An Arg-Gly-Asp–directed Receptor on the Surface of Human Melanoma Cells Exists in a Divalent Cation-dependent Functional Complex with the Disialoganglioside GD2

David A. Cheresh,* Robert Pytela,‡ Michael D. Pierschbacher,‡ F. George Klier,‡ Erkki Ruoslahti,‡ and Ralph A. Reisfeld*

*Scripps Clinic and Research Foundation, and ‡La Jolla Cancer Research Foundation, La Jolla, California 92037

Abstract. The disialogangliosides GD2 and GD3 play a major role in the ability of human melanoma cells to attach to Arg-Gly-Asp–containing substrates such as fibronectin and vitronectin, since pretreatment of these cells with monoclonal antibodies to the oligosaccharide of GD2 and GD3 can inhibit their attachment and spreading on such adhesive proteins. This report demonstrates that human melanoma cells (M21) synthesize and express a glycoprotein receptor that shares antigenic epitopes with the vitronectin receptor on human fibroblasts and is capable of specifically recognizing the Gly-Arg-Gly-Asp-Ser-Pro sequence. In the presence of calcium, GD2, the major ganglioside of M21 cells, colocalized with this receptor on the surface of human melanoma cells and their focal adhesion plaques as demonstrated by double-label transmission immunoelectron microscopy and indirect immunofluorescence. Biochemical evidence is presented indicating that the vitronectin receptor on M21 human melanoma cells contains associated calcium and GD2. This ganglioside copurified with the glycoprotein receptor for vitronectin on affinity columns containing either an Arg-Gly-Asp–containing peptide, concanavalin A, or lentil lectin. This major Arg-Gly-Asp–directed receptor on M21 cells could be metabolically labeled with $^{45}Ca^{2+}$. Chelation of this ion with EDTA caused the dissociation of GD2 from the receptor and rendered the remaining glycoprotein incapable of binding to an Arg-Gly-Asp–containing peptide. Reconstitution experiments demonstrated a requirement for calcium, and not magnesium, for receptor binding to Arg-Gly-Asp and indicated that addition of ganglioside can enhance this interaction.

CELL adhesion to the extracellular matrix is a complex process involving the capacity of receptors on the cell surface to recognize and bind to a variety of adhesive matrix proteins. A class of divalent cation-dependent cell surface receptors has now been defined (5, 19, 21, 22) and shown to recognize the sequence Arg-Gly-Asp (RGD) (37, 43) found in a variety of adhesive proteins, including fibronectin (39, 40), vitronectin (42), fibrinogen, von Willebrand factor (24, 38, 41), and possibly laminin (26). The evolutionary significance of RGD-dependent, cell-substratum interactions is underscored by the fact that such interactions have also been reported for slime mold (16, 48), and platelets (17, 20, 40). In addition to the RGD receptors, other molecular species on cell surfaces have been implicated in the capacity of cells to attach to extracellular matrix proteins. These include proteoglycans (18, 31), and gangliosides (10, 11, 28, 32, 34, 36, 47, 53). In fact, gangliosides (i.e., sialic acid–containing glycolipids) have been proposed to serve as the cell surface receptor for fibronectin (28, 36, 53). This concept was based on the fact that exogenous addition of gangliosides to cultured cells inhibited cell attachment and spreading on a fibronectin substrate. The active moiety of such gangliosides was shown to reside in their oligosaccharide and particularly their sialic acid moiety, since periodate oxidation of terminal sialic acid destroyed the inhibitory effects on cell attachment (28).

