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Modulation of Vitronectin Receptor Binding by Membrane Lipid Composition*

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The vitronectin (Vn) receptor belongs to the integrin family of proteins and although its biochemical structure is fully characterized little is known about its binding affinity and specificity. We report here that Vn receptor binding to different matrix proteins is influenced by the surrounding lipid composition of the membrane. Human placenta affinity purified Vn receptor was inserted into liposomes of different composition: (i) phosphatidylcholine (PC); (ii) PC+phosphatidylethanolamine (PE); (iii) PC+PE+phosphatidylserine (PS) + phosphatidylinositol (PI) + cholesterol (chol). The amount of purified material that could be incorporated into the three lipid vesicle preparations was proportional to the efficiency of the vesicle formation that increased from PC (38%) to PC+PE and PC+PE+PS+PI+chol (about 50%) vesicles. Electron microscopy analysis showed that the homogeneity and size of the three liposome preparations were comparable (20-nm diameter) but their binding capacity to a series of substrates differed widely. Vn receptor inserted in PC liposomes bound only Vn, but when it was inserted in PC+PE and PC+PE+PS+PI+chol liposomes it also attached to von Willebrand factor (vWF) and fibronectin (Fn). Vn receptor had higher binding capacity for substrates when it was inserted in PC+PE+PS+PI+chol than PC+PE liposomes. Antibodies to Vn receptor blocked Vn receptor liposome binding to Vn, vWF, and Fn. The intrinsic emission fluorescence spectrum of the Vn receptor reconstituted in PC+PE+PS+PI+chol liposomes was blue-shifted in relation to PC liposomes, suggesting a conformational change of the receptor in the membranes. These data provide direct evidence that the Vn receptor is "promiscuous" and can associate with Vn, vWF and Fn. The nature of the membrane lipid composition surrounding the receptor could thus influence its binding affinity, possibly by changing its conformation or exposure or both.

The receptor for vitronectin (Vn)[1] has been isolated and characterized in different cell types (1, 2). It belongs to a superfamily of cell adhesion receptors, known as integrins, that recognize a number of extracellular matrix components and mediates a series of cell-cell adhesion phenomena (2, 3). These receptors are formed of two noncovalently associated subunits (α and β chains) with limited homology and at least some of them recognize the sequence Arg-Gly-Asp (RGD) present in the ligand matrix proteins (2, 3).

Attempts have been made to subdivide the integrin superfamily based on the fact that some of the member receptors share the same β chain but have different α chains (2, 3). The Vn receptor shares the same β chain ($\beta 3$) as the platelet glycoprotein IIb-IIIa (GpIIb-IIIa) complex but has a different α chain (4–6).

The biochemical structure and the amino acid sequence of the Vn receptor and of many other integrin receptors have been fully defined (5, 6) but little is known about the modulation of their affinity and specificity for the ligand proteins. The isolated GpIIb-IIIa complex appears to be promiscuous, being able to bind fibronectin (Fn), fibrinogen, von Willebrand factor (vWF), and Vn upon platelet activation (7). The Vn receptor isolated from human placenta and inserted in phosphatidylcholine (PC) liposomes bound only to Vn and not to other matrix proteins (7). However, some cell types such as endothelial cells (EC) or melanoma cells were able to adhere to vWF, fibrinogen, and thrombospondin in a way fully inhibitable by monoclonal antibodies directed to the Vn receptor (8–12). Since the Vn receptor of these cell types is apparently structurally identical to the placenta Vn receptor (6, 9), this suggests that when the receptor is inserted in a complex cellular biomembrane its specificity might be different from that in PC liposomes. In the present study we addressed this question by inserting the Vn receptor isolated from human placenta in liposomes of different phospholipid composition. When the Vn receptor was inserted in PC+PE, and even more effectively in PC+PE+PS+PI+chol liposomes, it was much more able to recognize Vn and it also became able to bind other proteins such as vWF and Fn.

EXPERIMENTAL PROCEDURES

Materials—Chemical reagents were purchased from the following sources: phenylmethylsulfonyl fluoride (PMSF), digitonin, *n*-octyl β-

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[1] The abbreviations used are: Vn, vitronectin; EC, endothelial cells; Fn, fibronectin, vWF, von Willebrand factor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; chol, cholesterol; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; mAb, monoclonal antibody; GRGDSP, Gly-Arg-Gly-Asp-Ser-Pro; GRGDSPK, Gly-Arg-Gly-Asp-Ser-Pro-Lys; GRGESP, Gly-Arg-Gly-Glu-Ser-Pro; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; VLA, very late antigen; GpIIb-IIIa, glycoprotein IIb-IIIa; octylglucoside, *n*-octyl-β-glucopyranoside.

glucopyranoside, L-α-phosphatidylcholine type III from soybean, L-α-phosphatidylinositol from soybean, cholesterol (5-cholesten-3β-ol), glucose oxidase, and bovine serum albumin from Sigma; EDTA from Merck-Schuchardt (Darmstadt, Federal Republic of Germany); SDS-PAGE standards (M_r 200,000 myosin, - M_r 116,200 β-galactosidase, M_r 97,400 phosphorylase *b*, M_r 66,200 bovine serum albumin, M_r 42,600 ovalbumin) and all electrophoretic reagents from Bio-Rad; $Na^{125}I$, [^{3}H]phosphatidylcholine, and ^{14}C-methylated proteins molecular weight standards (M_r 200,000 myosin, M_r 100,000–92,500 phosphorylase *b*, M_r 69,000 bovine serum albumin, M_r 46,000 ovalbumin, M_r 30,000 carbonic anhydrase, M_r 14,300 lysozyme) from Amersham International (Buckinghamshire, United Kingdom); Sephadex G-25, CNBr-activated Sepharose 4B, Sepharose 2B, and protein A-Sepharose 4B from Pharmacia (Uppsala, Sweden); rabbit anti-mouse IgG from Miles (Biochemicals and Immunochemicals, Milano, Italy); PE, PS (purity > 95%) was a kind gift from Fidia (Abano Terme, Padova, Italy); microtitration plates from Flow Laboratories Ltd. (Irvine, Scotland); carboxylate-modified polystyrene latex spheres (0.69-μm diameter) from Polysciences Inc. (Warrington, PA); lactoperoxidase from Calbiochem; cytochrome *c* from Serva (Heidelberg, FRG); uranyl acetate from Fluka (Buchs, Switzerland).

