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Research Article

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Reconstitution of Platelet Proteins into Phospholipid Vesicles

Functional Proteoliposomes

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Abstract

Platelet membrane glycoproteins IIb and IIIa were reconstituted into liposomes containing phosphatidylcholine. The reconstituted vesicles bound antiplatelet antibodies and showed specific binding to thrombin-activated platelets. Prostacyclin, a known inhibitor of thrombin-activated platelet aggregation, inhibited the binding of the proteoliposomes to thrombin-activated platelets. The reconstituted vesicles also specifically bound ^{125}I -labeled fibrinogen. This binding was insensitive to ADP but dependent on calcium ions. These data indicate that platelet glycoproteins IIb and IIIa have been successfully reconstituted into phospholipid vesicles such that their behavior is similar to that in intact platelets.

Introduction

Platelet aggregation culminates as a result of a complex series of interrelated processes. Intact platelets are activated by the binding of agonists (e.g., thrombin, ADP, and epinephrine) to receptors on the platelet surface. Activation results in the unmasking of specific binding sites for fibrinogen (1–3). Although the binding of fibrinogen is known to be essential for aggregation, the mechanism by which fibrinogen binding leads to aggregation is not known.

Several lines of evidence suggest that platelet membrane glycoproteins IIb and IIIa are important in fibrinogen binding to platelets and in platelet aggregation. Platelets from patients with Glanzmann's thrombasthenia which lack these two proteins do not aggregate or bind fibrinogen (4). In addition, antibody directed against these glycoproteins inhibits fibrinogen binding and platelet aggregation (5). Finally, a specific photoaffinity derivative of fibrinogen has been shown to bind covalently to glycoprotein IIIa (6).

One approach to the study of platelet aggregation is to reconstitute purified platelet glycoproteins into liposomes. By preselecting proteins to be incorporated into a lipid bilayer one could study the processes necessary for platelet aggregation in a relatively controlled way. In this study we report the successful reconstitution of glycoproteins IIb and IIIa into phospholipid vesicles, and we demonstrate that these proteoliposomes have receptors that mediate binding of the vesicles to activated intact platelets.

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Methods

Materials. Na^{125}I and [choline-methyl- ^3H]L- α -dipalmitoyl phosphatidylcholine (40 Ci/mmol) were purchased from New England Nuclear (Boston, MA). The scintillation mixture 3A70 was obtained from Research Products International Corp. (Mt. Prospect, IL). Thrombin and prostacyclin (PGI_2)¹ were purchased from Sigma Chemical Co. (St. Louis, MO). Phosphatidylcholine was isolated from crude egg phosphatidylcholine by silicic acid chromatography (7). Antiplatelet monoclonal antibodies were prepared as previously described (8), and a monoclonal antibody against glycoprotein IIb was a gift from Dr. Robert Allen, American Red Cross, St. Louis, MO. Human anti- PI^{A1} antisera were obtained from mothers of neonates with thrombocytopenia; the specificity of the antisera was confirmed by its reactions against a panel of platelets from PI^{A1} positive and PI^{A1} negative donors. Purified fibrinogen was obtained from Dr. L. Sherman, American Red Cross, St. Louis, MO. All other reagents were obtained from Sigma Chemical Company.

