Biochem. J. (1991) 280, 9–17 (Printed in Great Britain)

A vitronectin-receptor-related molecule in human placental brush border membranes

Oluseyi A. VANDERPUYE,* Carlos A. LABARRERE and John A. McINTYRE
Center for Reproduction and Transplantation Immunology, Methodist Hospital of Indiana, 1701 N. Senate Boulevard, Indianapolis, IN 46202, U.S.A.

The heterodimeric vitronectin receptor (VNR) and platelet glycoprotein IIb/IIIa (GPIIb/IIIa) are two members of the integrin family of cell adhesion receptors that share the same β subunit (GPIIIa). These proteins are involved in binding to vitronectin, fibrinogen and fibronectin and in cytoskeleton–membrane interactions. The present study shows that the human placental syncytiotrophoblast brush border membrane contains a heterodimer of subunit M_r values of 140000 and 90000 (non-reduced) or 125000 and 100000 (reduced). This protein was recognized by a monoclonal antibody to GPIIIa, rabbit antisera to the VNR and a human alloantiserum to GPIIIa. Brush border VNR-related protein bound to an immobilized peptide containing the Arg-Gly-Asp sequence and, less avidly, to immobilized fibrinogen. Only a small fraction of brush border VNR was associated with a cytoskeleton fraction. Membrane-bound brush border GPIIIa was distinct from that of platelets in its resistance to digestion by trypsin and *Staphylococcus aureus* V8 protease, and had a slightly lower mobility on SDS/PAGE. In addition, lectin-binding studies indicate glycosylation differences between microvillar and platelet GPIIIa heterodimers. Thus, although placental syncytiotrophoblast expresses a β_3 integrin in its apical brush border, differences in protease sensitivity and carbohydrate content suggest that it may lack or mask certain antigenic determinants. This may be beneficial in avoiding harmful maternal alloantibody responses during pregnancy. Immunohistology showed that the VNR was present in syncytiotrophoblast apical but not basal plasma membranes, and was absent from other forms of trophoblast. The brush border VNR could function in localizing Arg-Gly-Asp-sequence-containing plasma proteins to the materno–trophoblastic interface.

INTRODUCTION

Efforts to understand the molecules and mechanisms underlying cell adhesion processes have led to the recognition of important roles for members of the integrin family of proteins (Hynes, 1987; Akiyama *et al.*, 1990). Integrins are characterized by a heterodimeric structure, and three main subfamilies have been defined on the basis of sharing a common β subunit but having differing α subunits (Hynes, 1987; Akiyama *et al.*, 1990). The β_3 subfamily includes platelet glycoprotein IIb/IIIa (GPIIb/IIIa) and one form of vitronectin receptor (VNR) (Ginsberg *et al.*, 1987; Fitzgerald *et al.*, 1987). More recently, the receptor α subunit has been found associated with β subunits other than β_3, notably in carcinomas, epithelial cells and monocytes (Cheresh *et al.*, 1989; Toin *et al.*, 1989; Krissansen *et al.*, 1990; Ramaswamy & Hemler, 1990). In addition, an alternatively spliced variant of β_3 or GPIIIa has been reported, and the VNR α subunit has been described in association with the β_1 subunit, which defines a different integrin subfamily (Vogel *et al.*, 1990; Bodary & McLean, 1990). Thus the full details of VNR subunit structures in different cells are still being established. Functional properties of GPIIb/IIIa include binding to fibrinogen, fibronectin, von Willebrand factor and vitronectin (Phillips *et al.*, 1988). GPIIIa is also notable in that it bears alloantigenic determinants which are important in transfusion reactions (Kunicki & Aster, 1979).

The VNR (containing subunits αV and β_3) is also expressed on platelets and binds to vitronectin, fibrinogen and von Willebrand factor (Charo *et al.*, 1987; Cheresh, 1987; Lam *et al.*, 1989). Interestingly, the VNR has also been implicated in cytoskeleton–membrane interactions (Burridge *et al.*, 1988; Dejana *et al.*, 1988). The VNR has a wide tissue distribution and has been purified from homogenates of total placenta (Pytela *et al.*, 1987). However, the placental cell type of origin was not defined.

Recently the VNR was reported to be localized by immunohistology to the syncytiotrophoblast in a study of the osteoclast functional antigen (Davies *et al.*, 1989). Additional investigations are required to extend this significant observation, inasmuch as the placenta was not the focus of that study. Important questions remained as to the distribution of the VNR between the two cell membranes of the polarized syncytiotrophoblast and on other forms of trophoblast. It was thus of interest to characterize a potential brush border VNR, since structural and functional features of this protein can vary in different cell types (Cheresh *et al.*, 1989; Smith *et al.*, 1990). The syncytiotrophoblast epithelium is specialized for materno–fetal interactions during pregnancy (Truman & Ford, 1984), and maternal plasma contains potential VNR ligands. Maintenance of brush border structure also appears to involve a prominent microfilament core or cytoskeleton with which a VNR might interact (Edwards & Booth, 1987). The present study characterizes the placental syncytiotrophoblast VNR and examines its binding to potential ligands and to the cytoskeleton. We suggest that differences between the placental microvillar GPIIIa and platelet GPIIIa are helpful in avoiding adverse effects of maternal alloantibodies during pregnancy.

MATERIALS AND METHODS

Materials

Monoclonal antibodies to vimentin (V9), complement receptor type 1 (To 5) and platelet glycoproteins GPIIIa (Y2/51) and

Abbreviations used: GPIIIa, platelet glycoprotein IIIa; GPIIb, platelet glycoprotein IIb; α slow and α fast, M_r 140000 proteins associated with trophoblast GPIIIa; VNR, vitronectin receptor; PBS, phosphate-buffered saline (150 mM-NaCl/10 mM-sodium phosphate, pH 7.4); PMSF, phenylmethanesulphonyl fluoride; L-PHA, leucophytohaemagglutinin; JCA, PNA, ECA and UEA-1, lectins from *Artocarpus integrifolia*, *Arachis hypogaea*, *Erythrina cristagalli* and *Ulex europaeus* respectively.

* To whom correspondence should be addressed.

GPIb (AN51), and horseradish peroxidase conjugates of antibodies to rabbit and mouse immunoglobulins, were from Dako Corporation, Santa Barbara, CA, U.S.A., as were rabbit anti-(human platelets) and anti-(human plasma) antibodies. Rabbit antiserum to the VNR, a monoclonal antibody to CD9 (FMC-8) and alkaline-phosphatase-conjugated antibodies to mouse IgG and IgM were obtained from Chemicon, Temencula, CA, U.S.A. Another rabbit antiserum to the VNR and monoclonal antibodies AO50 and VNR147 to the VNR α subunit were from Telios, San Diego, CA, U.S.A. A monoclonal antibody to GPIIb (SZ-22) was from GenTrak, Plymouth Meeting, PA, U.S.A., and a monoclonal antibody to β_2-microglobulin was from Hybritech, San Diego, CA, U.S.A. Human alloantiserum to the PL^{A1} GPIIIa determinant was provided by Dr. P. Newman, Wisconsin Center for Blood Research, Milwaukee, WI, U.S.A.

Agarose-immobilized leucophytohaemagglutinin (L-PHA) and *Ulex europaeus*-1 and wheat-germ lectins were from E-Y Lab, San Mateo, CA, U.S.A. *Erythrina cristagalli* and *Artocarpus integrifolia* lectins coupled to agarose were obtained from Vector Laboratories, Burlingame, CA, U.S.A.

