Purification and Identification of the Lipoprotein-binding Proteins from Human Blood Platelet Membrane*

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As reported previously, homologous plasma lipoproteins specifically bind to the plasma membrane of human blood platelets. The two major lipoprotein-binding membrane glycoproteins were purified to apparent homogeneity and identified by their mobilities in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, both in the nonreduced and reduced state, by specific antibodies against glycoproteins Iib (GPIIb) and IIIa (GPIIIa), respectively, including the alloantibody anti-PI* and monoclonal antibodies. Furthermore, antigen binding to intact platelets is also inhibited in a dose-dependent fashion by preincubation of the platelets with antibodies against these glycoproteins. From these experiments it can be concluded that lipoproteins bind to both components of the glycoprotein IIb-IIIa complex in isolated membranes and intact platelets.

High density lipoprotein and low density lipoprotein binding to GPIIb complexes blotted to nitrocellulose in a way that binding of one species interferes with the binding of the other. Addition of fibrinogen significantly inhibits this binding. The specific binding of fibrinogen to GPIIb is strongly inhibited in the presence of either of the two lipoproteins. LDL and HDL are specifically bound by isolated GPIIb, too. In our bloting experiments fibrinogen shows no binding to this membrane glycoprotein. On the other hand, fibrinogen significantly interferes with the interaction between GPIIb and the lipoproteins.

Various hyperlipoproteinemic conditions are associated with a high risk for the development of atherosclerosis and thrombosis (1, 2). In recent years extensive in vitro investigations revealed that homologous plasma lipoproteins modulate in a concentration-dependent way the susceptibility of isolated human blood platelets to ADP and thrombin (3-8). These data strongly support the hypothesis that increased concentrations of lipoproteins in vivo, especially of the low density class, render platelets hyperreactive which in turn might enhance atherogenesis (9). Furthermore, at very high concentrations, LDL triggers platelet aggregation itself in vitro even in the absence of any other platelet effector (10). There is some evidence that this stimulation rather proceeds via direct interaction of lipoproteins with specific membrane receptors than effects due to lipid transfer or exchange.

Several groups have reported on specific binding of various lipoprotein subclasses to isolated intact platelets (11-15); there is, however, considerable disagreement about the properties of these binding sites (11, 15), and very little is known about the molecular events following binding in vivo (10, 16). Recently, we were able to demonstrate by ligand-blotting techniques up to three platelet membrane protein entities exhibiting saturable binding of LDL and HDL (17); their molecular properties, however, are not related to known lipoprotein receptor proteins from other mammalian cells (18). On the other hand, the electrophoretic pattern of these bonds strikingly resembles those of the components of the glycoprotein (GP) IIb-IIIa complex, one of the major membrane constituents of platelets (19, 20). It was therefore the aim of this study to purify and identify the lipoprotein-binding proteins from human platelet membranes. In fact, it could be shown that they are likely to be identical with the components of the GPIIb-IIIa-complex, the inducible platelet fibrinogen receptor.

EXPERIMENTAL PROCEDURES

Materials—Peroxidase-coupled antibodies against rabbit IgG, nitrocellulose 121 (Amersham Int. plc, United Kingdom; gelatine (Bio-Rad); anti-sera against fibrinogen, human whole serum, human von Willebrand factor and human fibronectin (Behringwerke AG, Marburg, Federal Republic of Germany); monoclonal antibodies against GPIIb-IIIa and against GPIIIa (Dakopatts, Glostrup, Denmark); fibrinogen (Kabi, Stockholm, Sweden); SDS (Merck, Darmstadt, F. R. G.); high molecular weight standard for SDS electrophoresis, 1-O-cetyl-β-D-glucopyranoside, human albumin, ovalbumin, phosphatidylcholine (Sigma); fluorescein-labeled lectins: concanavalin A, wheat germ agglutinin, peanut agglutinin, Dolichos biflorus, Ulex europeaus, Ricinus communis (Vector Laboratories Inc., Burlingame) were obtained as indicated. Anti-PI* was a generous gift of Dr. S. Panzer, First Department of Medicine, University of Vienna, Austria (21). Polyclonal rabbit anti-GPIIb and anti-GPIIIa, respectively, were generously donated by Dr. D. Pidard, INSERM, Hopital Lariboisiere, Paris, France. Antisera and monoclonal antibodies were purified by Protein A-Sepharose chromatography (22). Suramin was kindly provided by Bayer-Austria, Vienna.