Solubilization and reconstitution of platelet membrane glycoproteins IIb and IIIa. Platelet membrane glycoproteins IIb and IIIa were obtained in relatively pure form by the procedure described by Newman et al. (9) (Fig. 1). This method employs a one-step extraction procedure which uses the nonionic detergent Triton X-114. To achieve reconstitution, the Triton X-114 solubilized material was applied to a DEAE cellulose column at 4°C and eluted with buffer containing 30 mM *n*-octyl glucoside 10 mM Tris/HCl buffer, pH 7.4. In a standard reconstitution assay, 0.5 ml of the *n*-octylglucoside protein solution (1 mg/ml) was added at room temperature to 1.0 ml of a phosphatidylcholine dispersion. The phosphatidylcholine dispersion was prepared by sonication of phosphatidylcholine (20 mg/ml) in 10 mM Tris/HCl pH 7.4 in an ultrasonic bath sonifier for ~1 h. The clear phosphatidylcholine-protein mixture was dialyzed overnight at 4°C against 10 mM Tris/HCl buffer, pH 7.4, and then passed through a G-50 Sephadex column (1 × 18 cm) which had been equilibrated at room temperature with the same buffer. Unincorporated protein was separated from proteoliposomes by passage of the protein-lipid mixture through a Sepharose 4B column (1 × 20 cm) at room temperature. Under these conditions the proteoliposomes were found in the void volume of the column. Reconstitution of the glycoproteins into the liposomes was assayed by passage of vesicles containing ^{125}I -labeled proteins (procedure described below) through the Sepharose 4B column (15 × 0.750 ml) and determination of whether the ^{125}I -glycoproteins cochromatographed with the proteoliposomes (Fig. 2). Protein that did not reconstitute did not cochromatograph with the liposomes. In a typical experiment, >90% of the protein reconstituted into the phospholipid vesicles (Fig. 2).

Preparation of ^{125}I -labeled platelet membrane proteins. 10–50 μg of solubilized platelet membranes was labeled with 1 mCi of Na^{125}I by the use of lactoperoxidase (Enzymobeads; Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions. Unbound ^{125}I was removed from the reaction mixture by gel filtration through a 4 ml Sephadex G-25 column equilibrated in 150 mM NaCl, 50 mM NaH_2PO_4 , and 30 mM *m*-octylglucoside, pH 6.5. Fractions containing >80% bound radioactivity (5–10 $\mu\text{Ci}/\mu\text{g}$) as determined by trichloro-

1. Abbreviation used in this paper: PGI_2 , prostacyclin.

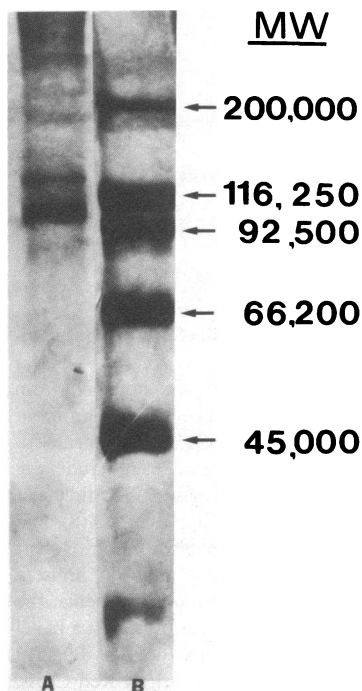


Figure 1. Triton X-114 extract of platelet membrane proteins. Glycoproteins IIb and IIIa were isolated from human platelet membranes according to the procedures described in reference 9. The isolated proteins were applied to a 5–15% polyacrylamide slab gel and stained with silver. Lane A, detergent phase proteins reduced with 5% 2-mercaptoethanol. Lane B, molecular weight standards.

acetic acid precipitation were pooled and stored at -70°C and used within 1 mo of preparation.

Preparation of ^{125}I -labeled fibrinogen. Fibrinogen was labeled with ^{125}I by the use of chloramine T as described by Marguerie et al. (2) with the following modification: unincorporated isotope was removed by gel filtration through Sephadex G-25. Approximately 95% of the radioactivity precipitated in 10% trichloroacetic acid and $\sim 90\%$ of the trichloroacetic acid-precipitable activity clotted when treated with thrombin. Fibrinogen concentrations were determined by using $E_{1\text{cm}}^{1\%}$ 280 of 15.1 (10).

Proteoliposome binding to intact platelets. Washed platelets (11) at a concentration of $\sim 5 \times 10^7$ platelets/ml in 0.5 ml of Tyrode's buffer were incubated at 37°C for ~ 1 min with or without thrombin (0.5 U/ml). ^{125}I - or ^3H -labeled proteoliposomes (or liposomes) in 50 μl (5 μg protein) were then added and the mixture was incubated for 30

