surface of the detergent extracts, and solubilized proteins were partitioned into aqueous and detergent phases (Bordier, 1981). The supernatant was warmed to 30 °C in a water bath and then centrifuged at 1,000 x g for 10 min at room temperature. Centrifugation of the aqueous phase yielded a second detergent phase and a small pellet of insoluble material. The two detergent phases and the accompanying insoluble material were then diluted in homogenization buffer and centrifuged at 100,000 x g for 1 h. The resulting supernatant was frozen at -20 °C.

Bovine platelet membranes were prepared from platelets isolated from blood collected in acid/citrate/dextrose. After centrifugation at 1,600 x g for 6 min, the supernatant was reconstituted at 4,000 x g for 10 min. The platelet pellet was resuspended in citrate buffer, sonicated, and centrifuged at 10,000 x g. The supernatant was then centrifuged at 100,000 x g for 1 h to obtain the final platelet membrane preparation. Extraction of the platelet membranes in Triton X-114 was as described above. Human platelets isolated from blood bank outdated human platelet preparations were similarly processed. Both human and bovine platelet CD36 were partially purified by Q-Sepharose ion-exchange chromatography of the detergent phase of platelet extracts (McGregor et al., 1989).

Immunopurification Chromatography—E-1, a monoclonal antibody to bovine PAS IV (which recognizes bovine heart endothelial cell CD36 (Greenwall et al., 1985)), was purified from ascites fluid by precipitation with ammonium sulfate (40% w/v) and chromatography on protein G-Sepharose. The purified monoclonal antibody was coupled to CNBr-activated Sepharose 4B (5 mg of protein/g of gel) according to the manufacturer's instructions and equilibrated in Tris-buffered saline containing 0.1% Triton X-100 (TBST). The detergent phase proteins of the solubilized heart microsomes were then chromatographed on the immunopurification column. After extensive washing in 0.1 M NaCl/TBST, specifically bound protein was eluted with 100 mM glycine, pH 2.7, containing 0.1% Triton X-100 and immediately neutralized with Tris, pH 9.0.

Gel Electrophoresis and Immunoblotting—Proteins were separated by SDS-PAGE in 0.75-mm minigel units as described by Laemmli (1970). The molecular weight standards were as follows: myosin (200,000), &beta-galactosidase (116,000), phosphorylase b (97,400), BSA (66,000), ovalbumin (45,000), and carbonic anhydrase (29,000). For immunoblotting, samples were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose paper (Towbin et al., 1979). After transfer, the protein bands were stained with Ponceau S, and the nitrocellulose was marked with a pen. The stain was removed with 2% BSA/Triton-buffered saline, and the nitrocellulose was incubated with affinity-purified bovine PAS IV-specific antibody (Greenwall et al., 1990) diluted 1:100 in Tris buffered saline, 0.1% BSA. The nitrocellulose was washed with a series of high salt and detergent solutions as previously described (Greenwall and Mather, 1985) and then incubated for 1 h in 0.1% mouse IgG-conjugated goat anti-rabbit IgG and washed a second time. The reactive protein bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate as the phosphate substrate. Control blots utilized 1:50 control sera.

Enzyme-linked Immunosorbent Assays—These were done according to procedures described previously (Mather et al., 1982) with slight modifications. Bovine heart CD36, human platelet CD36, and BSA were coated onto the plastic wells of enzyme-linked immunosorbent assay plates at 1 μg/well. Primary antibodies against human CD36 were used at a final dilution of 1:50, and horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse secondary antibodies were used at final dilutions of 1:1000. Bound secondary antibody was quantitated by cleavage of the peroxidase substrate 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid).

IRBC Binding—Asexual blood stage P. falciparum parasites of the Malayan Camp strain were derived from in vitro culture in human erythrocytes (Handunnetti et al., 1990). Mature trophozoite and schizont stage infected erythrocytes expressed surface knob protrusions and were adherent to C32 amelanotic melanoma cells in vitro. The adherent phenotype of these parasites was maintained by in vitro selection (Magowan et al., 1988). Infected blood of 75% parasitemia mature asexual parasites was obtained by sedimentation through gelatin (Jensen, 1978) and incubated on plastic Petri dishes coated with 10-μl spots of either bovine or human CD36 diluted in PBS. Coating was done at room temperature for 1 h. The plates were then blocked with 1% BSA in PBS for 30 min at room temperature. Binding was performed in RPMI 1640 medium containing 25 mM HEPES and 0.1% fetal calf serum, pH 6.8, at 1% hematocrit. The plates were washed three times with binding medium, fixed with 1% glutaraldehyde in PBS, and stained with Giemsa. Bound parasite infected erythrocytes were counted using light microscopy.