Plasma lipoproteins were isolated as described earlier (11) by means of sequential flotation of normal plasma at 100,000 g in a Sorvall OTD Combi ultracentrifuge (Sorvall Du Pont Company, Wilmington). Lipoproteins were washed during flotation by a KBr solution of the respective lipoprotein density. No contaminating plasma proteins were detectable by immunoelectrophoresis against rabbit anti-human serum, rabbit anti-human fibrinogen, rabbit anti-human von Willebrand factor, and goat anti-human fibronectin, respectively. Concentrations of lipoproteins are given as their protein content as determined by the method of Lowry et al. (23). According to their respective densities, lipoproteins were divided into very low

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§ The abbreviations used are: LDL, low density lipoprotein; GPIIb, glycoprotein Iib; GPIIIa, glycoprotein IIIa; HDL, high density lipoprotein; LDLr, high density lipoprotein-3; S-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Density lipoprotein, low density lipoprotein, high density lipoprotein-1, and high density lipoprotein-3 (HDL3) (24).

HDL-Sepharose was prepared from CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. Using 20 mg of HDL3-protein/g of dry gel, we obtained a final concentration of 3.6 mg of covalently attached lipoprotein/ml settled gel. LDL-Sepharose was prepared in an analogous manner reducing the final concentration to 0.5 mg/ml settled gel.

Fluorescein, eventually contaminating the fluorescence preparation used, was removed by chromatography on gelatine-Sepharose 4B (Pharmacia) (25). As judged by SDS-PAGE after reduction with 5% (v/v) β-mercaptoethanol no contaminating protein was detectable.

Preparations were labeled with iodine by the iodogen method (26).

Specific radioactivities of 3.5 (LDL), 2.2 (HDL3), and 1.9 (fibrinogen) Bq/ng of protein were obtained.

Human platelet membranes were prepared as published previously (17) by means of sucrose gradient centrifugation of sonified washed platelets obtained from normal blood donors. Washed human platelets were prepared as described previously (11).

Purification of Receptor Protein—50 ml of the crude plasma membrane preparation (1–2 mg of protein/ml) were diluted with an equal volume of 0.5 M Tris-maleate buffer, 2 mM phenylmethylsulfonyl fluoride, pH 6.0, or solution twice at 25 °C at 0 °C. The suspension was then made 1% (v/v) in Triton X-100, kept at 4 °C for 30 min, and then centrifuged for 1 h at 100,000 × g. The supernatant was dialyzed overnight at 4 °C against 0.05 M Tris-maleate, 1% (v/v) Triton X-100, and then applied to 40 ml of DEAE-Sephadex A-25 equilibrated with the same buffer. The column was washed with 56 ml of 0.05 M Tris-maleate, pH 6.0, 0.04 M octyl glucoside and thereafter eluted with a linear gradient of NaCl (0–0.2 M, total volume 80 ml) in the washing buffer. Fractions were screened for lipoprotein binding by means of the blotting experiments described below. The lipoprotein-binding proteins (150–310 μg protein/ml) eluted between 0.13 and 0.18 M NaCl. Active fractions were pooled and further purified by affinity chromatography on HDL-Sepharose. The pooled fractions were slowly applied to 2.5–3.5 ml of HDL-Sepharose, equilibrated with 0.05 M Tris-maleate, pH 6.0, 0.04 M octyl glucoside, 0.15 M NaCl. Following washing with equilibration buffer without NaCl, elution was performed with a total of 8 ml of 0.015 M sodium in 0.05 M Tris-maleate, pH 6.5, 0.04 M octyl glucoside. The column effluent was screened as above; active fractions were pooled and used as purified lipoprotein-binding proteins.