Antibodies—Anti-GpIIIa rabbit serum was prepared in this laboratory as described (13). Anti-αEβ4 rabbit serum was a kind gift from Dr. V. Quaranta (Scripps Clinic and Research Foundation, La Jolla, CA) (14). Affinity-purified monoclonal antibody (mAb) LM609 directed to an α/β complex-dependent epitope of Vn receptor and mAb LM142 directed to the α subunit of the Vn receptor (15) were kindly donated by Dr. D. A. Cheresh (Scripps Clinic and Research Foundation). Affinity-purified mAb 7E3 directed to platelet GpIIb-IIIa that also recognizes the Vn receptor and mAb 10E5 directed to GpIIb-IIIa but which does not recognize the Vn receptor (10) were kind gifts from Dr. B. S. Coller (Stony Brook, NY). mAb J143 ascitic fluid directed to a very common antigen (VCA2) or very late antigen 3 (VLA3) (16) was a gift from Dr. A. P. Albino (Memorial Sloan-Kettering Cancer Center, NY). mAb 12F1 directed to the VLA2 α subunit (17) was kindly provided by Dr. V. L. Woods (University of California Medical Center, San Diego, CA). mAb A1A5 ascitic fluid directed to the VLA β subunit (18) was donated by Dr. M. E. Hemler (Dana Farber Cancer Institute, Boston, MA). Anti-Fn rabbit serum (19) was obtained through the courtesy of Dr. G. Marguerie (Laboratoire d'Hematologie-Centre d'Etude Nucleaire, Grenoble, France). Rabbit affinity-purified antibodies directed to Vn were a gift from Dr. K. T. Preissner (Max-Planck-Gesellschaft, Justus-Liebig-Universitat, Giessen, FRG). mAbs 152B-20 directed to the synthetic peptide 1737–1750 of mature vWF including the RGD sequence and mAb 152B-18 directed to the adjacent RGD-free sequence (20) were kindly donated by Dr. Z. Ruggeri (Scripps Clinic and Research Foundation). An irrelevant mAb ascitic fluid and preimmune rabbit serum were used as control.

Proteins and Peptides—Human plasma Vn (21), Fn, and fibrinogen were prepared as previously described (22). Human vWF, purified as described (23), was a kind gift from Dr. Z. Ruggeri. Purified human thrombospondin (24) was kindly donated by Dr. D. Mosher (University of Wisconsin, Madison, WI). Fn was routinely analyzed by SDS-PAGE (gel gradient = 8–18%) for small molecular weight fragments. In some experiments purified Fn was used after dialysis against PBS at 25 °C (2 cycles, 1 liter each) in order to avoid smaller fragments undetectable in SDS-PAGE.

Synthetic hexapeptides Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) and Gly-Arg-Gly-Glu-Ser-Pro (GRGESP) were synthesized as previously described (22). Gly-Arg-Gly-Asp-Ser-Pro-Lys (GRGDSPK) coupled to Sepharose 4B, prepared as described (25), was kindly donated by Dr. R. Pytela (Gladstone Foundation, San Francisco, CA).

Affinity Chromatography—Two human placentas (820 g total weight) were extensively washed with cold PBS (4 liters) and then with cold PBS/1 mM PMSF (4 liters), cut in small pieces, and frozen. Thawed material was washed twice with 20 mM Tris-buffered saline (TBS) containing 1 mM Ca^{2+}/Mg^{2+}, 2 mM Mn^{2+}, 0.005% digitonin, 1 mM PMSF and centrifuged each time for 15 min at 5,000 rpm in a Beckman JA14 rotor at 4 °C. The last pellet was extracted with 800 ml of 100 mM octyl glucoside for 60 min at 4 °C and then centrifuged for 30 min at 10,000 rpm in a Beckman JA14 rotor at 4 °C. The resulting extract was applied on a 24-ml Fn fragment-Sepharose column as previously described for cells, except that 10 mM EDTA was used for elution and all buffers contained 2 mM Mn^{2+} (22). The flow-through was applied in succession on a 30-ml GRGDSPK-Sepharose column (25) equilibrated with TBS containing 1 mM Ca^{2+}/Mg^{2+}, 2 mM Mn^{2+}, 1 mM PMSF, 50 mM octyl glucoside, pH 7.4. All procedures followed the protocol for Fn receptor purification as described previously (22, 25).

Sixteen 7.5-ml fractions were collected. Aliquots of each fraction were run in SDS-PAGE and revealed by Coomassie Blue staining. Fractions were grouped in two pools: pool fractions 4 and 5 and pool fractions 6–16 dialyzed against TBS containing 1 mM Ca^{2+}/Mg^{2+}, 8 mM octyl glucoside, 1 mM PMSF, pH 7.4 (2 cycles, 5 liters each) and then tested by SDS-PAGE fractionation and revealed by silver staining. Pools were then lyophilized, resuspended in distilled H_2O, and dialyzed against TBS/16 mM octyl glucoside before being used.

Iodination of Vn Receptor—Lactoperoxidase was covalently coupled to carboxylate-modified polystyrene-latex spheres (lactoperoxidase-latex) as described by Muller *et al.* (26). Radioiodination of the affinity-purified pooled material (fractions 6–16) (10 μg in PBS, 8 mM octyl glucoside) was carried out at room temperature for 20 min in a PBS/glucose solution containing 10 mCi/ml carrier-free $Na^{125}I$ 12 microunits/ml glucose oxidase, and a 1:100 dilution of lactoperoxidase-latex stock (26). Iodination was stopped by adding 10 μl of 2% NaN_3 for 5 min followed by centrifugation for 10 min at 4 °C in a microcentrifuge to remove the lactoperoxidase-latex. The supernatant was brought to 50 mM octyl glucoside by adding concentrated octyl glucoside. Free ^{125}I was separated from ^{125}I-Vn receptor by gel filtration on a Sephadex G-25 column (0.3 × 25-cm disposable pipette). About 63% of the ^{125}I was incorporated into the Vn receptor as determined by trichloroacetic acid precipitation. The specific activity of the ^{125}I-Vn receptor was of 3.8 μCi/μg, as calculated by the percentage of the radionuclide incorporated into the protein.

Immunoprecipitation—^{125}I-Affinity-purified pooled material (fractions 6–16) was immunoprecipitated with polyclonal or monoclonal antibodies related to the integrin superfamily. mAbs LM609 and LM142 were coupled to CNBr-activated Sepharose-4B (5 mg/ml) following the manufacturer's directions. Anti-platelet GpIIIa (20 μl), anti-αEβ4 (25 μl), nonimmune serum (25 μl), and mAbs J143 (20 μl), A1A5 (10 μl), and an irrelevant mAb (20 μl) were all coupled to protein A-Sepharose. For mAbs an intermediate rabbit anti-mouse IgG (5 μg/ml) was used. All procedures were carried out as previously described (22). Samples were fractionated by 7.5% SDS-PAGE and then visualized by autoradiography on X-Omat XAR film.

Preparation and Characterization of Liposomes—Three types of liposomes with different phospholipid composition and either with or without cholesterol were prepared as follows: (i) PC alone was mixed with [^{3}H]PC; (ii) PC (58%) + PE (42%) were mixed with [^{3}H]PC; (iii) PC (40%) + PE (32%) + PS (12%) + PI (6%) + chol (10%) were mixed with [^{3}H]PC. Lipids were dissolved in chloroform and then dried under a stream of N_2 and dissolved in TBS (500 μl) containing 50 mM octyl glucoside alone or 50 mM octyl glucoside containing the Vn receptor. In some experiments ^{125}I-Vn receptor was added to the unlabeled Vn receptor. Detergent was removed by dialyzing against TBS/1 mM PMSF for 24 h at 4 °C, resulting in the formation of liposomes (27). Liposomes obtained from the dialysis step were then passed down a Sepharose 2B column (total volume = 23 ml) equilibrated with TBS/0.02% NaN_3 to minimize the amount of residual detergent and to remove free Vn receptor. The elution profile was followed by counting an aliquot of each fraction in a β counter and in some experiments also in a γ counter. Aliquots of all three types of liposomes were fractionated by SDS-PAGE before and after gel filtration on Sepharose 2B and then revealed by silver staining.