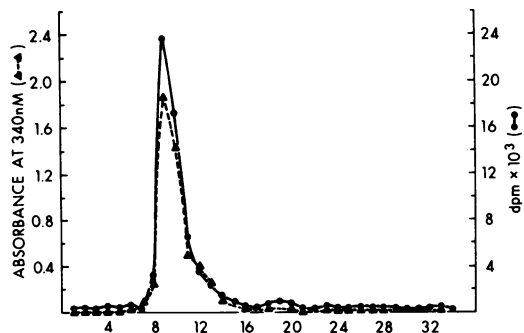


Figure 2. Elution profile of phospholipid vesicles and ^{125}I -labeled platelet glycoproteins. Vesicles were prepared as described in Methods and were applied to a column (915×0.75 cm) of Sepharose 4B and eluted with 10 mM Tris/HCl buffer, pH 7.4. Chromatography was performed at room temperature. Fractions (0.5 ml) were collected and monitored at 340 nm for phospholipid vesicles (\bullet), and analyzed for protein by measurement of the ^{125}I content (\blacktriangle).

min at 37°C without agitation. Intact platelets with attached radiolabeled proteoliposomes were then separated from unbound vesicles by centrifugation of the mixture through 1.0 ml of an oil containing *n*-butylphthalate plus corn oil (9/1) for ~ 3 min with a microfuge (Beckman Instruments, Inc., Spinco Div., La Jolla, CA). The top layer containing free vesicles was removed and the pellet containing $>80\%$ of the platelets was counted for radioactivity.

Fibrinogen binding to proteoliposomes. ^{125}I -labeled fibrinogen binding to reconstituted vesicles was measured by a method that involved rapid and complete separation of the vesicles from the medium. Proteoliposomes containing 0.5 mg/ml platelet protein were added to a solution (final volume 200 μl) containing 10^{-3} M CaCl_2 , 0.175 M NaCl, 0.02 M Tris/HCl pH 7.4, 2% bovine serum albumin (fraction V; Calbiochem-Behring Corp., San Diego, CA), and $1-3 \times 10^{-8}$ M ^{125}I -fibrinogen. After 1 h of incubation at 37°C 100- μl aliquots were removed and centrifuged either in an airfuge (Beckman Instruments Inc., Spinco Div.) according to the method of Phillips and Baughan (12) or through a Sepharose 4B column. For the latter method, columns of Sepharose 4B were poured to the 1-ml mark in disposable 1-ml plastic syringes. The columns were washed with a solution containing 10^{-3} M CaCl_2 , 0.175 M NaCl, 2% bovine serum albumin (fraction V; Calbiochem-Behring Corp.), and 0.02 M Tris, pH 7.4. Excluded medium was removed from the columns by centrifugation at 500 g for 3 min in a clinical centrifuge. In a control experiment in which a solution of ^{125}I -labeled fibrinogen without vesicles was applied to the column, $<0.1\%$ of the labeled fibrinogen was found in the eluate. Recovery of the vesicles as measured by the amount of protein and phospholipid in the eluate was $\sim 90\%$.

Total binding of fibrinogen was defined as the amount of ^{125}I -labeled fibrinogen bound to proteoliposomes in the absence of unlabeled fibrinogen. Nonspecific fibrinogen binding was defined as the amount of labeled fibrinogen bound in the presence of 3×10^{-7} M unlabeled fibrinogen. The latter concentration was determined from Fig. 3 which shows that concentrations of unlabeled fibrinogen $> 2 \times 10^{-8}$ M saturated the specific fibrinogen binding sites. Nonspecifically bound fibrinogen also consisted of molecules that were trapped between vesicles. [^{14}C]inulin was used as a measure of the amount of trapping and indicated that $\sim 10\%$ of the nonspecifically bound ^{125}I -labeled fibrinogen was indeed trapped. Specifically bound ^{125}I -labeled fibrinogen was determined by subtracting that which was nonspecifically bound from the total amount of fibrinogen associated with the vesicles.

