EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 29, No 1, 31-34, March 1997

Purification of thrombospondin receptor (CD36) from human platelet membrane

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Accepted 24 January 1997

Abbreviations: WGA, wheat germ agglutinin; TTP, thrombotic thrombocytopenic purpura

Abstract

Thrombospondin receptor (CD36) has been recently identified in platelets and various cell lines as the receptor for thrombospondin, an adhesive protein required for irreversible aggregation of platelets as well as other adhesive processes. Thrombospondin receptor, one of major glycosylated platelet membrane proteins, is thought to play an important role as a cell adhesion molecule in blood coagulation system as well as intercellular signaling. In this work, thrombospondin receptor was purified to homogeneity from human platelet by wheat germ agglutinin (WGA)-affinity chromatography and size exclusion chromatography on Ultrogel-AcA44. The molecular weight of the purified thrombospondin receptor was about 88 kDa on SDS-PAGE and its identity was confirmed by immunoblot analysis and immunodiffusion assay.

Keywords: CD36, glycoprotein IV (GPIV), platelets, thrombosis, thrombospondin receptor

Introduction

Thrombospondin receptor, known as GPIIIb, glycoprotein IV (GPIV) or CD36, is a highly glycosylated platelet membrane protein that is not related to a known gene family (Huang *et al.*, 1991). Thrombospondin receptor or CD36 has been found in monocytes, endothelial cells, melanoma cells, and nucleated erythroid cells (Knapp *et al.*, 1989). The cDNA of CD36 has been cloned and sequenced from placenta, and deduced amino acid sequences predict an extracellular domain of 438 amino acid residues, a transmembrane domain of 24 residues, and a short cytoplasmic tail of 6 residues (Knapp *et al.*, 1989).

The CD36 has been recently identified in platelets and various cell lines as the receptor for thrombospondin. Thrombospondin is an adhesive protein that is required for irreversible aggregation of platelets as well as other adhesive processes (Asch *et al.*, 1987). Thrombospondin is secreted by platelets, endothelial cells as well as macrophages, and plays an important role in the consolidation of hemostatic plugs by being incorporated into fibrin clots. It has been shown that CD36 binds to collagen type I fibrils, and antibody against CD36 completely blocks collagen-induced platelet aggregation (Diaz-Ricart *et al.*, 1993; Saelman *et al.*, 1994). The CD36 has been also implicated in the adherence of *Plasmodium falciparum*-infected erythrocytes to the endothelium (Howard and Gilladoga, 1989). As suggested by these previous studies CD36 may have important functions as a cell adhesion molecule in blood coagulation system as well as intercellular signaling. In the current work, we purified human CD36 from platelet membrane by wheat germ agglutinin (WGA) affinity chromatography combined with size exclusion chromatography on Ultrogel-AcA44. Although partial biochemical characterization of CD36 has been documented, purified human CD36 would allow one to further characterize the protein.

Materials and Methods

Materials

Triton X-114, Triton X-100, thrombin, trypsin, soybean trypsin inhibitor, 4-chloro-1-naphthol, and anti-mouse antibody conjugated with horseradish peroxidase were purchased from Sigma (St. Louis, MO). WGA-sepharose was obtained from Vector Laboratories (Burlingame, CA), Ultrogel AcA-44 from LKB (Gaithersburg, MD), and CD36 mouse monoclo-nal antibody from Pharmingen (San Diego, CA).

Preparation of human platelet

Human platelets were isolated from platelet concentrates by differential centrifugation on Ficoll (Pharmacia) according to the method of Anderson and Gahmberg, 1978. Microscopic observation showed that the proportion of the platelets in this cell preparation was more than 99.5%.

Purification of CD36

Intact platelets were first proteolyzed to remove proteolysis-sensitive membrane proteins by incubating