GPIIb-IIIa was purified to homogeneity from platelet membranes according to Jennings and Phillips (20) using temperature-dependent solubility differences of membrane proteins in Triton X-100 solutions and Sephadex S-300 chromatography.

Electrophoresis—SDS-PAGE was performed in 8% polyacrylamide slab gels, containing 18% (v/v) glycerol. The buffer contained 0.1% SDS, 5% 2-mercaptoethanol. Samples were prepared by incubation with 2% SDS for 45 min at room temperature and without addition of 5% 2-mercaptoethanol. Separated proteins were stained with Coomassie Brilliant Blue. Preparative electrophoresis was run in 2.5-mm thick slab gels at conditions as above. The zones corresponding to the two lipoprotein-binding protein species were separately electroeluted from the preparative gel in a sample concentrator, model 1750, Issc, Inc., Lincoln, Nebraska.

Immunoblotting—Membrane proteins were transferred to nitrocellulose membrane according to Towbin et al. (27).

Binding experiments—Binding of lipoproteins (biotinylated or radio-labeled) was performed as reported previously (17). Bledted membranes were incubated with various concentrations of lipoproteins in the presence of 10 mg/ml gelatine as given in the figure legends for 2 h at room temperature. Binding of unlabelled fibrinogen was detected by incubation with biotinylated anti-fibrinogen antiserum (Behringwerke AG). Binding of antibodies against GPIIb and GPIIIa, respectively, was followed with biotinylated donkey anti-rabbit Ig. As controls the biotinylated antibodies were incubated with the blotted membrane proteins in replicate experiments replacing the specific ligands by 10 ng/ml gelatine. Visualization of the bound biotinylated species was achieved by incubation with streptavidin-biotinylated peroxidase complex (28). Binding of radiolabeled species was quantitatively determined with the TLC Linear Analyzer LB 2820 (Bertold, Wildbad, F. R. G.).

Binding of fluorescein-labeled lectins was performed at lectin concentrations of 50 μg/ml following the same incubation procedure as used for lipoprotein binding. Bound fluorescein-labeled lectins were visualized under UV light at 366 nm. Neuraminidase (Merck) treatment of 0.3 mg of lipoprotein-binding proteins was done at pH 5.5 for 20 min at 37 °C with 10 milligrams of enzyme (0.02 mg of enzyme/ml).

Competitive binding experiments were performed under the same conditions as above. Radioactively labeled LDL or HDL3 were simultaneously applied to the blotted membrane proteins together with unlabeled LDL, HDL3, and human serum albumin concentrations given in Table I. Also, ovalbumin (10 mg/ml), caseine (10 mg/ml), and phosphatidylcholine vesicles (0.2 mg/ml) were used as competitors. After 2 h of incubation, membranes were washed with Tris-buffered saline and binding of radioactively labeled lipoproteins was determined with the thin layer chromatography Linear Analyzer LB 2820 (Bertold, Wildbad, F. R. G.).

Binding of radioactively labeled lipoproteins to washed human platelets was studied in the presence and absence of antibodies against glycoproteins IIb and IIIa. Washed platelets (6.5–11.2 × 109 platelets resuspended in Tyrode’s solution containing 3.5 mg of human serum albumin and 1 mg of D-glucose/ml and 5 mM HEPES, pH 7.35, were incubated with different amounts of the respective antibodies as given in Fig. 5 for 5 min at room temperature. Thereafter, 4.5 μg of [125I]HDL or 1.9 μg of [125I]LDL were added, the total volume was adjusted to 0.6 ml with the platelet resuspension buffer, and incubation was continued for additional 25 min at room temperature. Thereafter, 500 μl were removed and platelets were separated from the incubation mixture by centrifugation (3 min at 16,000 × g) in an Eppendorf centrifuge 5415C (Hamburg, F. R. G.) through 0.8 ml of a 200 mg/ml sucrose solution in the platelet resuspension buffer. The platelet pellet was then recovered by cutting the tips of the plastic centrifuge vial, and the platelet pellet as well as the supernatant are counted for their respective radioactivities in a gamma counter (Beckman G-8000). Nonspecific binding was determined by replicate experiments in the presence of 25-fold excess of unlabelled ligands. Specific binding is expressed as percent of total radioactivity in the platelet pellet minus percent nonspecific bound radioactivity. Effects of added antibodies are expressed as the ratio of specific binding in the presence of the respective antibody to specific binding in controls without the specific antibody.