Sialic acid hydrolysis. The total sialic acid content of the proteoliposomes was determined after hydrolysis with 0.1 M HCl at 80°C for 50 min by use of the thiobarbituric acid assay as described by Warren

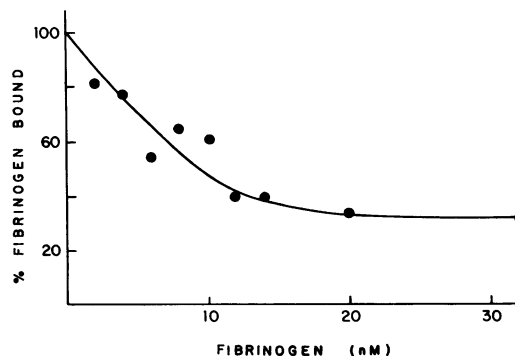


Figure 3. The effect of unlabeled fibrinogen on the binding of ^{125}I -labeled fibrinogen to reconstituted proteoliposomes. Proteoliposomes (0.5 mg protein/ml) were incubated with a 5×10^{-10} M concentration of ^{125}I -fibrinogen and 10^{-3} M CaCl_2 , as described in Methods, in the presence of various concentrations of unlabeled fibrinogen. 100% binding occurs in the absence of unlabeled fibrinogen. Each point represents the average of triplicate determinations.

(13). Neuraminidase was added to proteoliposomes (2 mg protein/ml) at the indicated concentrations and allowed to react for the described time on either intact vesicles or 0.05% Triton X-100 solubilized vesicles.

Immunoprecipitation and electrophoretic analysis. Proteoliposomes were precipitated with antibodies by addition of protein A *Staphylococcus aureus* cells (Cowan strain I) (14). The precipitated proteins were solubilized and then analyzed by one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as by Newman et al. (8). Protein concentration was determined by the method of Peterson (15).

Results

Orientation of the reconstituted platelet glycoproteins in the proteoliposomes. The functional activity of the reconstituted glycoproteins IIb and IIIa required that the glycoproteins be incorporated into the liposomes with their sialic acid residues exposed on the outer surface of the vesicles (16). Proteoliposomes were treated with neuraminidase to determine whether the sialic acid residues of the reconstituted glycoproteins were susceptible to hydrolysis. Table I compares the extent of sialic acid release from proteoliposomes and Triton X-100-treated proteoliposomes by *V. cholera* neuraminidase. Approximately 50% of the ³H-labeled sialic acid was removed by neuraminidase after 1 h of incubation. Longer incubations did not result in further release of sialic acid. Within 60 min after the addition of neuraminidase, ~90% of the labeled sialic acid was hydrolyzed from proteoliposomes solubilized with 0.05% Triton X-100. These results demonstrate that ≥50% of the sialic acid residues of the reconstituted glycoproteins were accessible to neuraminidase. Moreover, the data suggests that the proteins were randomly incorporated into the liposomes with ~50% of the glycoproteins oriented such that their sialic acid residues were exposed on the outer surface.

Immunoprecipitation of proteoliposomes with antiplatelet antibodies. Glycoproteins IIb and IIIa have been shown to be important for fibrinogen binding to platelets and platelet aggregation (4, 5). Thus it was important to determine whether these glycoproteins were incorporated into the liposomes in their native orientation before testing whether the proteoliposomes bound fibrinogen. Since Kunicki and Aster (17) have shown that glycoprotein IIIa is the carrier of the P1^{A1} deter-

minant, reconstituted proteoliposomes containing ¹²⁵I-labeled platelet membrane proteins were incubated with human alloantisera containing anti-P1^{A1} activity. Proteoliposomes were also incubated with mouse monoclonal antibodies raised against glycoprotein IIIa (18). As shown in Fig. 4, anti-P1^{A1} antibody and two antiplatelet monoclonal antibodies immunoprecipitated liposomes reconstituted with ¹²⁵I-labeled membrane proteins. A monoclonal antibody raised against glycophorin did not immunoprecipitate these proteoliposomes (Fig. 4, lane E). Control sera that did not contain either P1^{A1} or HLA antigenic activity immunoprecipitated very little labeled protein (Fig. 4, lane B). In addition, liposomes that did not contain platelet membrane proteins were also not immunoprecipitated with anti-P1^{A1} or any monoclonal antibody (data not shown). We believe the material at the top of the gels represents high molecular weight protein aggregates and is probably the result of either our method of ¹²⁵I-labeling of the glycoproteins or the immunoprecipitation procedure. SDS gels of unlabeled proteoliposomes that were not immunoprecipitated before solubilization with SDS did not show this high molecular weight aggregate (data not shown). This result suggests that the unlabeled proteoliposomes did not contain these aggregates. Since the anti-P1^{A1} antibody (17) and the antiplatelet monoclonal antibodies had previously been shown to react with glycoprotein IIIa on intact platelets (18), the results of these experiments indicated that the antigenic sites of glycoprotein IIIa, normally exposed on the platelet surface, were also exposed on the surface of the proteolipid vesicles.