with trypsin at 20˚C for 20 h followed by neutralization with soybean trypsin inhibitor. Washed platelets were then lysed by sonication and membrane fractions (62.4 mg of total protein) were solubilized in 30 ml of 1 % Triton X-114 containing 50 mM Tris-HCl (pH 7.4), 5 mM EGTA, and 0.5 mM phenylmethylsulfonyl fluoride as described (Tandon *et al.*, 1989) with slight modifications. Insoluble materials were removed by centrifugation at 100,000 *g* for 30 min. The Triton solution was warmed at 37˚C for 15 min for phase separation and then centrifuged at 1,800 *g* for 10 min. Detergent phase was loaded onto WGA-Sepharose column equilibriated with the buffer containing 50 mM Tris-HCl, 5 mM EGTA (pH 7.4) and 0.1% Triton X-100. Absorbed CD36 was then eluted with 3 column volumes of 250 mM *N*-acetylglucosamine. Fractions containing proteins were combined, dialyzed, and concentrated using an Amicon filtration assembly. For further purification concentrates were made 1 % with SDS, warmed at 80˚C for 2 min, cooled, and loaded onto an Ultrogel AcA-44 column equilibriated in 50 mM Tris-HCl (pH 7.4), 1 mM EGTA, 0.05 % Triton X-100, and 0.05 % SDS, which was subsequently eluted with the same buffer at a flow rate of 12 ml/h, and fractions of 3 ml collected. Fractions showing absorbance at 280 nm were analyzed by SDS-PAGE and those containing CD36 were pooled, dialyzed against 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and concentrated by Amicon filtration.

Electrophoresis and immunoblot analysis

Polyacrylamide gel electrophoresis in the presence of SDS was carried out by Laemmli system (Laemmli, 1970) on 7% polyacrylamide slab gel. After electrophoresis, the gels were stained with either Coomassie blue or periodic acid-Schiff reagent. For immunoblot analysis, proteins separated by electrophoresis were transferred to the membrane. And, the membrane was sequentially incubated with blocking solution of 2 % BSA, anti-CD36 monoclonal antibody, anti-mouse antibody conjugated with horseradish peroxidase, and chromogenic substrate, 4-chloro-1-naphthol.

Immunodiffusion assay

1.5% agar gels were poured onto slides and allowed to set. Wells are then punched in the gel and 10 μl of test solutions of antigens and antibodies were added to the wells. After solutions diffuse out, formation of precipitation lines was examined.

Results and Discussion

As outlined in the purification scheme (Figure 1), human platelet membrane was first extensively trypsinized to remove proteolysis-sensitive surface glycoproteins. This is based on the previous observation that CD36 is resistant to proteolysis in intact platelets (Okumura and Jamieson, 1976). Trypsinized platelet membrane was then solubilized using Triton X-114 and detergent phase was subjected to WGA-affinity chromatography (Figure 2). Lectins have been shown to specifically recognize and bind to certain glycoproteins and this specific interaction between lectins and glycoproteins has been successfully used in the purification of platelet membrane glycoproteins (Clemetson *et al.*, 1981, Leung *et al.*, 1981). A combination of various immobilized lectins with different binding characteristics was revealed to be very effective for the purification of glycoproteins.

Fractions eluted from WGA-affinity chromatography were analyzed by SDS-PAGE (Figure 3). Fractions 38 and 39 appeared to contain CD36 as a major protein and some lower molecular weight components. The major protein was first assumed as CD36 and further purifications proceeded, because the protein has the molecular weight similar to a known CD36 and two proteins were purified by similar procedures. The identity of the protein was confirmed after the final purification step. The minor contaminants in the fractions of WGA-affinity chromatography were removed by size exclusion chromatography on Ultrogel-AcA44. Again, CD36 was eluted and fractions electrophoresed (Figure 4). Fractions showing a single band of purified CD36 on a gel were pooled and concentrated for further analysis.

Purified CD36 was subjected to immunoblot analysis to confirm the identity and to test the antigenicity of the protein (Figure 5). The purified protein of 88 kDa was specifically recognized by anti-CD36 antibody. Proteins with higher molecular weights appear to be those that cross-reacted with anti-CD36 antibody. We currently cannot explain the nature of these proteins. Integrity of antigenic properties of purified CD36 was further

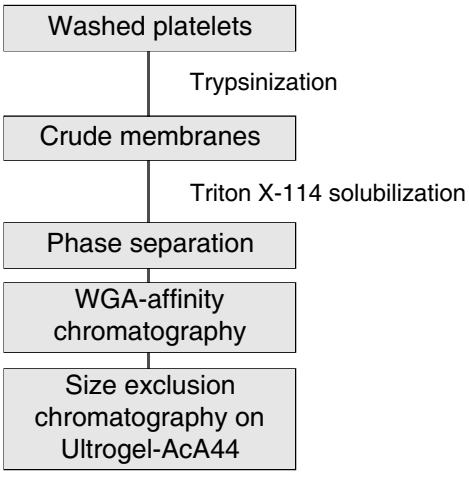

Figure 1. Purification scheme of CD36.