RESULTS

Table I summarizes the purification of the two major lipoprotein-binding proteins from human blood platelet membranes. Purification was followed by ligand blotting of SDS-PAGE-separated membrane proteins employing purified LDL. The same results were obtained when HDL was used instead of LDL. Data in the table represent relative amounts of the two lipoprotein-binding bands as compared with total stainable proteins. As can be seen from the table, both binding proteins copurified during the procedure and could only be separated upon SDS-PAGE. The yield after preparative electrophoresis was 27 and 22%, respectively, for the smaller and the larger binding moiety. Occasionally a third lipoprotein-binding band with a higher molecular weight was detectable.

Table I

| Purification of lipoprotein-binding proteins (114- and 140-kDa proteins, respectively) from platelet plasma membranes |
|---------------------------------------------------------------|
| Total protein | 114 kDa purification | 140 kDa purification |
|----------------|----------------------|----------------------|
| Plasma membrane preparation | 67.0 | 4.9 | 1.0 | 6.7 | 1.0 |
| Supernatant 100,000 × g | 46.0 | 5.9 | 1.2 | 7.6 | 1.1 |
| DEAE-Sephadex | 4.1 | 39.0 | 7.9 | 32.0 | 4.7 |
| HDL-Sepharose | 2.4 | 42.0 | 8.5 | 43.0 | 6.8 |

Preparative SDS-PAGE

| Fraction | 0.98 | 100.0 | 20.4 | 1.0 | 100.0 | 14.9 |

*% of total protein, determined by densitometry of Coomassie Brilliant Blue-stained proteins following SDS-PAGE.
in the starting material, but was removed by the DEAE-Sephadex step.

Fig. 1 shows SDS-PAGE of the purified preparation as compared with the starting material. Two stainable bands can be seen at the same position of the two lipoprotein-binding proteins (right panel, ligand blotting). The apparent molecular masses of the two bands were 140 ± 3 kDa and 114 ± 2 kDa, respectively.

Upon reduction (Fig. 2, lanes a and b) the apparent molecular mass of the slower migrating band decreased from 140 ± 3 to 130 ± 5 kDa, while the apparent molecular weight of the faster moving band increased from 114 ± 2 to 118 ± kDa; the lipoprotein binding ability of the reduced proteins was almost completely abolished (not shown).

The final two purification steps (affinity chromatography and preparative electrophoresis), however, were associated with partial loss of biological activity. Therefore, the non-homogeneous material obtained by ion exchange chromatography was employed in addition to the purified material in studies for characterization of the binding properties of the two lipoprotein-binding proteins.

The binding of radioactively labeled LDL and HDL₃, respectively, had no effect on the binding of the lipoproteins (Table II).

To analyze the presumptive glycoprotein nature of these two membrane proteins, the binding of fluorescein-labeled lectins to the blotted purified receptor preparations was evaluated (not shown). The 114-kDa species strongly bound concanavalin A, the 140-kDa species bound both concanavalin A and wheat germ agglutinin weakly. Regardless of prior treatment with neuraminidase, both receptor proteins did not bind peanut agglutinin, Dolichos biflorus, Ulex europaeus, and Ricinus communis lectins.