Electron Microscopy—For electron microscopy analysis Vn receptor-free PC, PC+PE, and PC+PE+PS+PI+chol liposomes were prepared as described above. After Sepharose 2B fractionation the liposomes were examined by negative staining. Small drops of liposome suspensions in TBS/0.02% NaN_3 were spread on carbon-coated grids wet with cytochrome *c* in PBS (20 μg/ml), stained with 1% uranyl acetate in water, and observed in a Siemens Elmiskop IA. Pictures were taken at × 20,000 magnification.

Liposome Attachment Assay—Microtiter wells were coated with increasing concentrations of purified Vn, vWF, Fn, thrombospondin, fibrinogen, and bovine serum albumin (1.25–40 μg/ml) in PBS Ca^{2+}/Mg^{2+} at room temperature overnight. The liposome adhesion assay was carried out as previously described (22). In liposome adhesion inhibition experiments appropriate mAbs were incubated together with the liposomes during the adhesion assay. When anti-Vn, -Fn, -vWF antibodies were used, they were incubated with the different substrates for 30 min at 37 °C and then removed before the adhesion assay. Preimmune IgG from rabbit serum, whole rabbit serum, and mAb 152B-18 were used as negative controls.

Tryptophan Fluorescence—Technical steady state fluorescence emission spectra were recorded in ratio mode with a Perkin-Elmer

MPF-4 spectrofluorometer using excitation and emission slits of 4 and 6 nm, respectively. The digitized spectra were then subtracted from the blank contribution. The steady state emission anisotropy was determined using a Polacoat dichroic polarizer installed in the excitation path and a linear polarizer Polaroid HNP'B in the emission path. The relative intensities for the four combinations of vertically (V) and horizontally (H) polarized beams (I_{VV}, I_{VH}, I_{HH}, I_{HV}) were recorded in the ratio mode using excitation and emission wavelengths of 295 and 340 nm, respectively. Steady state emission anisotropy was calculated as follows:

$$\langle r \rangle = \frac{I_{VV}(G) - I_{HV}}{I_{VV}(G) + 2I_{HV}}$$

$$G = I_{HH}/I_{VH}$$

where G is an instrumental normalization factor (28).

RESULTS

Affinity Chromatography on Sepharose-bound Heptapeptide Containing Arg-Gly-Asp—In order to purify the Vn receptor, octyl glucoside extract of human placenta was applied first to a Fn fragment-Sepharose column in order to remove Fn-binding material. The flow-through was then applied to a GRGDSPK-Sepharose affinity column. After lysate binding, the column was washed and then eluted with 10 mM EDTA.

As expected (Fig. 1*A*), the elution profile showed primarily two polypeptides of M_r 140,000/90,000 corresponding to the molecular weight of the placental Vn receptor α and β chains, respectively, under nonreducing conditions. Fractions 4 and 5 and fractions 6–16 were grouped in two separate pools for their different degree of purity and analyzed by SDS-PAGE. Samples run under reducing or nonreducing conditions are shown in Fig. 1*B*. Pool fractions 4 and 5 (*lane 3, R*) and pool fractions 6–16 (*lane 4, R*) migrated as two bands under reducing conditions of M_r 125,000/100,000. Pool fractions 6–16 showed an additional band running at M_r 135,000 which was absent in the pool fractions 4 and 5. The total yield of Vn receptor (fractions 4–16) was about 6.5 mg.

Immunological Analysis of the Affinity-purified Material—For the experiments reported below pool fractions 6–16 were used for insertion in liposomes because of their greater purity. Before inclusion in lipid vesicles the material was iodinated and analyzed by immunoprecipitation with a set of antibodies directed to the α or β chains of different integrins.

Rabbit polyclonal antibodies directed to platelet GpIIIa, mAb LM609 directed to the Vn receptor, and mAb LM142 directed to the α chain of the Vn receptor recognized the affinity purified material contained in pool fractions 6–16 (Fig. 2). All these antibodies precipitated two bands under nonreducing conditions of M_r 140,000/90,000 (Fig. 2*A*, *lanes 3–5*) corresponding to the molecular weight of the α and β chain of the Vn receptor. As expected, under reducing conditions the two bands migrated with a molecular weight of 125,000/100,000 (Fig. 2*B*, *lanes 12–14*). The third band of M_r 135,000, already seen in the starting material (Fig. 2*B*, *lane 11*), was apparent after immunoprecipitation with all the above antibodies. In contrast, the polyclonal antibodies to the integrin $\alpha E\beta 4$ (Fig. 2*A*, *lane 6*, and Fig. 2*B*, *lane 15*), mAb J143 raised against the $\alpha 3$ subunit of the VLA3 (Fig. 2*A*, *lane 8*, and Fig. 2*B*, *lane 17*) and mAb A1A5 which recognizes the β chain of the VLA subgroup (Fig. 2*A*, *lane 9*, and Fig. 2*B*, *lane 18*) did not precipitate any detectable material.

In two separate experiments the mAb 12F1 directed to the $\alpha 2$ subunit of the VLA2 integrin was also tested, with negative results (not shown). These data show that the material eluted from the GRGDSPK column was related to the Vn receptor with no apparent presence of the VLA or $\beta 4$ subgroup of integrins.

Insertion of the Affinity-purified Material in Liposomes—After immunological characterization, this material was included in lipid vesicles. Liposomes of different lipid composition were prepared, formed of PC alone, PC+PE, or PC+PE+PS+PI+chol. The material inserted in the different liposomes, before and after elution from a Sepharose 2B column, was analyzed by SDS-PAGE under nonreducing conditions (Fig. 3). The same type of material was included in the liposomes irrespective of their phospholipid composition. Two bands of M_r 140,000/90,000 corresponding to α and β chains of the Vn receptor molecular weight were detected after Sepharose 2B. A faint lower band running at M_r 55,000, already present in the starting material (Fig. 3, *lane 1*), was evident only before gel filtration of all three types of liposomes, indicating that it was not an integral membrane protein and could be separated from the liposomes by chromatography. The third band, at M_r 135,000 also observed in the starting material, was detected after Sepharose 2B too, when aliquots of liposomes were fractionated by SDS-PAGE under reducing conditions (Fig. 4, *insets*).