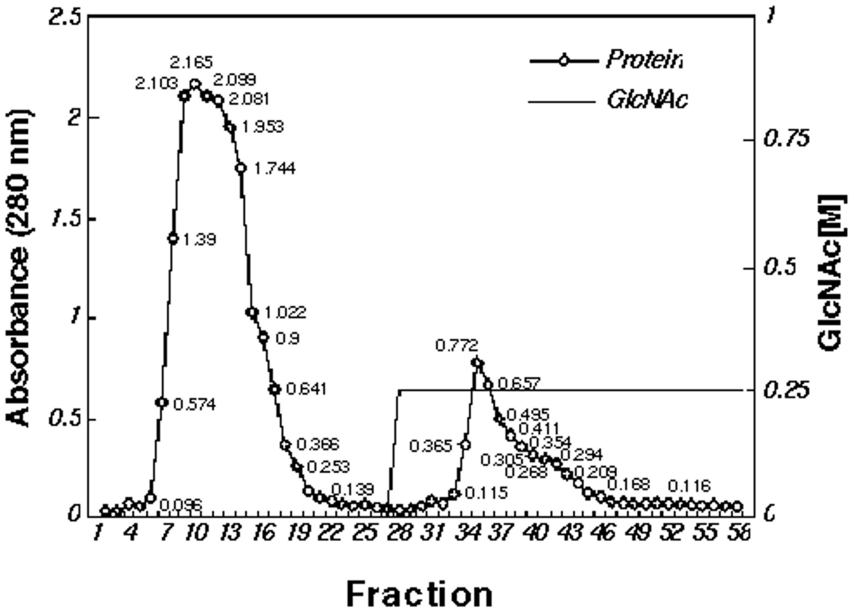

Figure 2. WGA-affinity chromato-graphy. Solubilized membranes of trypsinized platelets were loaded onto WGA-affinity column (1.2 × 12 cm). The column was first eluted with the buffer containing 50 mM Tris HCl, 5 mM EGTA (pH 7.4) and 0.1% Triton X-100, then absorbed proteins were eluted with the same buffer containing 250 mM *N*-acetylglucosamine (GlcNAc). Fractions were collected and absorbance measured at 280 nm. Solid line indicates absorbance values of each fraction and dotted line represents the time point where GlcNAc was added for the elution. The first peak of the solid line appears to be unbound proteins and the second peak seems to contain CD36.

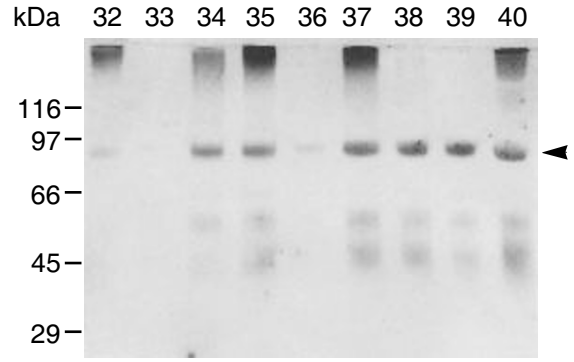

Figure 3. SDS-PAGE of fractions eluted from WGA-Sepharose column.Fraction eluted from WGA-Sepharose column was electrophoresed on 7% SDS-PAGE and stained with Coomassie blue. Lane numbers correspond to fraction numbers in Figure 2. The arrowhead indicates the protein that was assumed as CD36. The intensity of 88-kDa band in the fraction 36 is relatively weak because most of samples of this fraction was lost by mistake during experiment. This, however, did not significantly affect general purification scheme.

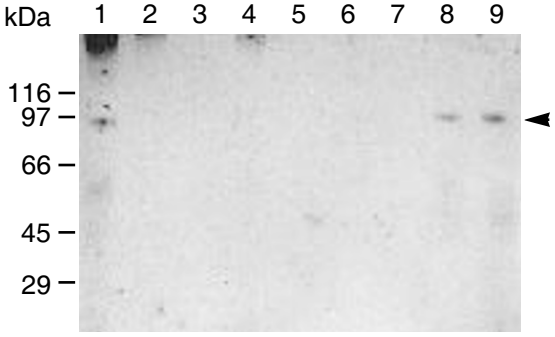

Figure 4. SDS-PAGE of fractions eluted from Ultrogel AcA-44 column. Pooled fractions containing partially purified CD36 (fractions 34 to 40 in Figure 3) were passed over Ultrogel AcA-44 column (fractionation range of 10-130 kDa), and fractions again analyzed by SDS-PAGE. Representative 9 fractions (lane 1 to 9) were electrophoresed on 7 % gel and stained with Coomassie blue. The arrowhead indicates the protein that was assumed as CD36.