The molecular weight pattern of the two solubilized proteins as well as their behavior in all of the experiments cited above suggest an involvement of the components of the major platelet membrane glycoprotein complex GPIIb-IIIa. To validate this assumption, immunoblotting experiments were per-

**Fig. 1.** Purification of lipoprotein-binding proteins: SDS-PAGE of total platelet lysate (derived from 1.7 × 10⁷ platelets, lanes a and d), of solubilized plasma membranes isolated by density gradient centrifugation (lanes b and e, 160 µg of protein), and of a preparation purified by DEAE-Sephadex and HDL₃-affinity chromatography (lanes c and f, 18 µg of protein). Coomassie Brilliant Blue-stained gel (lanes a-c). Ligand blotting experiments (lanes d to f); binding of biotinylated LDL (15 µg/ml) to transferred membrane proteins was visualized by a streptavidin/biotinylated peroxidase complex.

**Fig. 2.** Effect of reduction of disulfide bridges on electrophoretic behavior of lipoprotein-binding proteins. Lane a, SDS-PAGE of nonreduced receptor proteins purified by DEAE-Sephadex chromatography (95 µg of protein applied, stained with Coomassie Brilliant Blue); lane b, as lane a, but sample was reduced with 5% β-mercaptoethanol, 100°C, 10 min; lane c, nitrocellulose-transferred receptor proteins (13 µg, unreduced), incubated with 11 µg/ml of biotinylated LDL; lane d, nitrocellulose-transferred receptor proteins (10 µg reduced as in lane b) incubation with rabbit anti-GPIIb antibody (1/1000).

**TABLE II**

| ¹²⁵I-Labeled ligand | Competitor | Radioactivity bound to the |
|---------------------|------------|---------------------------|
|                     |            | 140-kDa band | 114-kDa band |
|                     |            | cpm | % | cpm | % |
| LDL                  | None       | 31,700 | 100.0 | 58,400 | 100.0 |
| LDL                  | LDL (440 µg/ml) | 5,670 | 17.3 | 13,800 | 23.4 |
| LDL                  | HDL₃ (89 µg/ml) | 4,560 | 14.4 | 11,200 | 18.9 |
| LDL                  | HSA (10 mg/ml) | 30,300 | 95.5 | 93,800 | 90.6 |
| HDL₃                 | None       | 14,900 | 100.0 | 20,300 | 100.0 |
| HDL₃                 | LDL (440 µg/ml) | 2,010 | 13.5 | 2,400 | 11.8 |
| HDL₃                 | HDL₃ (89 µg/ml) | 1,470 | 9.9 | 2,060 | 10.1 |
| HDL₃                 | HSA (10 mg/ml) | 14,500 | 97.9 | 19,200 | 94.6 |

*Percent of the ligand bound in the presence of competitors as compared to no competitors.

a 50.2 µg/ml; 0.82 kBq/µg.

b 7.6 µg/ml; 1.14 kBq/µg.
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FIG. 3. Binding of specific antibodies to GPIIb and GPIIIa, respectively, and of fibrinogen. A, blotting of 14 μg of DEAE-Sephadex-purified membrane proteins with lane a, anti-GPIIIa antibody (1/2000); lane b, 14 μg/ml biotinylated LDL; lane c, anti-GPIIb antibody (1/1000). Bound antibodies were visualized following incubation with biotinylated donkey anti-rabbit Ig. B, ligand blotting experiments with 20 μg of purified receptor proteins with lane a, 17 μg/ml HDL₃; lane b, 60 μg/ml anti-PI⁺⁺⁺ antiserum; lane c, 200 μg/ml fibrinogen; lanes d-f, 15 μg/ml LDL, in the presence of 0; 0.2; 2.0 mg/ml of fibrinogen. With the exception of fibrinogen, biotinylated ligands were applied in these experiments. A biotinylated antibody was used to visualize bound fibrinogen. No bands were visualized in controls.