The amount of purified material that could be incorporated into liposomes of different composition was checked using trace amounts of ^{125}I-Vn receptor. The radioactivity associated with ^{3}H-phospholipids and ^{125}I-Vn receptor was determined in the fractions collected from the Sepharose 2B column (Fig. 4). PC and PC+PE+PS+PI+chol liposomes were compared. The phospholipid combination was more effective in promoting vesicle formation, 42 *versus* 28% for PC liposomes alone. However, the ratio of ^{125}I to ^{3}H radioactivity in the peak fractions was essentially identical for both types of

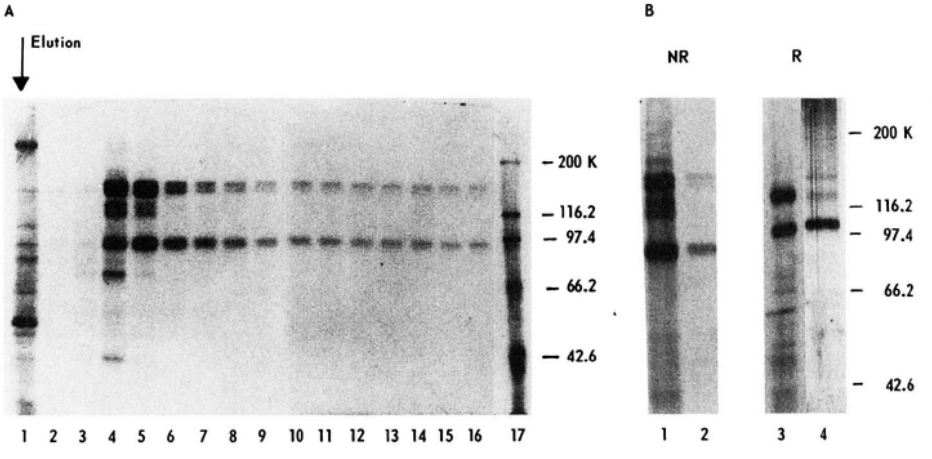

FIG. 1. **Affinity chromatography of human placental extract on GRGDSPK-Sepharose: analysis by SDS-PAGE.** Placental extract applied to the column (see "Materials and Methods") was eluted with 10 mM EDTA. *A*, aliquots (120 μl) of each fraction were visualized by Coomassie Blue staining (*lanes 1–16*); lane 17, molecular mass markers. Fractions 4 and 5 and fractions 6–16 were grouped in two separated pools. *B*, aliquots (60 μl) of pool fractions 4 and 5 (*lanes 1* and *3*) and pool fractions 6–16 (*lanes 2* and *4*) were revealed by silver staining under nonreducing (*NR*) and reducing (*R*) conditions.

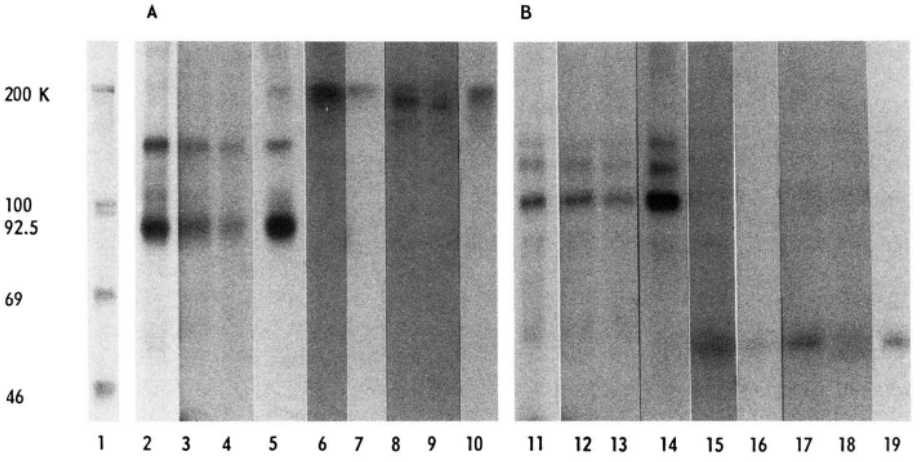

FIG. 2. **Immunoprecipitation analysis of the affinity purified material after iodination.** Ten μg of the affinity purified material (pool fractions 6–16) were radiolabeled with ^{125}I as reported under "Materials and Methods." 5×10^4 cpm associated to this material were immunoprecipitated (see "Experimental Procedures") using polyclonal or monoclonal antibodies related to the integrin receptors. The immunocomplexes were analyzed by SDS-PAGE (7.5% gel) and autoradiography under nonreducing (*A*) or reducing (*B*) conditions. *Lane 1*, molecular mass markers; *lanes 2* and *11*, starting material; *lanes 3* and *12*, mAb LM609; *lanes 4* and *13*, mAb LM142; *lanes 5* and *14*, anti-GpIIIa rabbit serum; *lanes 6* and *15*, anti-αEβ4 rabbit serum; *lanes 7* and *16*, nonimmune rabbit serum; *lanes 8* and *17*, mAb J143; *lanes 9* and *18*, mAb A1A5; *lanes 10* and *19*, irrelevant mAb.

liposomes. This indicates that the ^3H counts associated with phospholipids correspond to roughly the same amount of ^{125}I counts associated with the purified receptor for both liposome preparations. This result was consistently observed in a series of four different liposome preparations in which the range of vesicle formation was 28–48% for PC, 47–57% for PC+PE, and 40–60% for PC+PE+PS+PI+chol. The three types of liposomes were also analyzed by electron microscopy (Fig. 5). The liposomes appear comparable for homogeneity and size. In all samples, 50–200-nm-wide globules were always observed together with an enormous amount of small spherical particles. These latter formed clusters and chains; their mean diameter was 17.6 ± 5.8 nm for PC (Fig. 5*a*), 17.2 ± 4.1 nm for PC+PE (Fig. 5*b*), and 18.3 ± 3.6 nm for PC+PE+PS+PI+chol (Fig. 5*c*). On comparing the amounts of the two populations, the great majority of the phospholipids appeared to be organized as small 20-nm-wide liposomes.

Liposome Binding to Different Matrix Proteins—The three types of liposome preparations were then tested for their adhesive properties on a set of substrates. As reported previously (7), when the Vn receptor-containing PC liposomes were used, they significantly bound only to Vn, in a concentration-dependent way, while no specific interaction was observed with vWF, Fn, thrombospondin, and fibrinogen (Fig. 6*A*). However, when the receptor was included into PC+PE, and even more effectively in PC+PE+PS+PI+chol liposomes, it bound not only about five times more efficiently to Vn but was also able to recognize other substrates such as vWF and Fn (Fig. 6, *B* and *C*). When the three types of liposomes were prepared in absence of proteins no significant binding was observed to any substrate (Fig. 6, *A–C, dotted lines*).

In no experiment were we able to detect any significant binding to thrombospondin and fibrinogen of any preparation of liposomes (Fig. 6, *A–C*). This was true even when in a few experiments the fibrinogen concentration in coating was increased up to 250 μg/ml (not shown). When bovine serum albumin or laminin were used (1.25–40 μg/ml in coating) no binding of any type of Vn receptor liposomes was observed (not shown). Dialysis of the Fn (see "Materials and Methods") did not change the binding of the Vn receptor-containing liposomes to this protein (not shown). In some experiments using Vn receptor-containing PC, PC+PE, and PC+PE+PS+PI+chol liposomes, Mn^{2+} (1 mM) was included during the binding assay (Fig. 7). This markedly increased the binding of the three types of liposomes to Vn, vWF, and Fn (20 μg/ml in coating). Slight binding of PC+PE+PS+PI+chol liposomes to fibrinogen was detected. In contrast, in this condition also, no significant attachment to bovine serum albumin (Fig. 7) or to thrombospondin and laminin at 20 μg/ml was present. Similarly Mn^{2+} did not modify the binding of protein-free liposomes to any substrate (not shown).