The peptides Gly-Arg-Gly-Asp-Ser-Pro-Lys (GRGDSPK) and Gly-Arg-Ala-Asp-Ser-Pro-Lys (GRADSPK) were from Peninsula Laboratories, Belmont, CA, U.S.A. Fibrinogen and fibronectin coupled to agarose were purchased from Calbiochem, La Jolla, CA, U.S.A. All other materials were the highest quality available from Sigma Chemical Co., St. Louis, MO, U.S.A.

Preparation of trophoblast microvilli and platelets

Platelets were isolated as previously described (Fox & Phillips, 1982), washed five times with phosphate-buffered saline (PBS) containing 0.1% (w/v) BSA and 1 mM-EDTA and once with PBS, and then resuspended at $(2–5) \times 10^9$ platelets/ml in PBS. A platelet surface membrane fraction was prepared by sonicating the platelets at level 6 for 2×10 s in an Ultrasonic Inc. sonicator (Plainview, NY, U.S.A.). The suspension was centrifuged at 2000 *g* for 10 min and the supernatant was resonicated and centrifuged as above. Combined supernatants were centrifuged at 10000 *g* for 10 min, and platelet membranes were pelleted from the resulting supernatant at 40000 *g* for 60 min and resuspended to 2–5 mg of protein/ml in PBS containing 0.2 mM-phenylmethanesulphonyl fluoride (PMSF) and leupeptin, aprotinin, antipain and benzamidine, all at 10 μg/ml. Trophoblast microvilli were purified as reported (Booth *et al.*, 1980) and have been previously shown to be free of significant contamination by non-trophoblast and basal plasma membranes (Booth *et al.*, 1980; Kelley *et al.*, 1983). Microvillar microfilament core structures (microvillar cytoskeletons) were isolated as previously described (Edwards & Booth, 1987).

SDS/PAGE and immunoblotting

Immunoblotting and SDS/PAGE were performed as previously described (Vanderpuye *et al.*, 1988) using 8% (w/v) polyacrylamide gels with non-reduced samples. The incubation and washing buffer was PBS/0.05% (v/v) Tween 20. Monoclonal antibodies were diluted to 2 μg/ml and secondary detection antibodies were diluted 1000-fold in PBS containing 10 mg of BSA/ml. Alkaline-phosphatase-conjugated secondary antibodies were used with a 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium kit from Bio-Rad, Richmond, CA, U.S.A.

Crossed immunoelectrophoresis was performed as previously described (Booth *et al.*, 1979).

Immunohistology

Tissue blocks from 10 clinically normal placentae were embedded in optimal cutting temperature (OCT) compound (Miles Diagnostics, Elkhart, IN, U.S.A.) and snap-frozen in liquid nitrogen for study. Immunofluorescence localization of proteins in 4 μm-thick cryostat sections of tissues was performed without exposure to any fixatives. Experiments included buffer, isotype-matched control monoclonal antibodies and conjugate controls to confirm specificity, as previously described (Labarrere *et al.*, 1990). Monoclonal antibody to GPIIIa was used at a dilution of 3.75 μg/ml, anti-($\alpha V\beta_3$ complex) (23C6) at a 1:2–1:4 dilution of culture supernatant, rabbit antiserum to VNR at 1:100 dilution and fluorescein-isothiocyanate-conjugated anti-mouse or anti-rabbit IgGs at 1:40 dilutions.

Immunoprecipitation

Microvilli or platelet membranes were resuspended in 150 mM-NaCl/20 mM-$NaHCO_3$, pH 8.3, at 3–5 mg of protein/ml. The suspensions were incubated for 25 min at 25 °C with sulphosuccinimidyl-6-biotinamido hexanoate (NHS-LC-Biotin; Pierce Chemical Co., Rockford, IL, U.S.A.) at a final concentration of 0.1 mg/ml. Lysine was added to 10 mM (final concentration) and the membranes were pelleted at 100000 *g* for 10 min and washed once with PBS/10 mM-lysine. Triton X-100-solubilized proteins were obtained by addition of Triton X-100 to 2% (v/v) final concentration and centrifugation as above. Preclearing was performed by addition of 200 μl of a 50% (v/v) slurry of Protein G–Sepharose/ml of Triton-solubilized proteins followed by incubation at 4 °C for 1 h and centrifugation to remove the matrix. Aliquots of 30–50 μl of monoclonal antibody (2–5 μg of protein) were added to 1 ml of Triton X-100-solubilized proteins followed by incubation at 4 °C for 1 h. Protein G–Sepharose or anti-mouse-Ig–agarose [100 μl of a 50% (v/v) slurry] was added and the matrix, after a 1 h incubation, was washed four times with 10 vol. of PBS containing 0.1% (v/v) Triton X-100. Bound proteins were eluted with 1% (w/v) SDS, electrophoresed by SDS/PAGE and electrotransferred to nitrocellulose. Blots were probed with alkaline-phosphatase-conjugated avidin (1 μg/ml) in PBS/BSA using the procedures for immunoblotting described above. In certain experiments antibody was added to intact cells followed by processing as above.

E.l.i.s.a.

Immulon II microtitre plates (Dynatech Labs, Alexandria, VA, U.S.A.) were coated with 50 μl of platelet membranes, platelets or intact microvilli (1 mg of protein/ml) in PBS for 16 h at 4 °C. The wells were emptied, rinsed with PBS containing 0.05% (v/v) Tween 20 and blocked for 30 min with 20 mg of BSA/ml in PBS. Incubation with 50 μl of monoclonal antibody (1–10 μg/ml) diluted in 10 mg of BSA/ml in PBS was performed for 1 h and followed by four rinses in PBS/Tween 20. Wells were incubated with horseradish-peroxidase-conjugated anti-mouse IgG diluted 1000-fold in PBS/BSA for 30 min, followed by washing as above. The substrate was 4.2 mM-tetramethylbenzidine in 0.1 M-sodium acetate/citric acid, pH 6.0. Absorbance at 450 nm was measured with a MultiSkan microtitre plate reader (Flow Laboratory, Helsinki, Finland) after a 15 min incubation and termination of the reaction with 30 μl of 2 M-H_2SO_4. Rabbit antisera were used at a starting dilution of 200-fold and secondary antibodies at 1000-fold in the above procedure.