TABLE III
Specificity of binding of radiolabeled human fibrinogen to the 114-kDa protein species

| Competitor                  | Radioactivity bound to the 114-kDa band |
|-----------------------------|----------------------------------------|
| None                        | 46,200 ± 1,900                          |
| Fibrinogen (4 mg/ml)        | 6,680 ± 520                            |
| LDL (1.8 mg/ml)             | 2,310 ± 210                            |
| HDL₃ (3.7 mg/ml)            | 1,870 ± 130                            |
| Human albumin (10 mg/ml)    | 41,400 ± 2,000                          |
| Ovalbumin (10 mg/ml)        | 42,700 ± 2,100                          |
| Phosphatidylcholine (2.0 mg/ml) | 44,400 ± 1,950                      |

*Percent of the ligand bound in the presence of competitors as compared with no competitors.

FIG. 4. Comparison of lipoprotein-binding proteins in two differently prepared platelet membrane preparations. Lane a, SDS-PAGE of 26 μg/ml (unreduced) protein prepared according to Jennings and Phillips (20), after Sephacryl S-300 chromatography, stained with Coomassie Brilliant Blue; lane b, 18 μg/ml protein, prepared as described under “Experimental Procedures” after DEAE-Sephadex chromatography, Coomassie Brilliant Blue stained; lanes c and d, nitrocellulose-transferred proteins corresponding to the sample shown in lanes a and b, respectively, blotted with 11 μg/ml biotinylated LDL.

formed. Results are shown in Fig. 3. As can be seen, polyclonal antibodies specifically directed against isolated GPIIb and GPIIIa, respectively, bind to both the 140 and 114 kDa proteins, of the purified preparation (Fig. 3A). Upon reduction, binding of anti-GPIIb antibody to two bands corresponding to molecular masses of 130 and 23 kDa, respectively, could be seen (Fig. 2, lane d). Employing the alloantibody anti-PI⁺⁺⁺, which is an antibody against PI⁺⁺⁺ antigen located on GPIIIa (29), the same pattern as with the anti-GPIIIa antibody was seen (Fig. 3B, lane b).

Furthermore, the faster moving protein exhibited specific binding of fibrinogen, the major GPIIb-IIIa ligands. Fig. 3B shows binding of biotinylated LDL (lane d), fibrinogen (lane...
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Fig. 5. Inhibition of lipoprotein binding to washed platelets by antibodies against GPIIb (■—■) or antibodies against GPIIIa (●—●). Percent inhibition of specifically bound radioactively labeled HDL₃ is plotted against micrograms of purified antibody added to the platelet suspension 5 min before addition of the lipoprotein (4.5 μg of HDL₃ protein/ml; 1.42 Bq/ng; concentrations given are final).

c, and biotinylated HDL₃ (lane a) to the blotted preparation. As can be seen, binding of fibrinogen as visualized by labeled anti-fibrinogen antibody and the two lipoprotein classes bind to the same position of the blotted gel. Furthermore, specificity of fibrinogen binding was analyzed by competition experiments summarized in Table III. An excess of unlabeled fibrinogen markedly inhibits binding of labeled fibrinogen. The binding of fibrinogen is even more inhibited by both LDL and HDL. It was virtually unaffected by addition of a 20-fold molar excess of unrelated proteins (human serum albumin and ovalbumin) and phosphatidylcholine vesicles, respectively.

To prove whether the GPIIb-IIIa is in fact the lipoprotein binding moiety of the purified preparation, competition experiments between fibrinogen and LDL were performed (Fig. 3B, lanes a–f). Increasing amounts of fibrinogen decreased binding of LDL in a dose-dependent fashion to the 114 kDa band. Although we could not detect any binding of fibrinogen to the larger of the two purified lipoprotein-binding proteins, fibrinogen strongly inhibits also binding of LDL to this species. Furthermore, when blotted membranes were incubated simultaneously with LDL and anti-GPIIb and anti-GPIIIa, no lipoprotein binding at all was detectable (not shown). Identity of the lipoprotein-binding proteins with the components of the GPIIb-IIIa complex was further evaluated by studying the lipoprotein binding of GPIIb-IIIa purified by a method generally used for the GPIIb-IIIa complex. Comparison of our receptor preparation, and GPIIb-IIIa prepared according to the method of Jennings and Phillips (20) reveals that both preparations obviously contain an identical pair of LDL-blotting membrane proteins (Fig. 4).