Sequence Analysis—Unmodified bovine CD36 was subjected to N-terminal sequence analysis using an Applied Biosystems 470A Gas-Phase Sequencer with an on-line Model 120A phenylthiohydantoin analyzer (Hewick et al., 1981). Initial yields were calculated to be 57-62%, with a repetitive yield of 90-95%.

Protein Determinations—Protein concentrations were analyzed with the bicinchoninic acid reagent and BSA as a standard (Smith et al., 1985). When Triton X-114 was present, all samples and standards were adjusted to a final concentration of 0.1% SDS prior to the addition of the bicinchoninic acid reagent.

RESULTS

Purification of Bovine CD36—Bovine CD36 was purified from bovine heart microsomes by a procedure which involved detergent partitioning and immunopurification chromatography (Fig. 1). The success of the procedure was facilitated by the amphiphilic nature of CD36. When heart microsomes were solubilized in the nonionic detergent Triton X-114 and phase-partitioned at 30 °C, three different fractions were obtained (Fig. 2A). Immunoblot analysis of the aqueous, detergent-rich, and insoluble/aggregated fractions demonstrated that CD36 was present only in the detergent-rich fraction (Fig. 2B, lane 1). The phase partitioning step provided a simple

FIG. 1. Protocol for purification of CD36 from bovine heart microsomes. Heart tissue was homogenized and solubilized in 1% Triton X-114 (step 1). The soluble extract was partitioned (step 2) into aqueous and detergent phases. The aqueous phase was recovered and again partitioned (step 3) after the addition of detergent. The two detergent phases (steps 4 and 5) were then combined and centrifuged. The resulting supernatant was chromatographed on an antibody-activated platelet CD36 immunoaffinity column (step 6).
and rapid way of enriching for CD36. The immunoaffinity step of the purification of bovine CD36 is based on the similarity of CD36 to the mammary epithelial cell membrane protein PAS IV (Greenwalt et al., 1990). Use of buffer containing 0.5 M NaCl in the washing of the immunoaffinity column prior to elution resulted in the absence of nonspecific binding and the elution of pure CD36 (Fig. 3). Purified CD36 migrated on an SDS-polyacrylamide gel as a broad band of ~85 kDa. Recovered CD36 constituted 0.1% of the total microsomal membrane (Table I).

**Immunological Cross-reactivity with Human CD36**—Evidence that the protein isolated from bovine heart was CD36 was obtained by examination of its reactivity with antisera against purified human platelet CD36 (Fig. 4A). Binding of monospecific rabbit anti-CD36 to bovine CD36 was 90% of that obtained with equivalent amounts of human CD36 and ~4 times that found in BSA control wells. Monoclonal antibody 8A6 (Barnwell et al., 1989) against human CD36 did not bind to bovine CD36, indicating the presence of a human platelet CD36-specific epitope (Fig. 4B). Further evidence for species-specific epitopes was provided by the observation that monoclonal antibodies OKM5 and OKM8, which also recognize human CD36 (Talle et al., 1983), did not bind to bovine CD36 (data not shown).

**N-terminal Sequence Analysis**—Additional evidence for the identification of the bovine protein as CD36 was provided by the homology of its primary structure to that of human CD36. Of the 18 identifiable residues of the N terminus of bovine CD36 (Fig. 5), 17 conformed to the N-terminal sequence of human CD36 (data not shown). The one difference is the substitution of an asparagine for aspartic acid at residue 3. Residues 1, 2, and 6 could not be definitively identified.