Affinity chromatography

BSA (20 mg), deoxyribonuclease-1 (20 mg), GRADSPK (5 mg), GRGDSPK (5 mg), fibrinogen (20 mg) and fibronectin (5 mg) were coupled to Affigel 15 using the manufacturer's (Bio-Rad) protocol. Batchwise absorption was performed by incubating 0.5 ml of Triton-solubilized placental microvilli or platelets (2–5 mg of protein/ml) with 0.2 ml of a 50% (v/v)

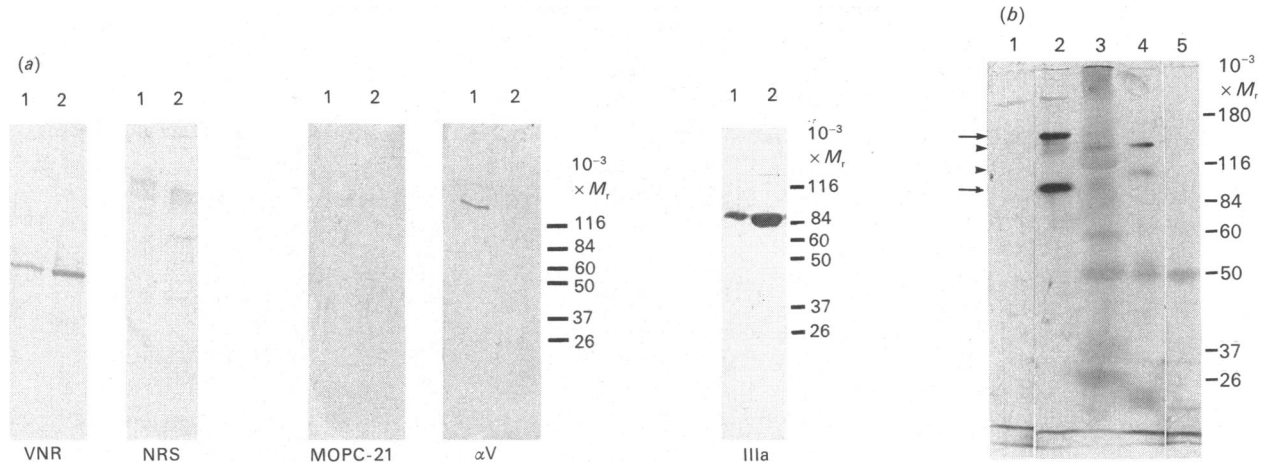

Fig. 1. Detection of placental brush border GPIIIa and VNR

(*a*) Non-reduced placental microvillar (lanes 1) or platelet (lanes 2) proteins (50 μg) were electrophoresed on SDS/8 %-polyacrylamide gels and electrotransferred to nitrocellulose. Blots were treated with monoclonal antibodies to VNR α subunit (αV) or platelet GPIIIa (IIIa), or with MOPC-21 as a negative control, and with rabbit antiserum to VNR or normal rabbit serum (NRS). (*b*) Biotinylated microvillar proteins immunoprecipitated by antibody MOPC-21 (lane 1) or anti-GPIIIa (lane 2) were analysed by enzyme–avidin blotting under non-reducing conditions. In addition, anti-GPIIIa (lane 4) and MOPC-21 (lane 5) immunoprecipitates were analysed after reduction. Arrows mark positions of non-reduced proteins and arrowheads indicate the reduced proteins. Lane 3 contained prestained M_r standards.

slurry of matrix for 16 h at 4 °C. The affinity matrices were washed four times with 10 vol. of buffer [75 mM-NaCl, 2 mM-$MgCl_2$, 2 mM-$MnCl_2$, 20 mM-Hepes/NaOH, pH 7.4, containing 0.1 % (v/v) Triton X-100] by centrifugation. Bound proteins were eluted with 1 % (w/v) SDS or 5 mM-EDTA with similar results and analysed by immunoblotting with monoclonal antibody to GPIIIa or with rabbit anti-VNR.

Lectin affinity chromatography was performed as described above, except that matrices were eluted with the inhibitory sugar: wheat-germ agglutinin and *Erythrina cristagalli* lectin (ECA), *N*-acetyl-D-glucosamine; concanavalin A and *Lens culinaris* lectin, α-methyl D-mannoside; *Arachis hypogaea* lectin (PNA) and *Artocarpus integrifolia* lectin (JCA), galactose; *Ulex europaeus*-1 lectin (UEA-1), fucose. Alternatively, bound proteins were eluted with 1 % (w/v) SDS. Analysis was by immunoblotting using anti-GPIIIa.

Limited proteolysis

Trypsin and *Staphylococcus aureus* V8 protease digestion of sealed right-side-out placental microvilli and of fresh intact platelets was performed by methods previously described (Sheng *et al.*, 1989). Immunoblotting with Y2/51 identified GPIIIa.

RESULTS

Immunoblot detection of GPIIIa and VNR α subunit in microvilli

Immunoblotting of non-reduced samples of membranes with a monoclonal antibody to GPIIIa (Y2/51) and a monoclonal antibody to VNR α subunit (AO50) was performed to detect VNR-related proteins. The results showed that placental microvilli contained a protein of subunit M_r 90000 which was similar to GPIIIa and a protein of M_r 140000 which was reactive with the antibody to the VNR α subunit (Fig. 1*a*). Control monoclonal antibodies such as anti-vimentin were unreactive. A rabbit antiserum to the VNR reacted with a microvillar protein of M_r 90000 under non-reducing conditions (Fig. 1*a*). This protein was not reactive with the antiserum after reduction (results not shown). No immunoreactivity was observed in immunoblots of reduced proteins with the monoclonal antibodies to VNR α subunit and GPIIIa. Microvillar proteins immunoprecipitated by the antibody to GPIIIa had M_r values of 140000 and 90000 under non-reducing conditions. After reduction the 140000-M_r protein displayed slightly increased migration and the 90000-M_r protein migrated in the region of M_r 100000 (Fig. 1*b*). Thus placental microvilli contain proteins of similar size and immunoreactivity to VNR subunits.

VNR is not merely a contaminant of trophoblast microvilli

In order to determine if microvillar VNR-related proteins were derived from platelet contamination, microvilli and platelet membrane proteins were analysed by crossed immunoelectrophoresis with an antiserum to human platelets (Fig. 2*a*). An intermediate gel containing anti-(human plasma) was used to keep plasma proteins from the upper gel. A number of membrane proteins were detected in platelets, including GPIIb/IIIa, identified by comparison with published reference patterns (Hagen & Solum, 1989). In contrast, microvilli contained only one major cross-reactive protein that formed an immunoprecipitate distinct in detailed appearance from platelet GPIIb/IIIa. The lack of several platelet membrane proteins in placental microvilli suggested that microvillar VNR-related proteins did not arise simply from contamination by platelets.

Possible contamination of microvilli by non-microvillar membranes was also investigated by immunochemical assay of specific marker proteins. CD9 was detected by immunoblotting in platelets but not in microvilli (Fig. 2*b*). Since CD9 is a major platelet protein which can also be expressed on endothelium (Favaloro *et al.*, 1990), contamination by these cell types is unlikely to account for microvillar VNR-related proteins. Furthermore, β_2-microglobulin was not detected in microvilli by e.l.i.s.a. (Table 1) or by immunoblotting (results not shown), in accordance with previous reports (Faulk & McIntyre, 1983; Truman & Ford, 1984). Since β_2-microglobulin is present on placental villus mesenchymal cells, VNR-related proteins are unlikely to be derived from contamination by non-trophoblast membranes. This proposal is supported by the similar levels of reactivity of platelets and microvilli with an antiserum to VNR (Table 1), which suggested an endogenous origin. Negligible