Results of competition experiments between radioactively labeled lipoproteins and antibodies against GPIIb and GPIIIa for binding to washed human platelets are shown in Fig. 5. A dose-dependent decrease of specific HDL₃ binding by an antibody directed against GPIIIa was observed which plateaued at about 50% binding; an antibody against GPIIb decreased HDL₃ binding only by less than 10% using similar antibody concentrations. Comparable results were obtained when radioactively labeled LDL was used and a monoclonal antibody against GPIIb-IIIa, resulting in a decrease of LDL binding by 46% at an antibody concentration of 12 μg/ml.

DISCUSSION

As reported earlier by us (11), unstimulated human platelets contain 1500 binding sites for LDL and 3200 for HDL₃, respectively. Upon SDS-PAGE of solubilized platelet plasma membranes, we could previously identify up to three protein bands capable of saturable, high affinity binding of both LDL and HDL₃ (17). In this paper we attempted to purify the lipoprotein-binding proteins by a method generally used for purification of the apoB/E lipoprotein receptor (30). This purification employs solubilization of the membranes in Triton X-100, followed by DEAE-Sepharose chromatography and affinity chromatography on LDL-Sepharose. In fact, it was possible by means of this method to purify two separate lipoprotein-binding proteins from platelet membranes. The two entities copurified during the whole procedure and only could be separated by SDS gel electrophoresis. The molecular weights, however, obtained for the two lipoprotein-binding proteins differed from that usually observed for the lipoprotein receptors obtained from fibroblasts (30). Furthermore, the pattern of the reduced apoB/E-receptor as visualized upon SDS-PAGE differs from that obtained with the purified platelet lipoprotein receptor preparation.

The predominant membrane protein in human platelet is the GPIIb-IIIa complex, two glycoproteins with apparent molecular masses of 142 kDa for GPIIb and 85–115 kDa for GPIIIa, respectively (19, 20). Reduced GPIIb exhibits two subunits of molecular masses of 123 and 22 kDa, respectively. Upon reduction, the molecular mass of GPIIIa raises by 4–10 kDa. In fact, our purified lipoprotein binding preparation resembles the molecular masses of the GPIIb-IIIa complex constituents before as well as after reduction. Although the apparent molecular masses of GPIIb-IIIa, based on their migration in gradient gels, differ from those obtained by us, virtually identical values are reported at nonequilibrium conditions at lower acrylamide concentrations, both for nonreduced and reduced samples (19, 31, 32). Furthermore, the glycoprotein nature of our purified receptor preparations was revealed by various lectin binding experiments and is compatible with the proposed identification as the glycoprotein IIb-IIIa complex (33).

This identification is substantially supported by the effects observed with different protein probes specific for GPIIIa (anti-P₃+₄, anti-GPIIIa), and for GPIIb (anti-GPIIb), respectively. The former two antibodies have been shown to bind to the 114-kDa protein, the latter strongly associates with the transferred 140-kDa protein and with both subunits obtained in the reduced protein (Fig. 2, lane d).

One of the natural ligands of the GPIIb-IIIa complex is fibrinogen (34). Fibrinogen binding is thought to be dependent on the presence of an associated GPIIIa complex in the presence of calcium. These data, however, were obtained in vitro and by two-dimensional electrophoresis in agarose gel (35, 36). No data are available for fibrinogen binding to blotted glycoproteins separated by SDS-PAGE. We could show that the 114-kDa band recognized by an antibody against GPIIIa in fact exhibits fibrinogen binding. This binding was specific because radiolabeled fibrinogen could be replaced by unlabeled material. Furthermore, unrelated proteins did not interfere with the binding of fibrinogen (Table III). Therefore it may be concluded that our purified lipoprotein binding preparation contains the major glycoprotein IIb-IIIa, whereby the GPIIIa moiety is present in such a conformation that it is still able to bind fibrinogen. This would be consistent with
data obtained by Bennet et al. (37) who found that the GPIIIa moiety becomes cross-linked to fibrinogen.