Studies from our laboratories have suggested that cell surface gangliosides play a major role in the attachment of human melanoma and neuroblastoma cells to various extracellular matrix components. Thus, monoclonal antibodies (mAbs) to the oligosaccharide portion of the disialogangliosides GD3 and GD2[1] could specifically localize these molecules on the surface and in substrate-attached focal adhesion plaques of human melanoma cells (7, 10, 11). Pre- or posttreatment of these cells with anti-GD2 or anti-GD3 could inhibit their attachment and spreading on a number of extracellular matrix proteins including fibronectin, vitronectin, collagen, laminin, or RGD-containing synthetic peptides (12), as well as an intact matrix laid down by bovine endothelial cells (8). Periodate oxidation of terminal cell surface sialic acid on these cells resulted in a dose-dependent

R. Pytela's new address is Basel Institute for Immunology, Basel, Switzerland.

1. Gangliosides are termed according to the nomenclature of Svennerholm (50).

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decrease in cell attachment and a parallel decrease in the reactivity of anti-ganglioside mAbs with the melanoma cell surface (12). An ultrastructural examination of the surface distribution of GD2 on M21 human melanoma cells revealed that this ganglioside was most heavily expressed on microprocesses emanating from the surface and making direct contact with a fibronectin substrate (10). Taken together these data suggested the possibility that gangliosides on the surface of human melanoma cells may play a key role in cell attachment, either independently or in cooperation with cell surface RGD-receptors.

This report presents direct ultrastructural and biochemical evidence that GD2 is part of a calcium-dependent functional complex, involving the vitronectin receptor (VNr)[2] which is expressed on the surface of human melanoma cells. Moreover, biochemical data indicate that calcium is required for VNr recognition of RGD and that GD2, although not required, augments receptor function in the presence of calcium.

Materials and Methods

Cell Lines

The M21 human melanoma cell line was kindly provided by Dr. D. L. Morton (University of California, Los Angeles). These cells were propagated at 37°C in 7.5% CO_2/92.5% air in RPMI 1640, supplemented with 10% FCS and were shown to be free of mycoplasma by repeated testing during the course of these studies. GD2 was shown to be the predominant ganglioside of M21 cells by intrinsic labeling with [^{3}H]glucosamine (11) and by quantitative surface staining using anti-GD2 mAb and flow cytometric analysis (12).

Monoclonal Antibodies

The various sources of monoclonal antibodies used in these studies are listed with their isotype denoted in parentheses. mAbs 14.18 (IgG3) and 126 (IgM) (10) are directed to the carbohydrate portion of ganglioside GD2. mAb 9.2.27 (IgG2a) is directed to a 250-kD core glycoprotein of a melanoma-associated chondroitin sulfate proteoglycan (6) expressed on the surface of M21 human melanoma cells. mAb W6/32 (IgG2a) anti-HLA (human lymphocyte antigen) was the gift of Dr. P. Parham (Stanford University). All IgG mAbs were isolated from murine ascites fluid and purified on a protein-A Sepharose (Pharmacia, Inc., Piscataway, NJ) immunoadsorbant by a procedure described previously (35). mAb of IgM isotype was isolated from murine ascites as previously described (57). The antibodies directed to GD2 used in this study were previously shown to be nonreactive with carbohydrate determinants on glycoproteins (10).

Indirect Immunofluorescence

M21 melanoma cells were allowed to attach for 60–90 min to glass coverslips previously coated for 24 h with human vitronectin at 5–10 μg/ml in PBS pH 7.2. After the cells had attached and spread, they were permeabilized with 0.1% Triton X-100 for 5 min at 4°C and were fixed with 3% paraformaldehyde for 15 min at room temperature. Each coverslip was then individually washed several times in Hanks' balanced salt solution (HBSS) (Gibco, Grand Island, NY), followed by an additional wash in HBSS containing 0.1 M glycine. The cells were then overlaid with an appropriate murine mAb or affinity-purified polyclonal rabbit anti-VNr at a concentration of ~5 μg/ml for 1 h at room temperature. After three washes in HBSS containing 1% BSA, the cells were overlaid with FITC-conjugated goat anti-rabbit IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) diluted 1:1000 and/or rhodamine-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Inc., Birmingham, AL) diluted 1:800 for 1 h at room temperature. After three additional washes, the coverslips were inverted and mounted on slides in a drop of Fluoromount-G (Fischer Scientific, Pittsburgh, PA). The stained cells were observed through a Zeiss epifluorescent microscopy and photographed.

2. *Abbreviations used in this paper*: HLA, human lymphocyte antigen; VNr, vitronectin receptor; WGA, wheat germ agglutinin.