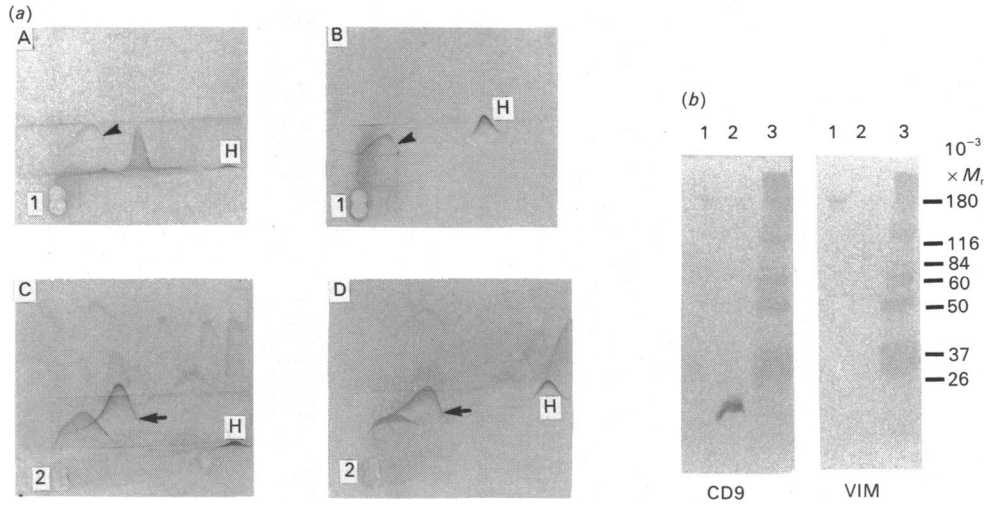

Fig. 2. VNR in trophoblast microvilli is not a platelet-derived contaminant

(*a*) Crossed immunoelectrophoresis of placental brush border and platelet membrane proteins against antibodies to human platelets. Proteins solubilized with Triton X-100 from placental microvilli (80 μg; 1), or platelet membranes (20 μg; 2) were applied to wells. Electrophoresis in the second dimension was through an intermediate gel containing no antibody (B and D) or 100 μl of anti-(human plasma) antibody/ml (A and C) and into a gel containing 100 μl of anti-(human platelets) antibody/ml. Arrows indicate the GPIIb/IIIa immunoprecipitates; arrowheads mark a cross-reactive microvillar protein, and H indicates human albumin. (*b*) CD9 is absent from placental brush border but not from platelets, as shown by immunoblot analysis. Microvillar proteins (lanes 1) and platelet membrane proteins (lanes 2) were immunoblotted with monoclonal antibody to CD9 or with a control isotype-matched monoclonal antibody to vimentin (VIM). Lane 3 contains prestained M_r standards.

Table 1. Reactivities of platelet and microvillar membranes with marker antibodies on e.l.i.s.a.

E.l.i.s.a. was performed as described in the Materials and methods section using 40 μg of platelet or microvillar protein per microtitre well. Monoclonal antibodies were diluted to 3 μg/ml and 0.75 μg/ml. Rabbit primary antisera were diluted 200-fold or 800-fold. The antibodies and antisera used were: anti-GPIIIa, anti-β_2-microglobulin (β_2M), anti-GPIb, anti-vimentin, antiserum to vitronectin receptor (VNR) and normal rabbit serum (NRS). Absorbance values shown are means ± S.D. of duplicates for a typical experiment. Three or more experiments with the various reagents varied by less than 20% and antisera or antibodies other than to VNR or GPIIIa yielded background values for microvilli ($\leqslant$ 0.042 and 0.253 absorbance units respectively).

	Absorbance (units)			
	Microvilli		Platelets	
Antibody	3.0 μg/ml	0.75 μg/ml	3.0 μg/ml	0.75 μg/ml
Anti-GPIIIa	0.205±0.023	0.180±0.037	0.579±0.131	0.549±0.128
Anti-β_2M	0.019±0.017	0.042±0.017	0.489±0.016	0.419±0.036
Anti-GPIb	0.011±0.003	0.019±0.018	0.629±0.027	0.447±0.042
Anti-Vimentin	0.026±0.008	0.018±0.002	0.047±0.017	0.035±0.003
Anti-VNR	1.062±0.088	0.611±0.068	0.894±0.082	0.397±0.055
NRS	0.253±0.011	0.054±0.02	0.245±0.003	0.041±0.018

contamination by platelets in particular was additionally indicated by failure to detect GPIb in microvilli.

Comparison by e.l.i.s.a. of amounts of GPIIIa in platelets and microvilli

Relative amounts of GPIIIa in platelet and microvillar membranes were compared by e.l.i.s.a. of serial 2-fold dilutions of membranes (Fig. 3). In the linear portion of the binding curves, absorbance values were on average 4-fold higher for platelet membranes than for microvilli, and plateau values were 3-fold higher for platelets. GPIIIa became undetectable in platelet membranes at a 1028-fold dilution of 1 mg of membrane protein/ml, compared with between 128- and 256-fold for placental microvilli. The results suggest that there are at least 4–8-fold higher concentrations of GPIIIa in platelet membranes than in placental microvilli. Inasmuch as GPIIb/IIIa is particularly abundant in constituting 20% of platelet membrane proteins, placental microvilli appear to contain significant amounts of GPIIIa.

Comparison of heterodimers containing GPIIIa in placental microvilli and platelets

Immunoprecipitation of biotinylated microvillar and platelet surface proteins was used to clarify microvillar GPIIIa subunit associations.

Monoclonal antibodies to GPIIIa, VNR $\alpha V\beta_3$ complex and VNR α subunit immunoprecipitated placental microvillar proteins of M_r 140000 (α slow) and 90000 (Fig. 4). These proteins were not recognized by control monoclonals, e.g. MOPC-21. A rabbit antiserum to VNR immunoprecipitated the 90000-M_r protein more efficiently than the 140000-M_r α slow subunit. A monoclonal antibody to GPIIb did not precipitate any microvillar proteins in this experiment. With some microvilli anti-GPIIb

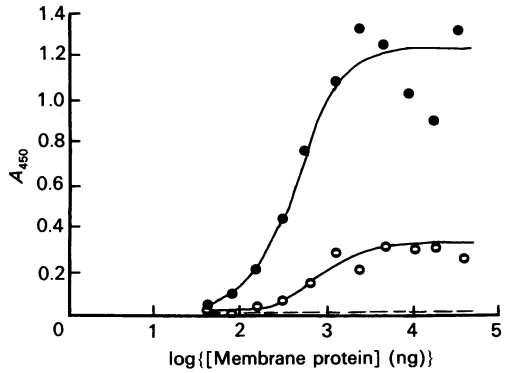

Fig. 3. Comparison by e.l.i.s.a. of relative amounts of GPIIIa in placental microvilli and platelet membranes

Binding of monoclonal antibody to GPIIIa (3 μg/ml) was determined by e.l.i.s.a. for 2-fold serial dilutions of microvilli (○) or platelet membranes (●), starting at 40 μg of protein coated per microtitre well. The broken line shows absorbance values obtained with a negative control monoclonal antibody to vimentin. Binding measurements were in duplicate and differed from the mean by less than 11%.

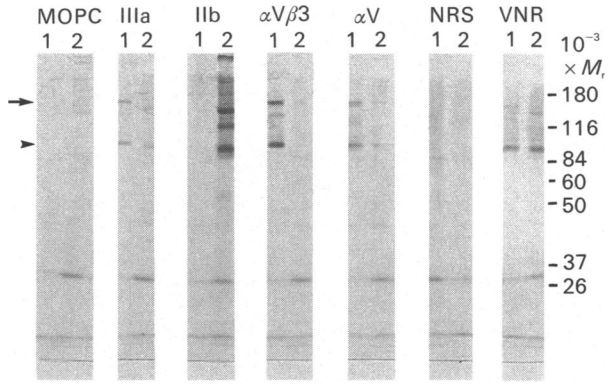

Fig. 4. Immunoprecipitation of placental brush border and platelet heterodimers containing GPIIIa

Immunoprecipitates from biotin-labelled microvilli (lanes 1) or platelet membrane proteins (lanes 2) were electrophoresed under non-reduced conditions in the same gel, transferred to nitrocellulose and probed with alkaline-phosphatase-conjugated avidin. Indicated are the positions of VNR α subunit (arrow) and β subunit (arrowhead). Antibodies used were a negative control monoclonal (MOPC-21), monoclonals to platelet glycoproteins IIIa (IIIa) and IIb (IIb), VNR complex ($\alpha V\beta_3$) and VNR α subunit (αV), rabbit antiserum to VNR and normal rabbit serum (NRS).

weakly immunoprecipitated a protein of M_r 140000, designated α fast, and a protein of M_r 90000.