As described above, the material included in liposomes (corresponding to pool fractions 6–16) was related to the Vn receptor by immunological analysis. However, a third band of M_r 135,000 was evident under reducing conditions (Fig. 1*B, lane 4*). In order to analyze its role, in some experiments the pool fractions 4 and 5 were included in PC+PE liposomes following the procedure described above. As shown in Fig. 1*B, lane 3*, this material did not present the M_r 135,000 band under reducing conditions but these vesicles did bind to Vn, vWF, and Fn.

Prevention of Liposome Adhesion by Antibodies to Integrin Molecules—The purified material to be included in liposomes appeared to be related to the Vn receptor as tested by immunoprecipitation with specific antibodies (see above). We then tested whether antibodies directed to the Vn receptor could block the binding of the purified material-containing liposomes to the different substrates.

The mAb LM609 and 7E3 reduced the binding of Vn receptor-containing liposomes to Vn by 73 and 81%, respectively (Fig. 8). Both antibodies blocked liposome interaction with vWF and Fn. As expected, mAb LM142 was barely active, as previously reported in cell adhesion assays (11). Similarly the platelet GpIIb-IIIa mAb 10E5 at a concentration fully blocking platelet aggregation and fibrinogen binding (29) did not change liposome binding to any substrate. This antibody was reported to interact selectively with platelet (GpIIb-IIIa (10).

GRGDSP peptide at a concentration of 75 μg/ml reduced binding of the Vn receptor-containing PC+PE liposomes to

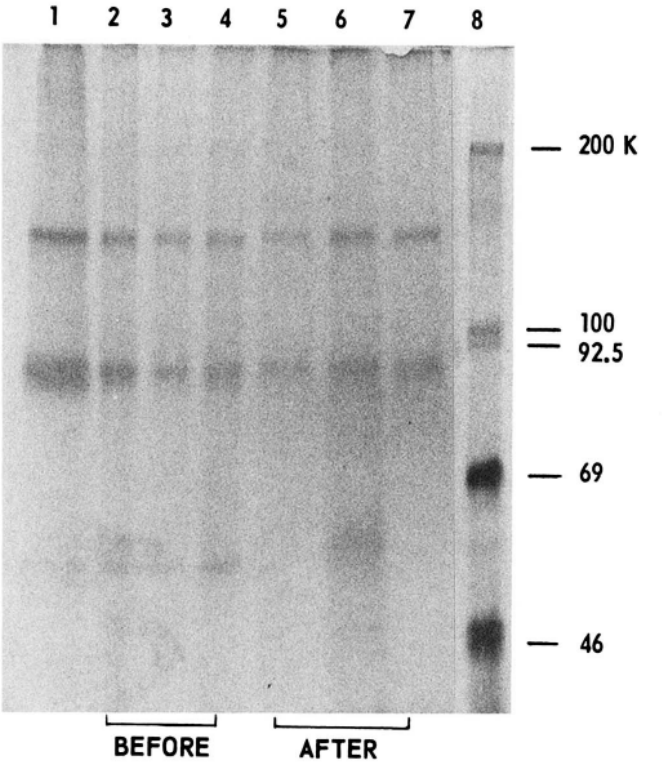

FIG. 3. **Incorporation of the affinity purified material in liposomes.** Pool material (fractions 6–16) immunologically related to the Vn receptor (60 μg) and [^{3}H]PC (16.5 $\times$ 10^6 cpm) were supplemented with three different lipid compositions (330 μg of total lipid for each preparation) as described in "Experimental Procedures." Liposomes formed by detergent dilution through dialysis were fractionated by chromatography on Sepharose 2B and 400-μl fractions were collected. The Vn receptor-containing liposomes (600 ng) for all three preparations were analyzed before and after gel filtration by SDS-PAGE and silver staining. *Lane 1*, starting material pool fractions 6–16 (600 ng); *lanes 2* and *5*, PC-[^{3}H]PC liposomes; *lanes 3* and *6*, PC+PE+PS+PI+chol-[^{3}H]PC liposomes; *lanes 4* and *7*, PC+PE-[^{3}H]PC liposomes; *lane 8*, molecular mass markers.

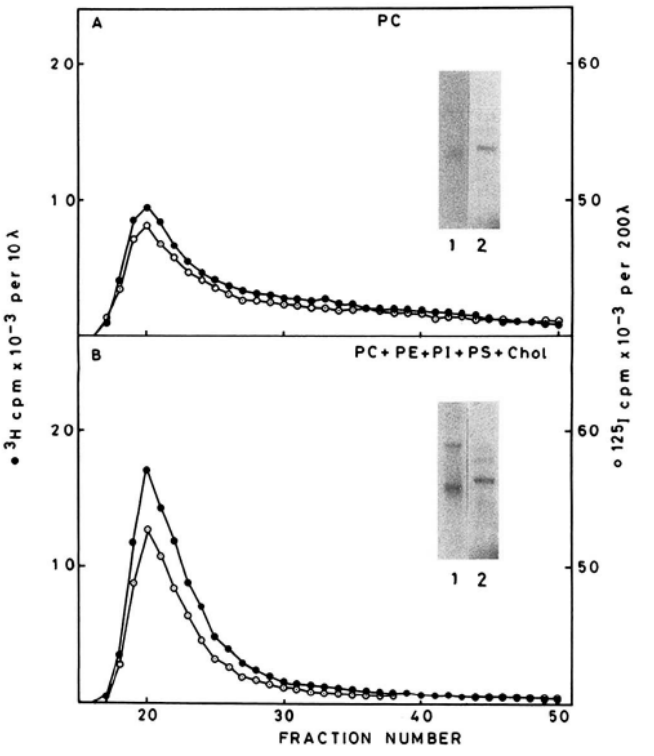

FIG. 4. **Elution profile of Vn receptor-containing liposomes on Sepharose 2B.** The labeled ^{125}I-Vn receptor was monitored by radioactivity; lipids were monitored by radioactivity for the presence of [^{3}H]PC. Vesicle formation was less efficient when pure PC (*A*) was used in comparison to the mixed lipid composition PC+PE+PS+PI+chol (*B*). 28% (*A*) and 41% (*B*) of the protein coeluted with the lipids at the void volume. Much of the remainder eluted for both types of liposomes around the 60th fraction. Final concentration of protein-lipid was 26 μg (+1.9 $\times$ 10^6 ^{125}I cpm) to 133 μg (+6.6 $\times$ 10^6 ^{3}H cpm) after detergent removal in a volume of 420 μl. *Insets* in *A* (PC liposomes) and *B* (PC+PE+PS+PI+chol liposomes) show autoradiograms of ^{125}I-Vn receptor-containing liposomes using aliquots of the peak fractions for each type of liposome after SDS-PAGE under nonreducing (*lane 1*) or reducing (*lane 2*) conditions.