Binding of proteoliposomes to thrombin-treated platelets.

In order to determine whether the proteoliposomes contain functional aggregation sites, reconstituted liposomes containing ¹²⁵I-labeled platelet glycoproteins IIb and IIIa were incubated with either thrombin-treated or control platelets at 37°C (Table II, A). Thrombin-activated platelets bound approximately five-fold more ¹²⁵I-labeled vesicles than did unstimulated platelets. The binding of proteoliposomes to activated platelets could be inhibited by pre-incubation of the platelets with EDTA, indicating that Ca⁺⁺ was required. Also, the presence of platelet

Table I. Hydrolysis of Sialic Acid in Intact Proteoliposomes and in Triton X-100 Solubilized Vesicles

	Neuraminidase U/ml	% Sialic acid hydrolyzed*		
		30 min	60 min	120 min
Proteoliposomes	0.05	39	54	51
	0.1	43	53	49
Proteoliposomes solubilized with 0.05% Triton X-100	0.05	76	93	87
		0.1	84	89

* Percentage of total determined by (nanomoles of sialic acid hydrolyzed per milligram of reconstituted protein divided by total nanomoles of sialic acid per milligram of reconstituted protein) × 100.

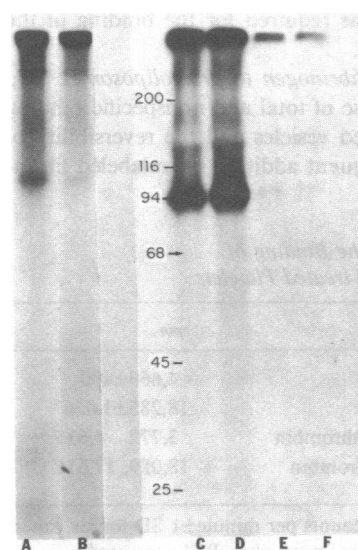


Figure 4. Immunoprecipitation of proteoliposomes with antiplatelet antibodies. Proteoliposomes containing ¹²⁵I-labeled platelet membrane proteins were reacted with 100 µg of antiplatelet antibodies overnight at 4°C. Immune complexes were precipitated and then analyzed by SDS-polyacrylamide gel electrophoresis (unreduced) and autoradiography. Lane A, proteoliposomes incubated with anti-PL^{A1} antisera or (lane B) with control sera. Lanes C and D, incubation with two antiplatelet monoclonal antibodies. Lane E, incubation with anti-glycophorin monoclonal antibody. Lane F, control medium.

Table II. Binding of Proteoliposomes to Thrombin-treated Platelets

Addition	cpm	
A None	4,669±750	
Thrombin (0.5 U/ml)	18,285±1,056	
EDTA (5 mm) + thrombin (0.5 U/ml)	4,461, 4,852	
	Liposomes	Proteoliposomes
	cpm	cpm
B None	3,206, 2,513	2,884, 3,472
Thrombin (0.5 U/ml)	4,651, 5,816	16,029, 17,197

EDTA was added before thrombin. Results are expressed as the mean counts per minute±1 SD for six experiments, or the actual counts per minute obtained in two experiments. Phospholipid vesicles and proteoliposomes were prepared in a similar manner (see text); both were labeled with [³H]phosphatidylcholine.

membranes in the mixture inhibited proteoliposome binding to activated platelets (data not shown).

