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RECONSTITUTION OF LIPOSOMES BEARING PLATELET RECEPTORS FOR HUMAN von WILLEBRAND FACTOR

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SUMMARY : The authors describe a method of reconstitution of lipidic vesicles bearing glycoprotein I isolated from human platelet membranes. Briefly, the glycoprotein is solubilized from plasma membrane with Na-deoxycholate and purified by wheat-germ agglutinin affinity chromatography. After replacement of deoxycholate by TRITON X 100, the glycoprotein is mixed with pure egg-phosphatidylcholine solubilized by the same detergent. Removal of Triton yields large, unilamellar lipidic vesicles as assessed by electron microscopy. Theses vesicles are reversibly agglutinable by wheat-germ agglutinin. When incubated with purified von Willebrand factor, a strong, ristocetin-independent, agglutination occurs, whereas ristocetin alone has no effect. This membrane model provides a new tool for studing the structure and function of the platelet membrane.

1. INTRODUCTION

Plasma membrane glycoproteins are implicated in essential platelet functions such as adhesion to subendothelium and aggregation (1). Although indirect evidence may attribute to some GP a receptor role for different agonists of platelet function (e. g. : von Willebrand factor, fibrinogen, thrombin), a functional study of isolated GP has been only carried out with the easily soluble glycocalicin (2). We describe here a method of reconstitution of large lipidic vesicles bearing an aqueous insoluble GP isolated from human platelet plasma membrane ; since this GP is the putative receptor for vWF, we examined the effect of purified vWF on these vesicles.

2. MATERIAL AND METHODS

2.1. Materials. Blood platelets were obtained from donors from the Centre Régional de Transfusion Sanguine, Toulouse. Gel filtration media, polyacrylamide gradient gel (PAA 4/30), WGA, DEAE-Sephacel, PERCOLL for density gradient

ABREVIATION : WGA, Wheat-germ agglutinin ; NAG, N-acetyl glucosamine ; GP, glycoprotein (s) ; vWF, von Willebrand factor ; PC, phosphatidylcholine ; DOC, Na-deoxycholate ; PAGE, polyacrylamide gel electrophoresis.

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centrifugation and WGA-linked Sepharose were from Pharmacia, Bio-Beads SM-2 from Bio-Rad, ristocetin sulphate from Lundbeck, NAG from Sigma, intermediate purity factor VIII concentrate from Cutter and other chemicals from Merck. Egg-phosphatidylcholines were purified according to (3) and determined as phosphorus by the procedure described in (4).

2.2. Isolation of platelet membranes. Membranes were isolated within one day of blood collection essentially according to the method of Perret et al. (5), with the following modifications : all the buffers contained 1 mM EDTA and the lysis buffer was 25 mM Tris-HCl (pH 7.35), 100 mM NaCl, 1 mM EDTA. Platelet disruption was performed using the nitrogen bomb decompression technique. Membranes were resuspended in 100 mM borate buffer (pH 8.00), 100 mM NaCl, 1 mM EDTA (buffer A) and kept at -80° C until use.

2.3. Glycoprotein I purification. Membranes were solubilized at room temperature with 2% (w/v) DOC. All the subsequent steps were performed at + 4° C. After removal of insoluble material and occasionally residual Percoll beads by centrifugation (100 000 X g for 60 min.), the DOC concentration was lowered to 0.1% by dilution with buffer A, and the soluble extract was applied to a column (0.9 X 15 cm) containing 9.5 ml of WGA - Sepharose equilibrated with buffer A. After washing the column through until the A 280 of the eluant was less than 0.01, the bound material was eluted with 2.5% (w/v) NAG in buffer A concentrated by ultrafiltration (Amicon procedure). Removal of NAG and glycolipids and replacement of DOC by Triton were performed by gel filtration of the concentrate through a G 50 (medium) column perfused with 20 mM Tris-HCl (pH 8.00), 150 mM NaCl, 1 mM EDTA (buffer B) containing 0.5% (v/v) Triton X 100. The fractions collected at the void volume were pooled, adjusted to pH 7.35 with HCl 0.1 N and appropriately concentrated.

2.4. Membrane reconstitution. GP I solution was mixed with pure PC dried from $CHCl_3$ (protein/lipid ratio : 1/8 (w/w), final concentration of PC : 2 mM). Removal of the detergent was performed according to the procedure of Gerritsen et al. (6). Briefly, the mixture was slowly rotated for 2 hours with 0.3 g wet biobeads/ml treated as described in (7) and the beads were removed by filtration through glass wool. This step was repeated twice.