Transmission Immunoelectron Microscopy

M21 cells grown in suspension culture were washed free of growth media and resuspended in PBS containing 10 mM EDTA for 15 min at room temperature. The cells were then washed, resuspended in HBSS with or without 1 mM Ca^{2+} and Mg^{2+} for 15 min at 37°C, and fixed for 30 min at room temperature with 0.5% glutaraldehyde in PBS. After a brief wash in HBSS containing 0.1 M glycine, the appropriately diluted murine monoclonal or affinity-purified rabbit polyclonal antiserum was added to each dish for 3 h at room temperature. After three washes, each sample was overlayed with goat anti-mouse IgG (Cappel Laboratories, Inc., Cochranville, PA) and/or goat anti-rabbit IgG coupled to colloidal gold (27). Gold was prepared by using the ether (5–7 nm) or the Na-citrate (20–30 nm) reduction of aurochloric acid methods (46). After conjugation, each preparation was centrifuged to remove aggregates and then tested for its ability to specifically stain the IgG band of immunoblotted mouse or rabbit serum after separation by SDS-PAGE. After incubation with gold-labeled secondary antibody, the cells were washed three times and fixed with 2.5% glutaraldehyde in PBS, washed, and postfixed in 1% OsO_4. To increase contrast, thiocarbohydrazide-osmium double fixation was performed. Samples were dehydrated using a graded series of ethanols from 50–100% and each dish was embedded in Epon 812, cured in vacuo for 48 h at 60°C (29), and sectioned on an ultrotome V (LKB Instruments, Inc., Gaithersburg, MD). Samples were viewed in an Hitachi H-600 STEM with a LaB6 or pointed filament. Transmission immunoelectron microscopy was also performed on vitronectin-adherent M21 cells as previously described (10).

Intrinsic Labeling and Indirect Immunoprecipitation

M21 human melanoma cells (10^7 cells in a 75-cm^2 flask) were labeled for 24 h with 1 mCi of [^{3}H]leucine (110 mCi/mmole; New England Nuclear, Cambridge, MA) in leucine-free growth media. Cell monolayers were washed and extracted at 4°C with RIPA lysis buffer (6), which consisted of 100 mM Tris-Cl, pH 7.2, 0.15 M NaCl, 1% (wt/vol) deoxycholate, 1% SDS, and 15 (wt/vol) trasylol (Sigma Chemical Co., St. Louis, MO). Cell lysates were cleared by ultracentrifugation at 35,000 rpm in an ultracentrifuge (L3-50; SW 50.1 rotor; Beckman Instruments, Inc., Palo Alto) for 40 min at 4°C and then were stored at −20°C until use. Lysates were also prepared from focal adhesion plaques remaining on substrate after cell removal with EDTA as described (13).

Affinity-purified anti-VNr antibody or mAb was adsorbed to protein A-Sepharose directly and washed. Immunoadsorbants, each consisting of 100 μl of 10% antibody-coated protein A-Sepharose were incubated with cell or adhesion plaque lysates overnight at 4°C, were washed five times in PBS containing 0.1% ovalbumin and 0.5% Tween 20, then resuspended and boiled in Laemmli sample buffer (30). Samples were analyzed by SDS-PAGE and subjected to fluoroautography as described previously (6).

Preparation of Vitronectin Receptor from M21 Human Melanoma Cells

VNr from M21 cells was purified by affinity chromatography essentially as described (41, 42). M21 cells were grown in suspension culture and harvested by centrifugation at 400 *g*. A 4-ml packed cell pellet was combined with 1 ml of cells, surface labeled with ^{125}I by lactoperoxidase and extracted with 5 ml of extraction buffer (PBS containing 1 mM $CaCl_2$, with or without 1 mM $MgCl_2$), 100 mM octylglucoside, and 3 mM phenylmethylsulfonyl fluoride) for 15 min at 4°C after which the extract was cleared by centrifugation at 25,000 *g* for 30 min. To isolate the VNr cell lysate was directly applied to a Gly-Arg-Gly-Asp-Ser-Pro-Cys-Sepharose column (bed volume, 5 ml) and allowed to bind at 4°C overnight. The column was washed with 25 ml of extraction buffer containing 50 mM octylglucoside and eluted with this buffer containing 1 mg/ml of Gly-Arg-Gly-Asp-Ser-Pro peptide. Fractions (1.25 ml) were collected after application of the elution buffer and 50 μl of each fraction was analyzed by SDS-PAGE as previously described (41, 42).