In contrast, monoclonal antibodies to VNR immunoprecipitated only trace amounts of VNR from platelets. Proteins of M_r 140000, 120000 and 90000 were strongly precipitated by the monoclonal antibody to GPIIb. These 140000- and 90000-M_r proteins were distinct in mobility from microvillar α slow and M_r 90000 protein.

These results indicate that GPIIIa is mainly associated with α slow in placental microvilli, but with GPIIb and a protein of M_r 120000 in platelets.

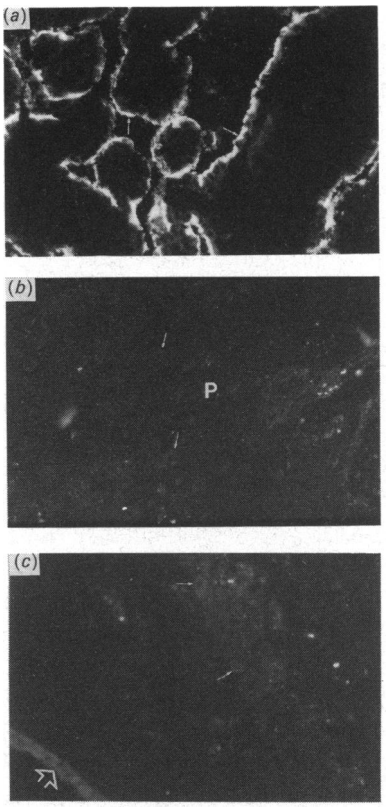

Fig. 5. Immunofluorescence with monoclonal antibody (23C6) to the VNR $\alpha V\beta_3$ complex

Cryostat sections of snap-frozen normal term placenta were incubated with monoclonal antibody to the VNR complex followed by fluorescein isothiocyanate-labelled goat anti-mouse IgG. Arrows in (*a*) indicate strong reactivity on the syncytiotrophoblast brush border. Interstitial cytotrophoblasts in the basal plate (P) are not reactive (arrows, *b*). Note the absence of reactivity in amniotic epithelium (large arrow) and chorion laeve cytotrophoblasts (small arrows, *c*). Magnification ×260.

Immunofluorescence localization of VNR in the placenta

The localization of VNR-related proteins in placenta was revealed by immunohistology (Figs. 5 and 6). In all chorionic villi, monoclonal antibody 23C6 to the VNR $\alpha V\beta_3$ complex bound to the syncytiotrophoblast apical brush border membrane. The antibody did not bind to the basal plasma membrane of the syncytium or to the underlying villous cytotrophoblast (Fig. 5*a*). Other forms of trophoblast, including interstitial cytotrophoblast in decidua, endovascular cytotrophoblast in maternal spiral arteries and chorion laeve cytotrophoblast, did not bind this antibody (Figs. 5*b* and 5*c*).

The antibody to VNR complex bound weakly to endothelial cells and mesenchymal cells of chorionic villi, but not to platelets. In contrast, a rabbit antiserum to VNR displayed occasional reactivity with platelets, but otherwise had an identical profile to the monoclonal anti-VNR ($\alpha V\beta_3$ complex) antibody (results not shown).

Interestingly, one monoclonal antibody (Y2/51) to GPIIIa or VNR β subunit reacted strongly with platelets but not with any other cells in placental sections. Platelets were typically present in areas of fibrin deposition, such as on or in damaged villi with fibrinoid necrosis lesions (Fig. 6*a*) (Labarrere *et al.*, 1990) and at the materno–placental junction (Fig. 6*b*). However, this antibody to GPIIIa invariably reacted with microvilli on immunoblots (Fig. 1). The lack of chemical fixation in immunohistology was

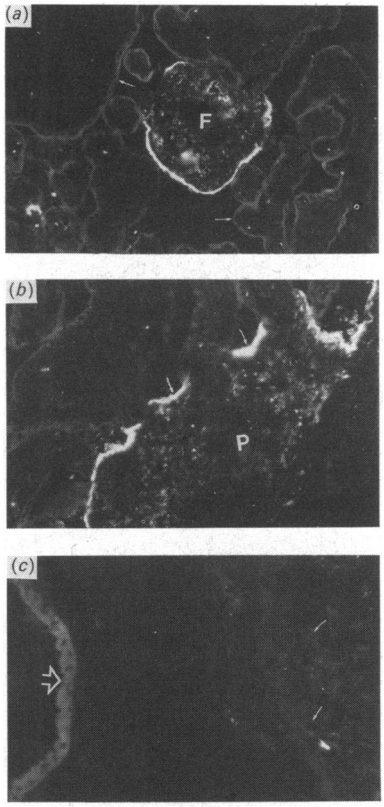

Fig. 6. Immunofluorescence localization with monoclonal antibody (Y2/51) to GPIIIa (VNR β subunit)

Reactivity is localized only to platelets adherent to a damaged villus with fibrin deposition (fibrinoid necrosis area, F). The syncytiotrophoblast is not reactive (arrows, *a*). In the basal plate (P), reactivity was limited to platelet aggregates at the materno–placental junction (arrows, *b*). The amniotic epithelium (large arrow) and chorion laeve cytotrophoblast (small arrows) were not reactive (*c*). Magnification ×260.

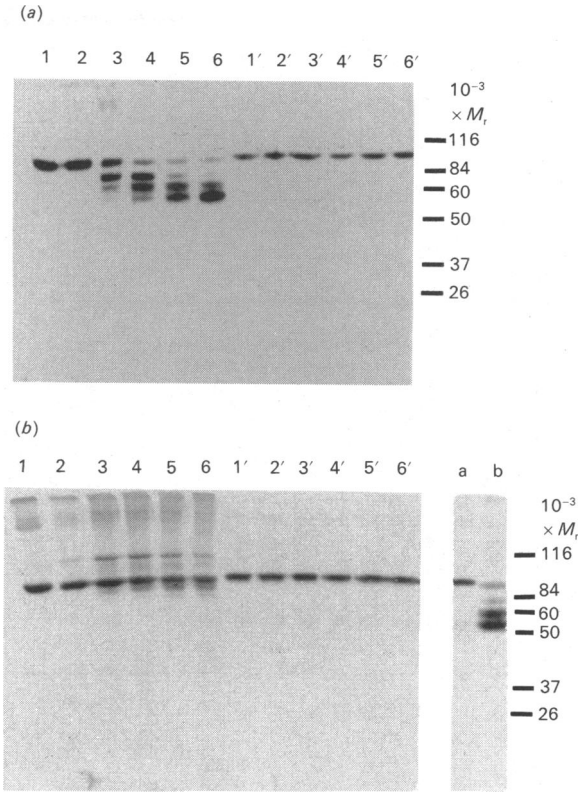

Fig. 7. Limited proteolysis of GPIIIa in sealed placental microvilli and intact platelets

Immunoblot analyses with monoclonal antibody to GPIIIa are shown. (*a*) Platelets (lanes 1–6) and microvilli (lanes 1′–6′) at a concentration of 5 mg of protein/ml were incubated in absence of trypsin (lanes 1) or with 0.1 mg of trypsin/ml in the presence of protease inhibitors for 60 min (lanes 2) or in the absence of inhibitors for 5 min (lanes 3), 15 min (lanes 4), 30 min (lanes 5) or 60 min (lanes 6). Digestion was stopped by addition of a cocktail of soybean trypsin inhibitor, PMSF, aprotinin and leupeptin and then washing the membranes. (*b*) Results of a similar experiment using V8 protease and labelling as above. Trypsin digestions (1 h) of microvilli (lane a) and platelets (lane b) loaded on the same gel are shown for comparison.

anticipated to leave proteins in the native state. The results therefore suggested that conformational factors or bound ligand mask this epitope for anti-GPIIIa on the syncytiotrophoblast in tissue sections.