Vn, Fn, and vWF by 92–95%. GRGESP peptide (75 μg/ml) was virtually inactive on these substrates. Antibodies to Vn (50 μg/ml), vWF (30 μg/ml), and Fn (1:30 dilution) (see "Experimental Procedures") blocked adhesion of Vn receptor-containing PC+PE and PC+PE+PS+PI+chol liposomes to their respective substrates but were inactive on the other substrates used (not shown).

Effect of Liposome Phospholipid Composition on Fluorescence Emission Spectra of the Vn Receptor—In order to analyze why the composition of liposomes caused such striking differences in the binding affinity of the Vn receptor we studied the intrinsic fluorescence of the protein reconstituted in either PC or PC+PE+PS+PI+chol vesicles. Fig. 9 presents the spectra obtained on exciting the sample in the absorption band of tryptophan (295 nm). The spectrum of the receptor reconstituted in homogeneous PC vesicles (*a*) is centered at about 350 nm, while Vn receptor in mixed liposomes (*b*) presents maximum emission fluorescence between 335 and 340 nm. The Vn receptor fluorescence is significantly blue-shifted compared to the previous spectrum.

The steady state anisotropy $\langle r \rangle$ measured in the tryptophan excitation and emission bands was 0.220 $\pm$ 0.01 with no detectable changes related to lipid vesicle composition.

These findings suggest that the tryptophan residues of Vn receptor in mixed liposomes are located in a more hydrophobic environment than the protein reconstituted in pure PC vesicles and that, in the first case, there is a stronger interaction between the lipid matrix and the peptide chains of the protein.

DISCUSSION

This study established two new findings on Vn receptor function and interaction with matrix proteins. First, we bring direct evidence that the purified Vn receptor is not selective for Vn but can also bind other substrates such as vWF and Fn. Second, but related to the first observation, we show that Vn receptor can be modulated by the surrounding phospholipid environment.

Considering the first point, we report here that when the purified human placenta Vn receptor is inserted in either PC+PE or PC+PE+PS+PI+chol liposomes it binds in a concentration-dependent way to Vn, vWF, and Fn. Binding to these substrates appears to be due to Vn receptor-related material being inhibited by two monoclonal antibodies directed against this receptor. In addition, RGD-containing peptides blocked the Vn receptor liposome interaction with Vn, vWF, and Fn.

We considered the possibility that other integrins, possibly contaminating the Vn receptor preparation and known to interact with some of the substrates tested (7), could contribute to liposome binding. The possibility that the Fn receptor

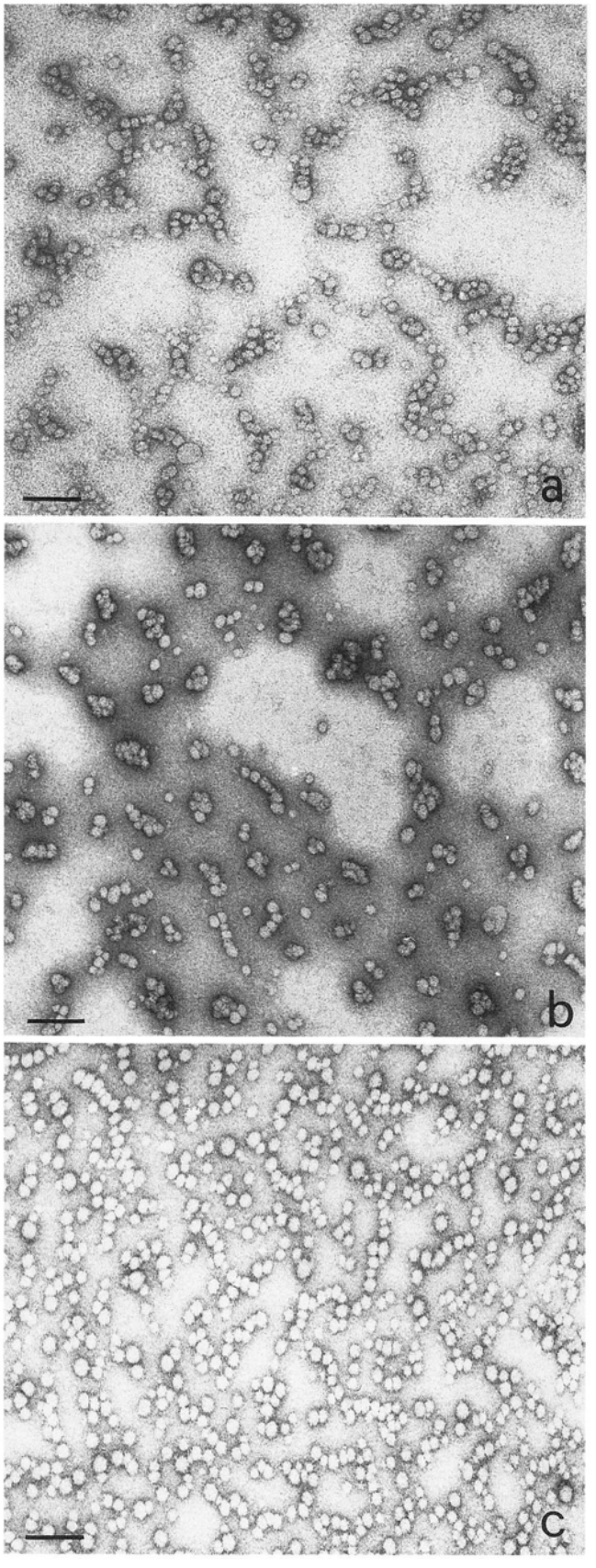

FIG. 5. **Electron micrographs of negatively stained phospholipid vesicles.** *a*, PC; *b*, PC+PE; *c*, PC+PE+PS+PI+chol. The specimens were stained with 1% uranyl acetate on the grid. In all cases, small chains and clusters of about 20-nm-wide spherical particles were observed. *Bars*, 100 nm.

(VLA5) could be present seems very unlikely. The preparation was depleted by Fn binding material through a Fn-fragment affinity column (see "Experimental Procedures") before purification on a GRGDSPK-Sepharose column. In addition, as reported by others (30), no Fn receptor from placental extracts could bind to a GRGDSPK column even in the presence of Mn^{2+}. Furthermore, when the Vn receptor purified from human placenta was analyzed by immunoprecipitation with different antibodies no evidence of $\beta 1$ or $\beta 4$ subgroups of integrins was found. The possibility of a platelet GpIIb-IIIa contribution to liposome adhesion seems unlikely too, because a specific anti-GpIIb-IIIa antibody at a concentration able to block platelet aggregation (29) was inactive on liposome binding to any of the substrates tested. In addition, mAb LM609 which fails to recognize GpIIb-IIIa (9), blocked Vn receptor-containing liposome adhesion to substrates.

Finally, no binding to any matrix proteins was observed when the liposomes were free of inserted proteins, thus excluding the possibility of a nonspecific interaction of the different phospholipids with the substrates.