Intrinsic Labeling of M21 Vitronectin Receptor with $^{45}Ca^{2+}$

M21 cells (1.5×10^8) grown in suspension culture were washed with PBS and resuspended in 10 ml of low Ca^{2+} (0.02 mM) containing RPMI 1640 supplemented with 0.043 mM of $^{45}Ca^{2+}$ (Spec. Act. 17.29 mc/mg; DuPont Co., Wilmington, DE) and dialyzed FCS. The cells were incubated at 37°C in suspension for 3.5 h and then diluted in this same media to 60 ml and incubated for 12 h. After labeling, the cells were washed free of exogenous

$^{45}Ca^{2+}$ and combined with 6×10^8 unlabeled M21 and extracted as described above.

ELISA Analysis of Vitronectin Receptor Fractions for Gangliosides

An aliquot (300 μl) of each column fraction (above) was extensively dialyzed against 1 mM $CaCl_2$ and lyophilized. Each lyophilized fraction was resuspended in 200 μl of methanol and subjected to mild sonication in a water bath and extensively vortexed. 15 μl of the methanol solution was added to individual wells of a polyvinyl chloride 96-well microtiter plate and allowed to dry at room temperature for 2 h. Wells coated with the peak column fraction were shown to contain 1–2 μg of total protein. The wells were then coated for 2 h at 37°C with 150 μl of PBS containing 5% BSA and 5% normal goat serum. The presence of ganglioside was determined by ELISA using anti-GD2 mAb 14.18 as primary antibody and peroxidase-conjugated goat-anti mouse Ig (Bio-Rad Laboratories, Richmond, CA) as secondary antibody as previously described (14). The vitronectin receptor was analyzed on these same microtiter plates using specific affinity-purified polyclonal rabbit antiserum to the receptor as primary antibody and diluted peroxidase-conjugated goat anti-rabbit Ig (Bio-Rad Laboratories).

Isolation of Wheat Germ Agglutinin (WGA)- or Concanavalin A (Con A)-binding Proteins from M21 Melanoma Cells

A 2 ml octylglucoside extract of M21 melanoma cells was prepared as described above, incubated overnight at 4°C with 2 ml of WGA-Sepharose or Con A-Sepharose (Pharmacia Fine Chemicals) and washed extensively with extraction buffer. Bound proteins were eluted with *N*-acetylglucosamine or α-methyl-D-mannoside (200 mM in extraction buffer) for WGA or Con A, respectively. The eluted material was analyzed by SDS-PAGE and the presence of various glycoproteins that were specifically bound to the lectin matrix was demonstrated. For analysis of protein-associated gangliosides, the eluted material was dialyzed against 1 mM $CaCl_2$, lyophilized, reconstituted in methanol, and assayed by ELISA as described above.

Depletion of Cations and GD2 from VNr on Lentil Lectin Sepharose

^{125}I- or $^{45}Ca^{2+}$-labeled VNr, isolated from M21 melanoma cells (10–50 μg) in 2 ml (50 mM) octylglucoside, was incubated for 2 h at room temperature with 1 ml of lentil lectin Sepharose (Pharmacia Fine Chemicals) and washed extensively with extraction buffer. Divalent cations were removed from the bound receptor by washing the column with 10 ml of 10 mM EDTA in extraction buffer. The receptor protein was eluted with α-methyl-D-mannoside (200 mM in extraction buffer) and assayed for receptor by SDS-PAGE and ELISA and for GD2 ELISA as described above.

Reconstitution of Vitronectin Receptor with Cations and/or GD2 and Other Lipids

The VNr depleted of cations and GD2 as described above was reconstituted in extraction buffer with or without 1 mM Ca^{2+}, Mg^{2+} and/or 5 μM ganglioside or phospholipid for 1 h at 37°C followed by 1 h at 4°C. This material was then allowed to bind to GRGDSPK-Sepharose as described above and eluted with the soluble GRGDSP peptide.