Limited proteolysis of GPIIIa in intact microvilli and platelets

In view of the differences in mobility of GPIIIa and associated proteins between placental microvilli and platelets, the characteristics of GPIIIa in these sources were further compared by proteolysis for timed intervals of sealed right-side-out microvilli and intact platelets. Immunoblotting showed that GPIIIa of platelet heterodimers was susceptible to both trypsin and V8 protease, in contrast with microvillar GPIIIa, which was completely resistant (Fig. 7). The pattern of fragments and precursor relationships observed for platelet GPIIIa were consistent with those in a previous study (Beer & Coller, 1989). Placental microvillar proteins were not generally protease-resistant, as cleavage of alkaline phosphatase and transferrin receptor was readily achieved (results not shown). The results indicated that certain peptide bonds involving lysine or arginine (trypsin-sensitive) and aspartate or glutamate (V8-protease-sensitive), which are located in a proposed *N*-terminal disulphide-bonded loop (Beer & Coller, 1989), are accessible in platelet GPIIIa but not in placental microvillar GPIIIa membrane-bound heterodimers. This is consistent with the proposal that microvillar GPIIIa heterodimers are distinct from those of platelets and with the difference in their reactivities in immunohistology.

Glycosylation differences in GPIIIa heterodimers of placental microvilli and platelets

Platelet glycoproteins reported to bear blood group A/B determinants include GPIIb/IIIa (Santoso *et al.*, 1989). Since blood group antigens are absent from the syncytiotrophoblast (Szulman, 1972), we compared glycans on GPIIIa heterodimers from trophoblast microvilli and platelets.

Immunoblotting of lectin-bound fractions showed that platelet GPIIIa heterodimers bound to lectins from *Artocarpus integrifolia* (JCA), *Arachis hypogaea* (PNA) and *Erythrina cristagalli* (ECA), whereas microvillar VNR proteins did not (Fig. 8). Unlike platelet GPIIIa-associated proteins, microvillar VNR bound poorly to *Ulex europaeus* 1 lectin (UEA-1) and L-PHA. The platelet and microvillar heterodimers were similar in binding to concanavalin A and to *Lens culinaris* and wheat-germ lectins.

These lectin-binding patterns indicated that trophoblast microvillar and platelet GPIIIa heterodimers differed in glycosylation.

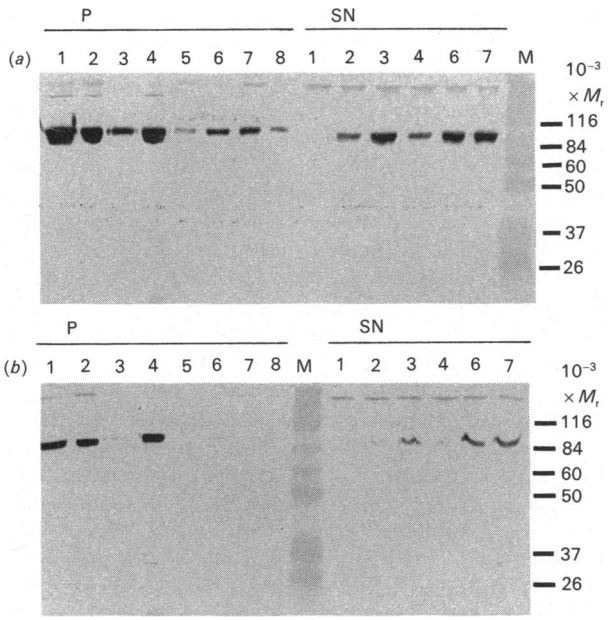

Fig. 8. Differences in lectin-binding properties between GPIIIa heterodimers of placental microvilli and platelets

Triton X-100-solubilized platelet membrane proteins (*a*) or microvillar proteins (*b*) were incubated with various immobilized lectins. A monoclonal antibody to GPIIIa was used to immunoblot proteins bound by the matrices (P) or non-retained (SN). Fractions were from columns containing the following lectins: concanavalin A (lanes 1), lectins from *Lens culinaris* (2), L-PHA (3), wheat-germ agglutinin (4), UEA-1 (5), ECA (6), JCA (7) or PNA (8). Because of space limitations, the non-retained fractions for UEA-1 (5) and PNA (8) were not analysed. Lane M contains M_r standards.

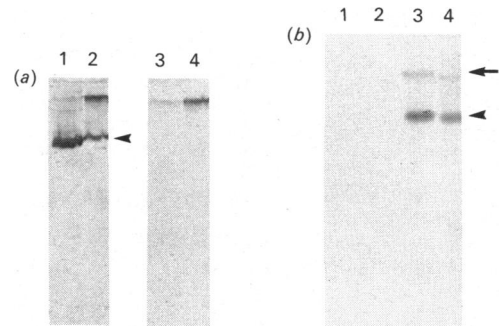

Fig. 9. Alloantibody recognition of brush border GPIIIa

(*a*) Human alloantiserum to the PL^{A1} alloantigenic determinant of GPIIIa (lanes 1 and 2) or normal human serum (lanes 3 and 4) were used to immunoblot non-reduced platelet membrane proteins (lanes 1 and 3) or placental microvillar proteins (lanes 2 and 4). The position of GPIIIa is indicated by the arrowhead. High M_r bands are endogenous human IgG that are cross-reactive with the anti-(human IgG)-conjugated antibody. (*b*) Biotinylated platelet proteins (lanes 2 and 4) and microvillar proteins (lanes 1 and 3) were immunoprecipitated with normal human serum (lanes 1 and 2) or anti-PL^{A1} antibody (lanes 3 and 4). The arrow and arrowhead indicate VNR α subunit and GPIIIa respectively.

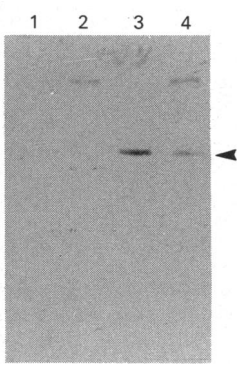

Fig. 10. Binding of brush border VNR to GRGDSPK–Sepharose

Triton X-100-soluble proteins bound by GRADSPK–Sepharose control matrix from platelets (lane 1) or microvilli (lane 2), or bound by GRGDSPK–Sepharose from platelets (3) or microvilli (4), were analysed by immunoblotting with a monoclonal antibody to GPIIIa. The arrowhead indicates GPIIIa.