Liposomes that did not contain platelet proteins showed a marked decrease in binding to thrombin-treated platelets as compared with proteoliposomes, but liposomes did bind to thrombin-treated platelets to a slight degree (Table II, B). It is unlikely that the different binding properties of proteoliposomes and liposomes was due to differences in the preparation of the vesicles, since those without protein were prepared in exactly the same fashion as those with protein except that during the *n*-octylglucoside incubation, glycoproteins IIb and IIIa were not included in the control vesicles.

To test whether the binding of proteoliposomes to thrombin-treated platelets was a result of factors normally involved in thrombin mediated platelet aggregation, intact platelets were preincubated with PGI₂, a known inhibitor of thrombin induced platelet aggregation (19), and proteoliposomes were then added. Table III shows that pre-incubation of intact platelets with PGI₂ inhibited the binding of proteoliposomes to thrombin-treated platelets. However, addition of PGI₂ after thrombin addition had little effect. Since PGI₂ has been shown to not affect thrombin binding to platelets (11), these results suggest that thrombin activation was required for the binding of the proteoliposomes to platelets.

Binding of ¹²⁵I-labeled fibrinogen to proteoliposomes. Fig. 5 shows both the time course of total and nonspecific binding of fibrinogen to reconstituted vesicles and the reversibility of this binding with the subsequent addition of unlabeled fibrin-

Table III. Effect of PGI₂ on the Binding of Proteoliposomes to Thrombin-treated Platelets

Addition	cpm
None	4,669±430
Thrombin (0.5 U/ml)	18,285±1,056
20 μM PGI ₂ 1 min before thrombin	3,772, 4,635
20 μM PGI ₂ 1 min after thrombin	18,089, 17,611

Results are expressed as mean counts per minute±1 SD for six experiments, or the actual counts per minute when PGI₂ was used.

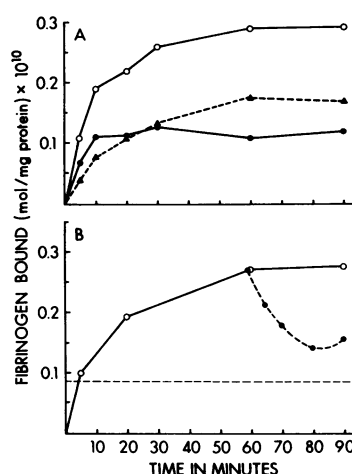


Figure 5. Time course of ¹²⁵I-fibrinogen binding to reconstituted vesicles and displacement with added unlabeled fibrinogen. (A) Reconstituted vesicles (0.5 mg/ml) were incubated with 4.2×10^{-9} M ¹²⁵I-fibrinogen and 3×10^{-3} M CaCl₂ as described in Methods. At the indicated times, aliquots (100 μl) of proteoliposomes were spun through Sepharose 4B columns as described in Methods. ○, total amount of fibrinogen bound in the absence of added unlabeled

fibrinogen; ●, nonspecific binding measured by inclusion of 3×10^{-7} M unlabeled fibrinogen to each assay; ▲, specific binding (total minus nonspecific). (B) Time course of total ¹²⁵I fibrinogen bound when proteoliposomes were incubated with 3.5×10^{-9} M ¹²⁵I-fibrinogen (○). Proteoliposomes were also collected after the addition of 3×10^{-7} M unlabeled fibrinogen (60 min) (●). The broken line represents nonspecific binding and was determined by addition of 3×10^{-7} M unlabeled fibrinogen at zero time and collection of the proteoliposomes after ~90 min.

ogen (10^{-7} M). Whereas nonspecific binding reached the maximum after ~10 min, specific binding took 60 min for maximum binding (Fig. 5 A). The addition of about a 50-fold excess of unlabeled fibrinogen displaced ~70% of the specific binding (Fig. 5, B). Liposomes without reconstituted protein showed only a fast binding component which could not be completed with unlabeled fibrinogen (data not shown). The binding of ¹²⁵I-labeled fibrinogen to proteoliposomes as a function of the amount of ¹²⁵I-fibrinogen is shown in Fig. 6. A Scatchard plot (20) shows a linear relationship which indicates a single class of receptors (Fig. 6). The dissociation constant (K_d) of 4.5×10^{-9} M was different than that obtained by Phillips and Baughnan (12) with isolated platelet membranes (K_d of 1.2×10^{-8} M). This difference probably does not reflect any important change of activity upon reconstitution of glycoproteins IIb and IIIa but more likely reflects intralaboratory differences, since we routinely obtained a K_d of 6.8×10^{-9} for fibrinogen binding to isolated membranes. In agreement with results obtained by Phillips and Baughnan (12) on the binding of fibrinogen to isolated human platelet membranes, the specific binding of fibrinogen to the proteoliposomes was insensitive to ADP, dependent on calcium, and completely inhibited by the addition of 5 mM EGTA (data not shown). These results suggest that the reconstituted vesicles have specific binding sites for fibrinogen.