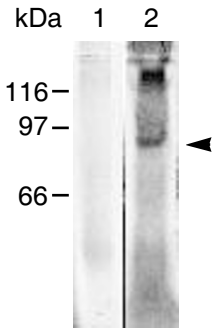

Figure 5. Immunoblot analysis. The purified protein of 88 kDa was subjected to immunoblot analysis. Proteins separated by electrophoresis were transferred to the membrane, which was subsequently incubated with blocking solution either without (lane 1) or with anti-CD36 mouse monoclonal antibody (lane 2). Then, the bound antibody was detected by sequential incubation with horse radish peroxidase-conjugated anti-mouse antibody and chromogenic\ substrate. The arrowhead indicates CD36.

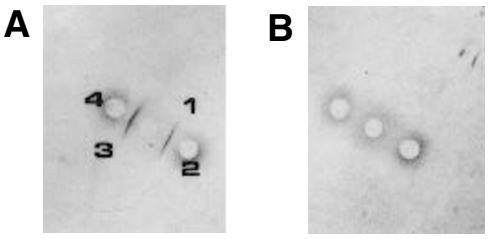

Figure 6. Immunodiffusion assay with purified CD36 and thrombin. **(A)**. Center well contained 5 μg of CD36 antibody and outside wells contained 7.5 μg of either purified CD36 (well 2 and 4), or thrombin (well 1 and 3). Only purified CD36 (well 2, 4) formed precipitation lines by complexing with anti-CD36 antibody. **(B)**. Center well contained 5 μg of thrombin antibody and outside wells contained 7.5 μg of purified CD36.

demonstrated by immunodiffusion assay (Figure 6). The purified CD36 protein showed a distinct precipitation band by forming complex with CD36 antibody. Thrombin, another important blood protein for platelet functions, was used as a negative control and did not react with CD36 antibody.

Taken together, CD36 was purified to apparent homogeneity by WGA-affinity chromatography and size exclusion chromatography. Although CD36 has previously been purified using lectin columns (Tandon *et al*., 1989, Tsuji and Osawa, 1986), the procedure employed in the current study is much simpler and time-saving without compromizing the purity and yield of the protein. The protein isolated by Tsuji and Osawa was described as CD36, but the protein showed proteolytic resistance, amino acid and carbohydrate analyses different from a known CD36 later purified (Tandon *et al*., 1989). Compared to purification procedures employed by Tandon group (Tandon *et al*., 1989), our current method does not involve DEAE chromatography or lectin-precipitation step between phase separation of Triton X-114 solubilized membranes and WGA-affinity chromatography (see purification scheme in Figure 1). The purification was initiated from 62.4 mg of total protein in platelets and the final recovery yield was 0.12 %, which is comparable to previously described methods (Tandon *et al*., 1989; Tsuji and Osawa, 1986).

Some of important functions of CD36 as a cell adhesion molecule have been previously reported (Asch *et al*., 1987; Howard and Gilladoga; 1989, Diaz-Ricart *et al*., 1993; Saelman *et al*., 1994). Recent findings on the involvement of CD36 in thrombotic thrombocytopenic purpura (TTP) and other disorders of hematopoietic system indicate its pathological implications and yet other critical functions (Byrnes and Moake, 1986; Lian *et al*., 1991; Siddiqui and Lian, 1992). Now, purified CD36 could be utilized for further characterization of the protein in biochemical as well as clinical aspects. For instance, we evaluated the possibility of CD36 binding to 37-kDa platelet agglutinating protein (PAP p37) found in TTP patient plasma. Although there has been a claim that platelet aggregation in TTP may be mediated by direct interaction between CD36 and PAP p37 (Lian *et al*., 1991), we were not able to confirm the results (data not shown). If indeed, however, platelet agglutination in TTP patient occurs through interaction between CD36 and PAP p37, inhibitors that block the interaction of two proteins can be designed for therapeutic purpose. For such applications, purified CD36 can be used for the generation of anti-CD36 antibody or searching for peptides that has such inhibitory effects. Elucidation of pathophysiological role of CD36 in TTP and other disorders is certainly an intriguing research area that awaits further works.

Acknowledgement

This work was supported by a grant from Korean Ministry of Science and Technology (HAN project, HS1140).

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