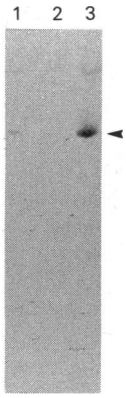

Fig. 11. Microfilament core content of brush border GPIIIa

A monoclonal antibody to GPIIIa was used to immunoblot microfilament core (cytoskeleton) fractions of microvilli prepared using 1% (v/v) Triton X-100 in 10 mM-Hepes, pH 7.4, containing 75 mM-NaCl, 1 mM-PMSF, 1 mM-$MgCl_2$, 1 mM-$CaCl_2$ and 1 mM-$MnCl_2$ (lane 1), or the same buffer containing 2 mM-EGTA instead of the cations (lane 2), or the same buffer but with whole microvilli (lane 3). The arrowhead indicates GPIIIa.

Alloantigen epitope on placental microvillar GPIIIa

Because trophoblast microvilli are directly exposed to the maternal immune system during pregnancy, it was of interest to assess the alloantigenicity of brush border GPIIIa. A human antiserum to the PL^{A1} alloantigen (expressed by more than 95% of individuals in studied populations) was observed to consistently react with placental microvillar GPIIIa by immunoblotting (Fig. 9*a*). This antibody also immunoprecipitated α slow and GPIIIa from microvillar detergent extracts and GPIIb/IIIa from platelets (Fig. 9*b*). However, this anti-PL^{A1} bound poorly to intact microvilli in flow cytometric analyses (results not shown).

Ligand binding by placental microvillar GPIIIa heterodimers

Immunoblotting of fractions retained in affinity chromatography experiments showed that microvillar GPIIIa, and thus presumably the VNR, bound to GRGDSPK–Sepharose but not to GRADSPK–Sepharose (Fig. 10). Platelet GPIIIa heterodimers displayed similar activity. Both receptors also bound to fibrinogen–Sepharose and fibronectin–Sepharose, but not to immobilized BSA or deoxyribonuclease 1 (results not shown). Placental microvillar VNR is therefore a functional receptor.

Microfilament association of placental microvillar GPIIIa heterodimers

Placental microvillar cytoskeleton fractions (Triton X-100-insoluble proteins) were analysed by immunoblotting with anti-

GPIIIa. Only trace amounts of GPIIIa were detected in the microfilament fraction (Fig. 11), which has previously been shown to be enriched in actin-binding proteins (Edwards & Booth, 1987). Rabbit anti-VNR was not reactive with the microvillar cytoskeleton fraction (results not shown).

DISCUSSION

Similar to other integrins, the VNR is of general significance as a transmembrane protein whose interactions with extracellular matrices or adhesion molecules may transduce signals, resulting in changes in cell behaviour. The importance of this role may be reflected in the wide distribution of these proteins. Our results and a previous study by Davies *et al.* (1989) provide clear evidence for a trophoblast brush border VNR with subunits αv and β_3. This may be noteworthy, since brush borders are not classical sites for the cell–extracellular-matrix interactions normally attributed to integrins. Because the syncytiotrophoblast is a polarized epithelium, selective expression of cell adhesion receptors at its basal aspect would be anticipated. Therefore the functions of a trophoblast brush border VNR may vary from those described for other cell types. The question arises as to whether a brush border localization of VNR is unique to trophoblasts or also occurs in analogously polarized epithelia. Antibodies to VNR did not react with, for example, renal proximal tubular epithelium (Davies *et al.*, 1989) or amniotic epithelium (the present study). The VNR was not detected by immunohistology in other subsets of the trophoblast such as villous, interstitial, endovascular and chorion laeve cytotrophoblasts. This particular VNR may thus represent a differentiation marker for syncytiotrophoblast in term placentae.

Two types of α subunit, herein designated α slow and α fast, were associated with GPIIIa in some trophoblast microvilli preparations. The minor component, α fast, was precipitated by a monoclonal antibody to GPIIb, but did not react with monoclonal antibodies to the VNR α subunit. A likely origin of α fast was occasional trace contamination by platelets. Unlike GPIIb/IIIa, the microvillar GPIIIa heterodimers lack *O*-linked Gal-GalNAc and also Gal-GlcNAc, which bind the JCA and ECA lectins respectively (Lis & Sharon, 1986; Krishna-Sastry & Surolia, 1988). The microvillar proteins also differed from GPIIb/IIIa in lacking a terminal fucose which is bound by UEA-1 and in having tri/tetra-antennary *N*-linked structures bound by L-PHA (Lis & Sharon, 1986; Merckle & Cummings, 1987). These results are consistent with the binding of UEA-1, ECA and PNA to connective tissue cells but not to syncytiotrophoblast brush border (Lee & Damjanov, 1984; Vierbuchen *et al.*, 1988). These data support a trophoblast origin for most of the GPIIIa heterodimer in preparations of trophoblast microvilli.

Candidate ligands for placental brush border VNR are maternal blood-borne proteins containing Arg-Gly-Asp sequences and include vitronectin and fibrinogen, which bind VNR in other cell types. Soluble vitronectin has regulatory activities in fibrinolysis (Declerck *et al.*, 1988) and the membrane attack complex of complement (Podack & Muller-Eberhard, 1979), and may convert plasminogen activator inhibitor 1 into an inhibitor of thrombin (Ehrlich *et al.*, 1990). If some of these functions persisted for receptor-bound vitronectin, the VNR could serve to beneficially localize certain of these activities to a membrane surface involved in maintaining fetal welfare. The recent report of VNR binding to complexes of vitronectin and C5b-9 (Biesecker, 1990) suggests that receptor-bound vitronectin activities may usefully be studied.

The reported involvement of VNR in cytoskeleton–membrane interactions (Burridge *et al.*, 1988; Dejana *et al.*, 1988) and the prominent cytoskeletal protein content of trophoblast microvilli (Vanderpuye *et al.*, 1986; Edwards & Booth, 1987) suggested a possible role for the VNR in microfilament–membrane interactions. Moreover, platelet GPIIb/IIIa is reported to specifically bind α-actinin (Otey *et al.*, 1990), which is abundant in trophoblast microvilli (Vanderpuye *et al.*, 1986). However, most of the trophoblast microvillar VNR was not constitutively associated with the cytoskeleton fraction. Trophoblast microvillar cytoskeletons are distinct in composition from those of platelets and adhesion plaques, and it is possible that other proteins compete with the VNR for binding to α-actinin.

This study is also relevant to materno–fetal immunological interactions. GPIIIa can express alloantigenic determinants, and maternal immune responses to non-self brush border VNR epitopes could potentially compromise placental function and also cause neonatal thrombocytopaenia after placental transport of IgG. The low frequency of the necessary genotype and the association of strong immune responses to PL^{A1} with certain human histocompatibility leucocyte antigen (HLA) types would serve to limit this possibility (Valentin *et al.*, 1990; Blanchette *et al.*, 1990). However, uneventful pregnancies can occur in the presence of anti-GPIIIa (anti-PL^{A1}), suggesting that other factors are involved. The present study provides evidence suggesting that GPIIIa in the placental microvillar VNR and platelet GPIIb/IIIa can differ in the amino acids exposed. It is possible that the relevant PL^{A1} epitope is cryptic in microvillar VNR and thus averts the consequences of alloantibody binding.