Analyzing the purified material by SDS-PAGE under reducing conditions we found a third band of M_r 135,000. We have no direct means of identifying this. However, it was immunoprecipitated by the three antibodies to Vn receptor (including the mAb LM142 which has been shown to specifically recognize the receptor α chain (15)) and not by the antibodies to $\beta 1$, $\alpha E\beta 4$, $\alpha 3$, or $\alpha 2$. This suggests that it is related to or closely associated with the Vn receptor. A possible explanation is that it corresponds to the M_r 128,000 Vn-receptor α-chain precursor recently described by Polack *et al.* (31). This pro-Vn receptor α appears to be a single chain protein whose migration velocity in SDS-PAGE does not change under reducing conditions. In addition as it is complexed with the mature β chain it can be immunoprecipitated by anti-Vn receptor β-chain antibodies. These characteristics correspond to those of the M_r 135,000 band described here. However other possibilities such as the presence of a new integrin related to Vn receptor cannot be excluded. Other authors (7, 25) did not find the same extra band in the purified Vn receptor from human placenta. Some differences in the purification procedure might justify this (the absence of Mn^{2+} during the affinity chromatography and an additional purification step on a wheat germ agglutinin-Sepharose column).

The presence of this material may arouse concern regarding its role in promoting Vn receptor liposome binding. For this reason in some experiments we included the pool fractions 4 and 5 of the affinity column, which are free of this material (see Fig. 1*B*, *lane 3*), in liposomes. The results on liposome binding were essentially the same as those with the pool fractions 6–16; *i.e.* the pool fractions 4 and 5 containing liposomes could bind Vn and also Fn and vWF.

An additional band of M_r 55,000 was found in pool fractions 6–16. It disappeared after gel filtration of Vn receptor-containing liposomes, thus indicating that it was not an integral membrane protein and it could not interfere with the binding of liposomes to different substrates.

The observation that the Vn receptor is promiscuous and binds more than one matrix protein is not surprising *per se* even though no direct data were available. vWF is probably a natural and important ligand for the Vn receptor. This protein is abundant in the subendothelium and is specifically synthesized by EC (32). It plays a pivotal role in platelet adhesion (33) but also acts as a matrix protein for EC promoting their adhesion and cytoskeletal organization through a RGD-mediated mechanism (34).

More surprising is the observation that the isolated Vn

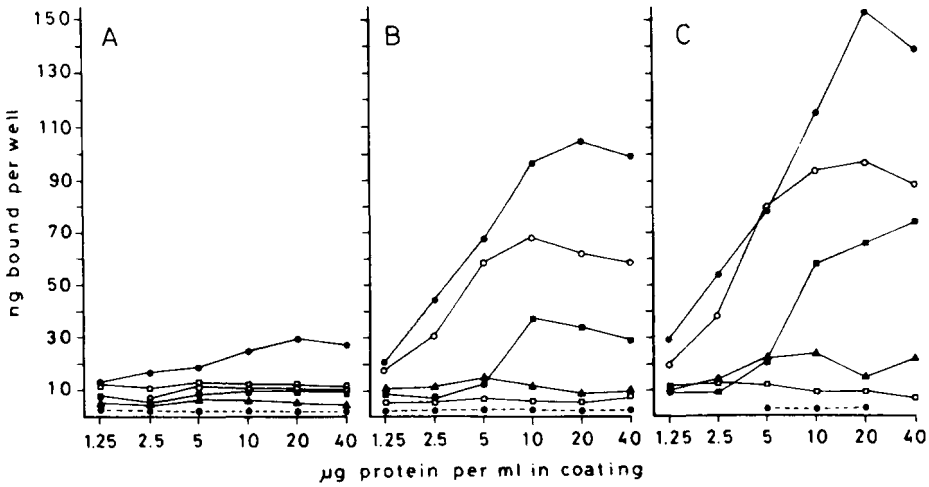

FIG. 6. **Lipid composition modulates the adhesion of Vn receptor-containing liposomes to different substrates.** Microtiter wells were coated with increasing concentrations of vitronectin (●), von Willebrand factor (○), fibronectin (■), thrombospondin (□), and fibrinogen (▲) in PBS Ca^{2+}/Mg^{2+}, overnight at room temperature. PC liposomes (*A*), PC+PE liposomes (*B*), and PC+PE+PS+PI+chol liposomes (*C*) were prepared as described in the legend to Fig. 3. Each preparation was diluted in TBS containing 1 mM Ca^{2+}/Mg^{2+}, 0.2% bovine serum albumin to obtain 3 μg of lipids = 100 μl/well and left to adhere for 5 h at 4°C. Supernatants were then removed and wells were washed twice with PBS. Bound liposomes were dissolved in 1% SDS (100 μl/well) and counted in a β counter. *Solid line*, Vn receptor-containing liposomes; *dotted line*, control liposomes formed in presence of detergent alone. Each point is the mean of three replicates from one typical experiment, S.E. never exceeded 10% of the mean. Comparable results were obtained in two additional experiments using different liposome preparations.

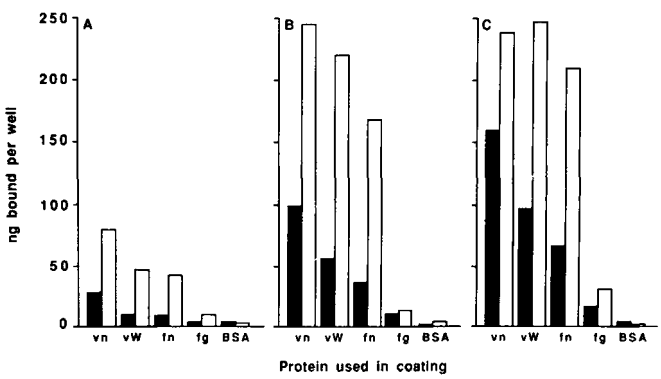

FIG. 7. **Effect of Mn^{2+} on the adhesion of Vn receptor-containing liposomes to different substrates.** Vn receptor reconstituted in PC (*A*), PC+PE (*B*), and PC+PE+PS+PI+chol (*C*) liposomes, were tested for attachment to vitronectin (*vn*), von Willebrand factor (*vW*), fibronectin (*fn*), fibrinogen (*fg*), and bovine serum albumin (*BSA*) (20 μg/ml in coating), either in the absence (*solid bars*) or in the presence (*open bars*) of 1 mM Mn^{2+}. Results are means of two replicates from one typical experiment out of three.

receptor could interact with Fn. This is apparently in contrast with the fact that Vn receptor antibodies do not prevent cell adhesion to Fn (10, 11, 13). These results might be explained considering that at least two other Fn receptors of the integrin family have been described together with other nonintegrin-binding sites (*i.e.* heparan sulfates) (35–37). The Vn receptor may therefore play only an accessory role in promoting cell adhesion to Fn.

Once blocked by specific antibodies the other receptors could account for normal cell binding to this substrate. Interestingly, in a recent work, Cheresh *et al.* (15) show that a novel integrin sharing the same α chain as the Vn receptor but a different β chain (β5) is also responsible for carcinoma cell attachment to Fn, besides attachment to Vn. The possibility that the purified Vn receptor could bind to low molecular weight RGD-containing fragments of Fn seems unlikely: no detectable low molecular weight bands were found in the Fn used, as visualized by SDS-PAGE fractionation (see "Experimental Procedures") and after dialysis Fn was equally active in supporting Vn receptor liposome adhesion.