**Binding of IRBC**—Because human CD36 has been identified as an endothelial receptor for *P. falciparum*-infected erythrocytes, it was of interest to examine the ability of bovine CD36 to bind IRBC. Whereas human CD36 bound IRBC, bovine CD36 when bound to the plastic wells of a Petri dish did not (Table II). The inability of bovine CD36 to bind IRBC was observed even in the presence of greatly increased levels of bovine CD36 when bound to the plastic wells of a Petri dish (Fig. 5). The one difference is the substitution of an asparagine for aspartic acid at residue 3. Residues 1, 2, and 6 could not be definitively identified.
concentration 5 times higher than that required for 100% IRBC binding to the human protein, bovine platelet CD36 failed to bind IRBC (data not shown).

**DISCUSSION**

A human CD36 cDNA has recently been sequenced and has revealed that the \( M_r \) of the polypeptide is \( \sim 53,000 \) (Oquendo et al., 1989). Since the apparent \( M_r \) of human CD36 on an SDS-polyacrylamide gel is 88,000, most if not all of the 10 potential N-linked glycosylation sites are probably glycosylated. The human sequence reveals two putative transmembrane regions, one at either end of the protein. Beyond this, however, nothing is known about the structure of CD36 or the mechanisms by which it binds IRBC, collagen, and thrombospodin. Whereas human CD36 has been purified from platelet membrane preparations, purification of this protein from a tissue such as heart tissue in which CD36 constitutes a very small percentage of the total protein would be expected to be more difficult. We have taken advantage of the observation that a membrane protein of mammary epithelial cells (PAS IV) is very similar to CD36 and that polyclonal and monoclonal antibodies to PAS IV react with CD36 (Greenwalt et al., 1985, 1990). Bovine heart tissue was chosen as the source of CD36 since previous studies have shown that the capillary endothelial cells of bovine heart tissue are specifically and intensely stained by antibody to PAS IV (Greenwalt and Mather, 1985). The purification protocol reported in this paper is both straightforward and efficient. The initial solubilization step resulted in the partitioning of CD36 into one of three fractions and provided a large enrichment of the amphiphilic endothelial cell protein. The E-1 antibody specifically bound CD36 and allowed removal of nonspecifically bound protein with a high salt buffer. The yield of 1.1 mg of CD36/1.1 g of microsomal protein was surprisingly high and suggests that the microsomal preparation may be enriched for endothelial cell membranes. Identification of the heart protein as CD36 was confirmed by comparisons of sequence data and immunological cross-reactivity. The bovine CD36 N-terminal amino acid sequence was similar to that of human CD36 and exhibited 94% identity. The conserved nature of the N-terminus may reflect the possible transmembrane nature of this region of CD36 (So, 1989; Tandon et al., 1989).

Although polyclonal antisera to human CD36 cross-reacted with bovine CD36, the presence of species-specific CD36 epitopes was demonstrated by the failure of monoclonal antibodies 8A6, OKM5, and OKM8 to recognize bovine CD36. The structural basis of the species-specific epitopes is not understood. Both proteins migrate on SDS-polyacrylamide gels as broad bands, usually an indication of carbohydrate-based heterogeneity. Greenwalt et al (1990) have shown that both bovine and human CD36 migrate on SDS-polyacrylamide gels as 57-kDa bands after deglycosylation with endoglycosidase F, which removed N-linked oligosaccharides. The 3-kDa difference in size and the species-specific epitopes may be the result of species- or cell type-specific glycosylation. Yet another difference between human and bovine CD36 is their contrasting patterns of tissue distribution. With few exceptions (e.g. glomerular capillary endothelial cells), CD36 has been localized to the microvasculature of most human tissues examined (Knowles et al., 1984). In contrast, the microvasculature of major bovine organs such as the lung and brain lacks CD36 (Greenwell and Mather, 1985). Interestingly, the endothelium of the brain is a preferential site of IRBC sequestration in human malaria (MacPherson et al., 1985; Oo et al., 1987), and occlusion of brain capillaries with infected erythrocytes is thought to contribute significantly to the pathology of cerebral malaria (Howard and Gilladoga, 1989).

Demonstration of IRBC-binding and IRBC-nonbinding forms of CD36 is important in that studies of the structural differences between the two proteins may reveal the molecular basis of IRBC binding. Final analysis of the differences between bovine and human CD36 awaits cloning and sequencing of a bovine CD36 cDNA.

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D E Greenwalt, K W Watt, T Hasler, R J Howard and S Patel


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