In summary, the VNR ($\alpha V\beta_3$) has been characterized from an apparently novel brush border location in which it is likely to have biological significance in ligand binding, materno–fetal immunology and the nature of cytoskeleton–membrane interactions.

We thank Dr. Peter J. Newman for the anti-PL^{A1} sera, Dr. Michael Horton for monoclonal antibody 23C6 and Dr. Carl H. Smith for initial facilities and encouragement. We also thank Patty Gulley for technical assistance, Judie Aitken for preparation of this manuscript and Carol Schwarz for photography. This work was supported in part by the Methodist Health Foundation.

REFERENCES

Akiyama, S. K., Nagata, K. & Yamada, K. M. (1990) Biochim. Biophys. Acta **1031**, 91–110
Beer, J. & Coller, B. S. (1989) J. Biol. Chem. **264**, 17564–17573
Biesecker, G. (1990) J. Immunol. **145**, 209–214
Blanchette, V. S., Chen, L., Salomon de Friedberg, Z., Hogan, V. A., Trudel, E. & Decary, F. (1990) Br. J. Haematol. **74**, 209–215
Bodary, S. C. & McLean, J. W. (1990) J. Biol. Chem. **265**, 5938–5941
Booth, A. G., Hubbard, L. M. L. & Kenny, A. J. (1979) Biochem. J. **179**, 397–405
Booth, A. G., Olaniyan, R. O. & Vanderpuye, O. A. (1980) Placenta **1**, 327–336
Burridge, K., Fath, K., Kelly, K., Nuckolls, G. & Turner, C. (1988) Annu. Rev. Cell Biol. **4**, 487–525
Charo, I. F., Bekeart, L. S. & Phillips, D. R. (1987) J. Biol. Chem. **262**, 9935–9938
Cheresh, D. A. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 6471–6475
Cheresh, D. A., Smith, J. W., Cooper, H. M. & Quaranta, V. (1989) Cell **57**, 59–69
Davies, J., Warwick, J., Totty, N., Philp, R., Helfrich, M. & Horton, M. (1989) J. Cell Biol. **109**, 1817–1826
Declerck, P. J., De Mol, M., Alessi, M. C., Baudner, S., Paques, E. P., Preissner, K. T., Muller-Berghaus, G. & Collen, D. (1988) J. Biol. Chem. **263**, 15454–15461
Dejana, E., Colella, S., Conforti, G., Abbadini, M., Gaboli, M. & Marchisio, P. C. (1988) J. Cell Biol. **107**, 1215–1223
Edwards, H. C. & Booth, A. G. (1987) J. Cell Biol. **105**, 303–311
Ehrlich, H. J., Gebbink, R. K., Keijer, J., Linders, M., Preissner, K. T. & Pannekoek, H. (1990) J. Biol. Chem. **265**, 13029–13035
Faulk, W. P. & McIntyre, J. A. (1983) Immunol. Rev. **75**, 139–175

Favaloro, E. J., Moraitis, N., Bradstock, K. & Koutts, J. (1990) Br. J. Haematol. **74**, 385–394
Fitzgerald, L. A., Poncz, M., Steiner, B., Rall, S. C., Jr., Bennett, J. S. & Phillips, D. R. (1987) Biochemistry **26**, 8158–8165
Fox, J. E. B. & Phillips, D. R. (1982) J. Biol. Chem. **257**, 4120–4126
Ginsberg, M. H., Loftus, J., Ryckwaert, J., Pierschbacher, M., Pytela, R., Ruoslahti, E. & Plow, E. F. (1987) J. Biol. Chem. **262**, 5437–5440
Hagen, I. & Solum, N. O. (1989) in Platelet Immunobiology: Molecular and Clinical Aspects (Kunicki, T. J. & George, J. N., eds.), pp. 105–127, J. B. Lippincott, Philadelphia
Hynes, R. O. (1987) Cell **48**, 549–554
Kelley, L. K., Smith, C. H. & King, B. F. (1983) Biochim. Biophys. Acta **734**, 91–98
Krishna-Sastry, M. V. & Surolia, A. (1986) Biosci. Rep. **6**, 853–860
Krissansen, G. W., Elliott, M. J., Lucas, C. M., Stomski, F. C., Berndt, M. C., Cheresh, D. A., Lopez, A. F. & Burns, G. F. (1990) J. Biol. Chem. **265**, 823–830
Kunicki, T. J. & Aster, R. H. (1979) Mol. Immunol. **16**, 353–360
Labarrere, C. A., McIntyre, J. A. & Faulk, W. P. (1990) Am. J. Obstet. Gynecol. **162**, 515–522
Lam, S. T., Plow, E. F., D'Souza, S. E., Cheresh, D. A., Frelinger, A. L., III & Ginsburg, M. H. (1989), J. Biol. Chem. **264**, 3742–3749
Lee, M. C. & Damjanov, I. (1984) Differentiation **28**, 123–128
Lis, H. & Sharon, N. (1986) Annu. Rev. Biochem. **55**, 35–67
Merckle, R. K. & Cummings, R. D. (1987) Methods Enzymol. **138**, 348–368
Otey, C. A., Pavalko, F. M. & Burridge, K. (1990) J. Cell Biol. **111**, 721–729
Phillips, D. R., Charo, I. F., Parise, L. V. & Fitzgerald, L. A. (1988) Blood **71**, 831–843
Podack, E. R. & Muller-Eberhard, H. J. (1979) J. Biol. Chem. **254**, 9908–9914
Pytela, R., Pierschbacher, M. D., Argraves, S., Suzuki, S. & Ruoslahti, E. (1987) Methods Enzymol. **144**, 475–489
Ramaswamy, H. & Hemler, M. E. (1990) EMBO J. **9**, 1561–1568
Santoso, S., Kiefel, V. & Mueller-Eckhardt, C. (1989) Blood **74**, 226a (abstr. 843)
Sheng, Z., Vanderpuye, O. A., Hull, S. R., Carothers-Carraway, C. A. & Carraway, K. L. (1989) J. Cell. Biochem. **40**, 453–466
Smith, J. W., Vestal, D. J., Irwin, S. V., Burke, T. A. & Cheresh, D. A. (1990) J. Biol. Chem. **265**, 11008–11013
Szulman, A. E. (1972) N. Engl. J. Med. **286**, 1028–1031
Toin, H. M., van Kuppevelt, S. M., Languino, L. R., Gailit, J. O., Suzuki, S. & Ruoslahti, E. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 5415–5418
Truman, P. & Ford, H. C. (1984) Biochim. Biophys. Acta **779**, 139–160
Valentin, N., Vergracht, A., Bignon, J. D., Cheneau, M. L., Blanchard, D., Kaplan, C., Reznikoff-Etievant, M. F. & Muller, J. Y. (1990) Hum. Immunol. **27**, 73–79
Vanderpuye, O. A., Edwards, H. C. & Booth, A. G. (1986) Biochem. J. **233**, 351–356
Vanderpuye, O. A., Carothers-Carraway, C. A. & Carraway, K. L. (1988) Exp. Cell Res. **178**, 211–223
Vierbuchen, M., Uhlenbruck, G., Ortmann, M., Dufhues, G. & Fischer, R. (1988) J. Histochem. Cytochem. **36**, 367–376
Vogel, B. E., Tarone, G., Giancotti, F. G., Gailit, J. & Ruoslahti, E. (1990) J. Biol. Chem. **265**, 5934–5937

Received 13 May 1991/7 June 1991; accepted 18 June 1991