Identity of GPIIb-IIIa with the lipoprotein binding moiety migrating in the same position was analyzed by antibody competition of the blotted preparation. Coincubation of the blotted lipoprotein-binding proteins with both anti-GPIIb and anti-GPIIIa antibodies as well as with lipoproteins completely abolished binding of lipoproteins. Furthermore, fibrinogen itself decreased binding of biotinylated LDL and HDL in a dose-dependent fashion to both glycoproteins, indicating close relationship if not identity of the lipoprotein-binding proteins with GPIIb-IIIa.

The fact that fibrinogen binding could be shown only for the GPIIIa moiety, but fibrinogen interfered also with the lipoprotein binding of the GPIIb protein, is puzzling. In preliminary experiments an interaction of fibrinogen and lipoprotein binding of the GPIIb protein, is puzzling. In the GPIIIa moiety, but fibrinogen interfered also with the lipoproteins completely abolished binding of lipoproteins. Furthermore, fibrinogen itself decreased binding of biotinylated LDL and HDL in a relationship if not identity of the lipoprotein-binding proteins to or identical with the GPIIb-IIIa. It cannot completely be ruled out that GPIIb-IIIa present in rather large amounts and 1500 and 3200 binding sites for different epitopes on the GPIIb-IIIa membrane glycoprotein. As often seen with mixtures of membrane glycoproteins separated by PAGE, additional bands appear either by dimerization or association with different membrane proteins. Any of the two proteins identified however, is unlikely, because also by means of a method published for purification of GPIIIa, the lipoprotein binding ability was copurified (Fig. 4). Furthermore, in experiments using intact washed platelets, it could be shown that antibodies against GPIIIa interfered with specific lipoprotein binding in a dose-dependent fashion (Fig. 5). These data indicate that the GPIIb-IIIa complex contributes also to lipoprotein binding to intact platelets and is not restricted to the artificial conditions of the blotting experiments. It is noteworthy that an antibody directed against GPIIb had only limited effect on HDL₃ binding which is, however, consistent with the weaker binding of HDL₃ to the blotted GPIIb as shown in Fig. 3B.

The third lipoprotein-binding band occasionally present in membrane preparations was removed upon DEAE-Sephadex chromatography. This entity might not necessarily be a separate lipoprotein-binding protein. As often seen with mixtures of membrane glycoproteins separated by PAGE, additional bands appear either by dimerization or association with different membrane proteins. Any of the two proteins identified in this paper could give rise to such additional bands. On the other hand, the possible in vivo existence of an independent third receptor protein cannot be ruled out.

We have previously reported on 1500 and 3200 binding sites for LDL and HDL₃, respectively (11) in unstimulated platelets. Curtiss and Plow (12) found the number of binding sites in the same range (7000, 1500). If in fact the lipoprotein-binding sites are identical to GPIIb-IIIa receptor, the number of binding sites for the lipoproteins is by far exceeded by the number of immunologically determined GPIIb-IIIa. This, however, might be explained by the fact that a functional assay for lipoprotein binding on intact platelets was used in this study whereas the number of GPIIb-IIIa receptors was revealed by immunological methods (38) and might not reflect the number of functionally intact binding sites in unstimulated platelets. It should be noted that similar discrepancies are published using different monoclonal antibodies specific for different epitopes on the GPIIb-IIIa membrane glycoprotein (38, 39).

GPIIb-IIIa fulfills a key role in course of platelet aggregation. Consequently, the binding of lipoproteins to this membrane protein complex might have major effects on platelet function in vivo. Such an influence of lipoproteins on platelet function is reflected by several reports showing enhancement of platelet aggregation in patients with hyperlipoproteinemia (3, 4, 40). In fact, we could show that fibrinogen binding to ADP or thrombin-stimulated platelets is significantly modified in the presence of LDL (41).

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