Some indirect data suggest that the Vn receptor could also bind fibrinogen and thrombospondin. Different investigators (9, 10, 38) showed that antibodies directed to the Vn receptor blocked EC or melanoma cell adhesion to fibrinogen and it was found that the Vn receptor of these cells was organized in adhesion structures upon adhesion to these substrates (38–40). Lawler *et al.* (12) isolated by affinity chromatography on thrombospondin a complex with the same immunological and structural characteristics as the Vn receptor from EC. These authors also showed that anti-Vn receptor antibodies prevented endothelial cell adhesion to thrombospondin. In contrast with this observation in this work we were unable to show any direct binding of the isolated Vn receptor to fibrinogen or thrombospondin. Only in the presence of Mn^{2+} (see below) was limited binding of the PC+PE+PS+PI+chol Vn receptor-containing liposomes to fibrinogen observed. EC spreading and cytoskeletal organization on fibrinogen could take place only when the cells release and organize an endogenous matrix (39, 40). Similarly cell adhesion to thrombospondin is very weak, and most of the adherent cells do not spread (12). This suggests that the Vn receptor interaction with fibrinogen and thrombospondin, even if specific, is of low affinity; in a whole cellular system it can be strengthened by the synthesis and release of endogenous matrix proteins or by other binding sites (as GpIV (41) or heparan sulfates (42) for thrombospondin) while in a purified receptor system it might be difficult to detect.

When Mn^{2+} was added in the liposome binding assay we observed an increase in binding to Vn, vWF, and Fn with all types of liposomes used. Mn^{2+} was also very effective in increasing receptor binding to the GRGDSPK column, improving the yield of the receptor 3.5 times compared to previous findings (25). These data are in agreement with Smith and Cheresh (43) who showed that the cross-linking of GRGDSPK to Vn receptor was enhanced by Mn^{2+}. Gailit *et*

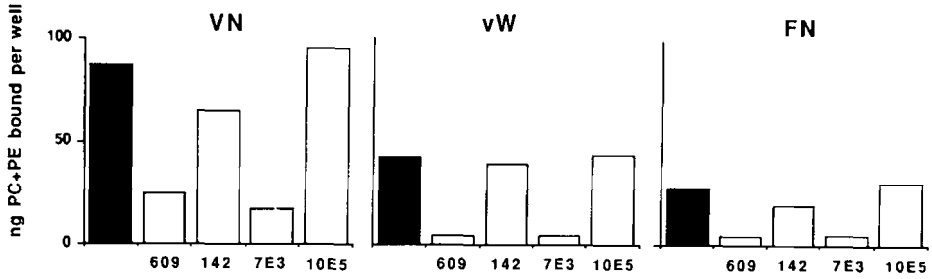

FIG. 8. **Binding of Vn receptor-containing liposomes to different substrates is immunologically inhibited by mAbs related to the Vn receptor.** PC+PE liposomes were prepared as described in the legend to Fig. 3. Microtiter wells were coated with 20 μg/ml of vitronectin (*VN*), von Willebrand factor (*vW*), and fibronectin (*FN*). Liposomes were added alone (*solid bars*), or mAbs (50 μg/ml) were added with the liposome suspension (*open bars*) as indicated. The adhesion assay was carried out as described in the Fig. 6 legend. The data are the mean of the results of two separate experiments using two different liposome preparations.

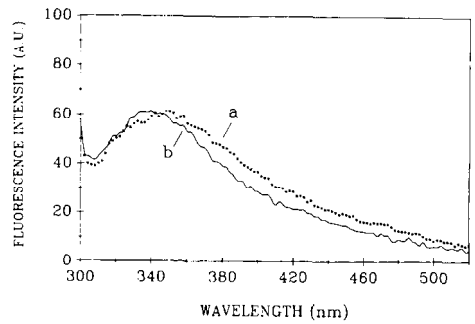

FIG. 9. **Intrinsic Vn receptor fluorescence after reconstitution in lipid vesicles.** Steady state fluorescence emission spectra of the intrinsic fluorescence of the Vn receptor were recorded in pure PC (*a*) and mixed PC+PE+PS+PI+chol (*b*) reconstituted vesicles in TBS buffer at 4 °C. The excitation wavelength was 295 nm.

al. (30) showed that the binding affinity of purified Fn receptor was greatly increased by Mn^{2+}. The results reported here indicate that the Vn receptor appears influenced by the addition of this cation too.

The second important observation of this work is that the Vn receptor affinity could be modulated by the phospholipid environment surrounding the receptor. Comparing the binding of Vn receptor reconstituted in PC liposomes to the binding of Vn receptor reconstituted in PC+PE and PC+PE+PS+PI+chol liposomes the difference was indeed dramatic. A significant difference was still apparent, however, when Vn receptor-containing liposomes composed of PC+PE were compared to PC+PE+PS+PI+chol.

The ability of membrane phospholipids to modulate receptor affinity has already been described for other types of receptors such as hormone receptors (see, for review, Ref. 44) and thrombomodulin (45), but this is the first time it is reported for an integrin type of receptor. In previous work Parise and Phillips (46) reconstituted platelet GpIIb-IIIa into liposomes of different phospholipid composition to study the efficiency of the receptor incorporation. Lipid-protein interaction may change not only the lateral (sideways) and rotational (on their own axis) mobility of the proteins but also their degree of exposure to the outer surroundings (44). The fluorescence data reported here for the Vn receptor showed a significant spectral shift of the emission fluorescence intensity in relation to the lipid vesicles composition. This suggests that different conformational states can be induced or maintained, depending on the lipids surrounding the receptor, this effect possibly being mediated by the number and distribution of the electrostatic charges involved in the lipid-protein interaction. We did not find any changes in steady state anisotropy of the receptor on changing the phospholipidic substrates, but at present we cannot exclude a role of the lipid environment on the protein rotational dynamics. It will be extremely informative in the future to study the mobility and the segmental flexibility of the protein by time-resolved fluorescence techniques.

Conformational changes such as those induced by Mn^{2+} on Fn receptor (30), by RGD peptide binding (47) or divalent cations (48) on platelet GpIIb-IIIa have been associated with modifications of the binding affinity of these integrin receptors. We propose that a conformational change may be another of the mechanisms by which the phospholipid environment could influence the Vn receptor's adhesive properties.

In conclusion, this work shows that it is possible to modulate the Vn receptor affinity and specificity. This was observed here in a purified system with artificial membrane vesicles. It is of course important to establish the biological relevance of these observations in a cellular system. It is now well documented that lipids move around the membrane and this dynamism plays a primary role in determining receptor binding affinity in the cells (44). It is attractive to speculate that the lipid composition around the Vn receptor might change in the same cell, for example during movement, and thus modulate its binding affinity to the matrix proteins.

It is also interesting to consider that different cell types (for example osteosarcoma and EC) have a Vn receptor that is structurally identical (6) but has different binding capacity, *i.e.* only EC can bind and fully spread on vWF (9, 11, 34). Differences in the lipid composition of the membrane might explain this functional difference of the receptor.

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