2.5. vWF purification. vWF was purified from intermediate purity concentrates by gel filtration at room temperature on Sepharose 4 B equilibrated with 20 mM imidazole-HCl buffer (pH 6.00), 150 mM NaCl, followed by ion exchange chromatography on DEAE-Sephacel equilibrated with the same buffer. The fractions showing platelet agglutination activity were eluted at about 0.25 M NaCl using a linear (0.15 M - 0.5 M) NaCl gradient. They were pooled and extensively dialysed against buffer B adjusted to pH 7.35. Fibrinogen concentration of the preparation was less than 1 µg/ml for a platelet agglutinating activity of 5 U/ml assayed by the method of (8). After reduction (5% mercaptoethanol for 15 min. at 60° C), the purified vWF showed a single 240 000 dalton protein band by SDS-PAGE.

2.6. Agglutination assays and electron microscopy. Unless otherwise stated, assays were performed at room temperature and examined after 5 min. of incubation. Agglutination of liposomes was assessed from electron microscopic examination since this method provides direct information about the morphology of lipidic structures. Moreover, it can be carried out with a minimal volume of sample (compared to the optical density measurement method which was also occasionaly used). Formwar-coated grids were impregnated with an aqueous solution of cytochrome c (6 µg/ml). The grids were floated on a drop of liposomal suspension and excess liquid was drained with filter paper. By the same procedure, the pre-

paration was contrasted with a 1% (w/v) solution of uranyl acetate. After air-drying, the grids were immediately observed with a Philipps 301 electron microscope operating at 80 KV.

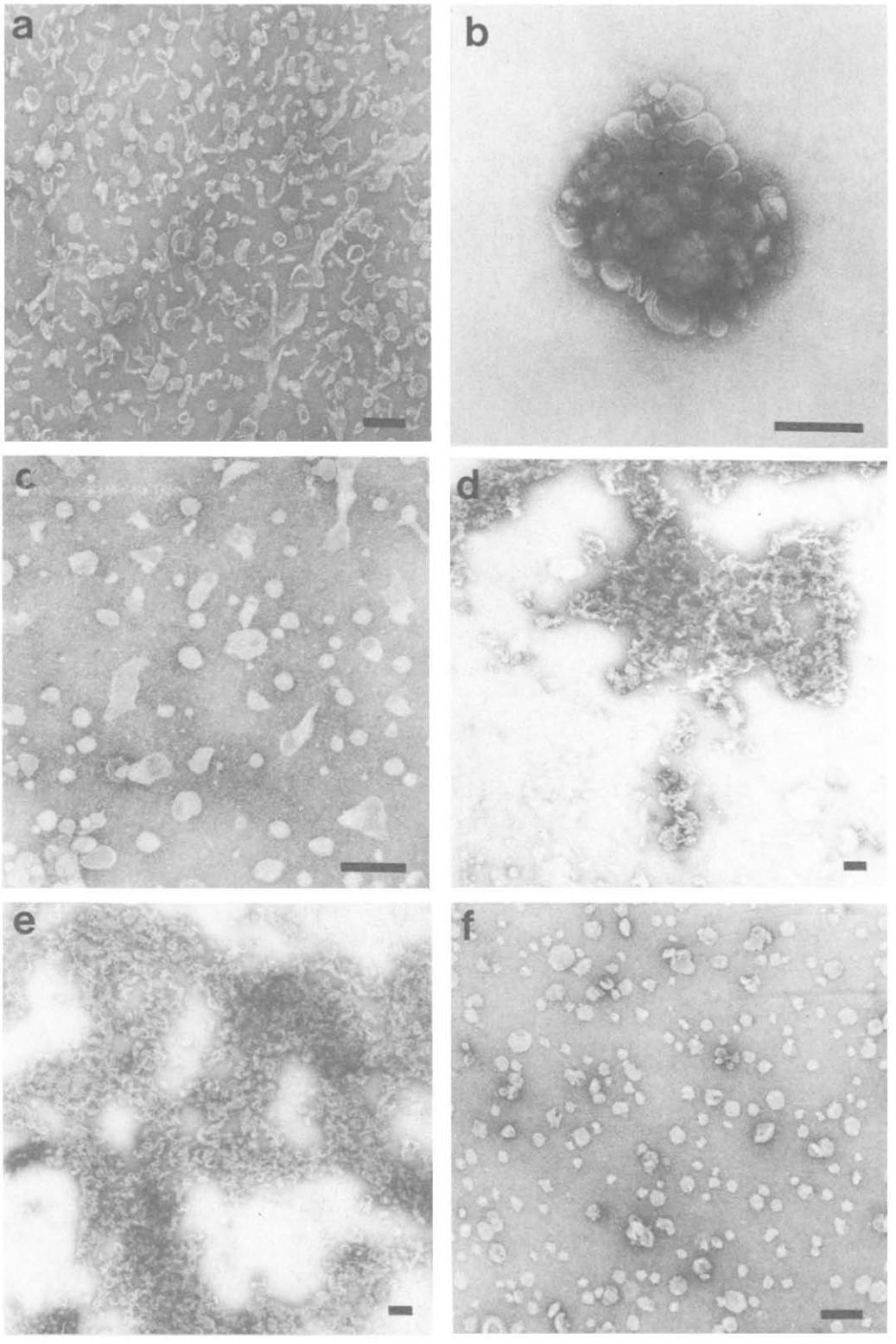
2.7. Miscellaneous. Protein was determined using the method of Lowry et al. (9), with 3% (w/v) SDS in the alkaline cooper reagent when Triton was present in the sample and with BSA as a standard. SDS-PAGE was performed on gel gradient PAA 4/30 and PAS-stained glycoprotein bands were identified according to the general procedure of Nurden and Caen (10).