Discussion

In this paper we described the successful reconstitution of platelet glycoproteins IIb and IIIa into lipid vesicles. We showed these proteoliposomes to have functional properties similar to those of intact platelets and isolated platelet membranes. Prasanna et al. (11) have shown that isolated platelet plasma membranes bind specifically to thrombin-activated

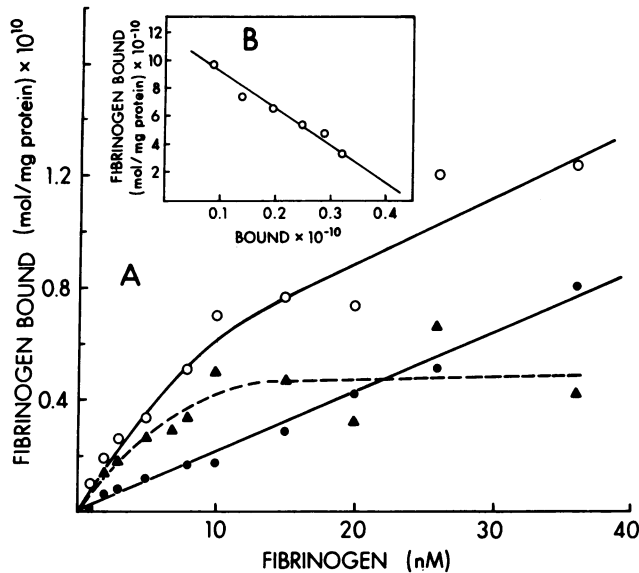


Figure 6. Scatchard analysis of ^{125}I -fibrinogen to reconstituted vesicles containing glycoproteins IIb and IIIa. (A) Proteoliposomes were incubated with the indicated concentrations of ^{125}I -fibrinogen with 10^{-3} M CaCl_2 . The uptake of ^{125}I -fibrinogen was determined as described in Methods in the absence of unlabeled fibrinogen to obtain total binding (○) and in the presence of 50 mM unlabeled fibrinogen to get nonspecific binding (●). The difference in the two sets of binding data was plotted to give the specific binding (▲). Each point represents the average of triplicate determinations. (B) Data from A plotted according to Scatchard (20). The slope corresponds to a K_d of 4.8×10^{-9} M.

platelets. They observed that the binding mimicked platelet-aggregation. Our data indicate that liposomes reconstituted with purified platelet proteins will also bind to thrombin-activated platelets. The binding was dependent on factors normally involved in thrombin stimulated platelet aggregation; binding required Ca^{++} and was inhibited by PGI_2 .

Proteoliposomes containing glycoproteins IIb and IIIa specifically bound fibrinogen. Although binding of fibrinogen to intact platelets requires activation of the fibrinogen binding site with ADP or thrombin (1, 2), fibrinogen binding to proteoliposomes occurred in the absence of ADP or thrombin but required the presence of calcium. Of note, Phillips and Baughan (12) also found that fibrinogen binding to isolated platelet membranes did not require ADP or thrombin. Our data and theirs suggest that fibrinogen receptors become unmasked during isolation of platelet membranes.

The successful reconstitution of platelet proteins into a lipid bilayer now raises the possibility of dissecting the mechanism of platelet aggregation in a physiological environment. For example, would incorporation of glycoprotein IIIa by itself be sufficient for the binding of fibrinogen to proteoliposomes? The reconstitution of platelet function also raises the possibility of clinically useful artificial platelets.

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