3. RESULTS AND DISCUSSION

Vesicles prepared by the method described above in the absence of GP consisted of uniform, small vesicles, frequently pilled up (not shown). In contrast, vesicles prepared in the presence of GP were irregular in size and shape, mainly unilamellar and large, and uniformly dispersed (fig. 1a). Addition of WGA (200 µg/ml) strongly agglutinated these vesicles (fig. 1b). Subsequent addition of NAG (20 mM) disrupted the aggregates (fig. 1c). Agglutination was also measured by following the change in turbidity of the liposome suspension (fig. 2). Such a reversible agglutination strongly argues for the incorporation of the GP in the liposomes and for the exposure on the surface of the oligosaccharide moiety.

SDS-PAGE revealed (not shown) that the main component eluted from the WGA-Sepharose column was band I glycoprotein (approximatively 150 000 daltons). This was in good agreement with the previously reported affinity of this lectin (11). The same profile was obtained when the vesicles were sedimented (100 000 X g for 30 min.) and the pellet analyzed by SDS-PAGE. Using a more sensitive detection technique, Mac Gregor et al. (12) indicated that WGA is able to bind 10 different glycoproteins, the major one corresponding to our GP I. So, we probably did not reach actual purification but simply enrichment and more specific methods of purification would be desirable.

Clinical and experimental evidence suggests that GP I on the surface of human platelets mediates ristocetin-dependent vWF-induced agglutination. In our model system (3 experiments), vWF alone (fig. 1d), vWF plus ristocetin (fig. 1 e), but not ristocetin alone (fig. 1 f), induced a strong agglutination. In one case, the time-course of agglutination was studied. The first agglutinates appeared at about 2-3 min. and grew until completion at about 10 min. Agglutination by vWF alone is rather surprising, since the binding of vWF is ristocetin-dependent in intact platelets (13), in contrast to that of bovine factor VIII which competes for the same binding sites (13) but agglutinates human platelets in the absence of ristocetin. According to a current hypothesis, ristocetin may act by changing the cell surface charges, so reducing the repulsive forces between vWF and platelets (14). Since the surface charges of natural and artificial membranes can be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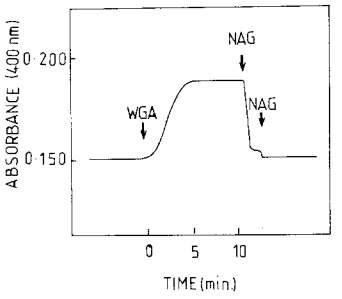

FIG. 2 - Time-course of agglutination of liposomes bearing GP with WGA (200 µg/ml) and effect of NAG (10 mM).

different, ristocetin could be no longer required for vWF action. An alternative hypothesis is that the molecular environment of GP I is of importance for its reactivity to vWF. Demonstration and characterisation of such and influence could clarify the mechanism of the interaction between vWF and platelets.

FIG. 1 - Electron micrographs of contrasted liposomal suspensions. Control (a). Suspensions incubated with : WGA (b), as in b but NAG was subsequently added (c), vWF (d), vWF and ristocetin (e), ristocetin (f). Final concentrations of reagents were as follows : WGA (200 µg/ml) ; NAG (20 mM) ; vWF (2 U/ml) ; ristocetin (1.25 mg/ml). The bar represents 0.1 u.

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