Results

Immunoprecipitation of the Vitronectin Receptor from M21 Human Melanoma Cells and their Focal Adhesion Plaques

A polyclonal antibody prepared against the VNr from human placenta (39) identified a protein in detergent extracts of metabolically labeled M21 cells or their focal adhesion plaques. As shown in Fig. 1 (lane *1*), this antiserum recognizes two distinct polypeptides of 130 and 105 kD as determined by SDS-PAGE analysis of immunoprecipitated detergent cell lysates under reducing conditions. These same polypeptides remained associated with the substrate in focal adhesion plaques after the cells were removed by mild detergent treatment of monolayers (Fig. 1, lane *2*). In contrast, a 250-kD melanoma cell-associated antigen immunoprecipitated with mAb 9.2.27 (6) could only be detected in a cell lysate (Fig. 1, lane *3*) and was not associated with the focal adhesion plaques (lane *4*). The anti-VNr antiserum bound functional receptors since it inhibited M21 cell attachment to immobilized vitronectin (data not shown).

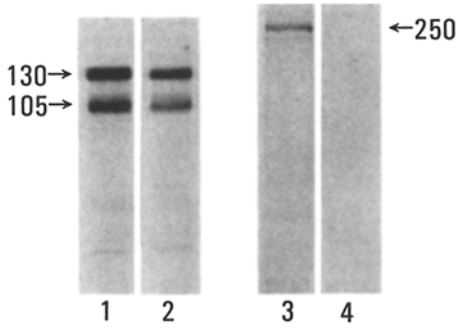

Figure 1. Immunoprecipitation of vitronectin receptor from M21 cells and their focal adhesion plaques. Detergent lysates prepared from [^3H]leucine-labeled M21 melanoma cells (lanes *1* and *3*) and their adhesion plaques (lanes *2* and *4*) were subjected to immunoprecipitation using rabbit anti-receptor antiserum (lanes *1* and *2*) or mAb 9.2.27 (lanes *3* and *4*). Immunoprecipitates were analyzed by SDS-PAGE on 10% gels under reducing conditions as described in Materials and Methods.

Colocalization of GD2 with the Vitronectin Receptor on the Surface of M21 Melanoma Cells

The possibility that complexes between GD2 and VNr may exist on the surface of M21 melanoma cells was examined by both indirect immunofluorescence microscopy and transmission immunoelectron microscopy. For this purpose, M21 cells attached to substrate-coated coverslips were fixed and allowed to react with monoclonal murine anti-GD2 and polyclonal rabbit anti-VNr antibodies, followed by species-specific secondary antibodies labeled with fluorescein or rhodamine. As shown in Fig. 2, the respective staining patterns for VNr (*A*) and GD2 (*B*) were strikingly similar, indicating significant colocalization (*arrows*). Moreover, focal adhesion plaques that remained after cell removal indicated colocalization (*arrows*) of the receptor and GD2. In contrast, murine mAb 9.2.27, which detects a 250-kD chondroitin sulfate proteoglycan core glycoprotein on these same melanoma cells (Fig. 2 *D*), failed to show an analogous staining pattern with the VNr (Fig. 2 *C*). In addition, this 250-kD antigen could not be detected in focal adhesion plaques of these cells (11).

Transmission immunoelectron microscopy also demonstrated colocalization between GD2 and VNr (Fig. 3). The presence of GD2 (Fig. 3, *D–F*) and class I histocompatibility HLA-antigens (Fig. 3, *A–C*) is indicated by a secondary anti-mouse antiserum coupled to large gold particles whereas the VNr is identified by secondary anti-rabbit antiserum coupled to small gold particles (Fig. 3, *A–F*). In controls (Fig. 3, *A–C*), HLA (*small arrow*) and the VNr (*large arrow*) showed no apparent colocalization on either the apical (*A* and *B*) or basal (*B* and *C*) surface of the vitronectin-attached cells. In contrast, colocalization between GD2 and VNr

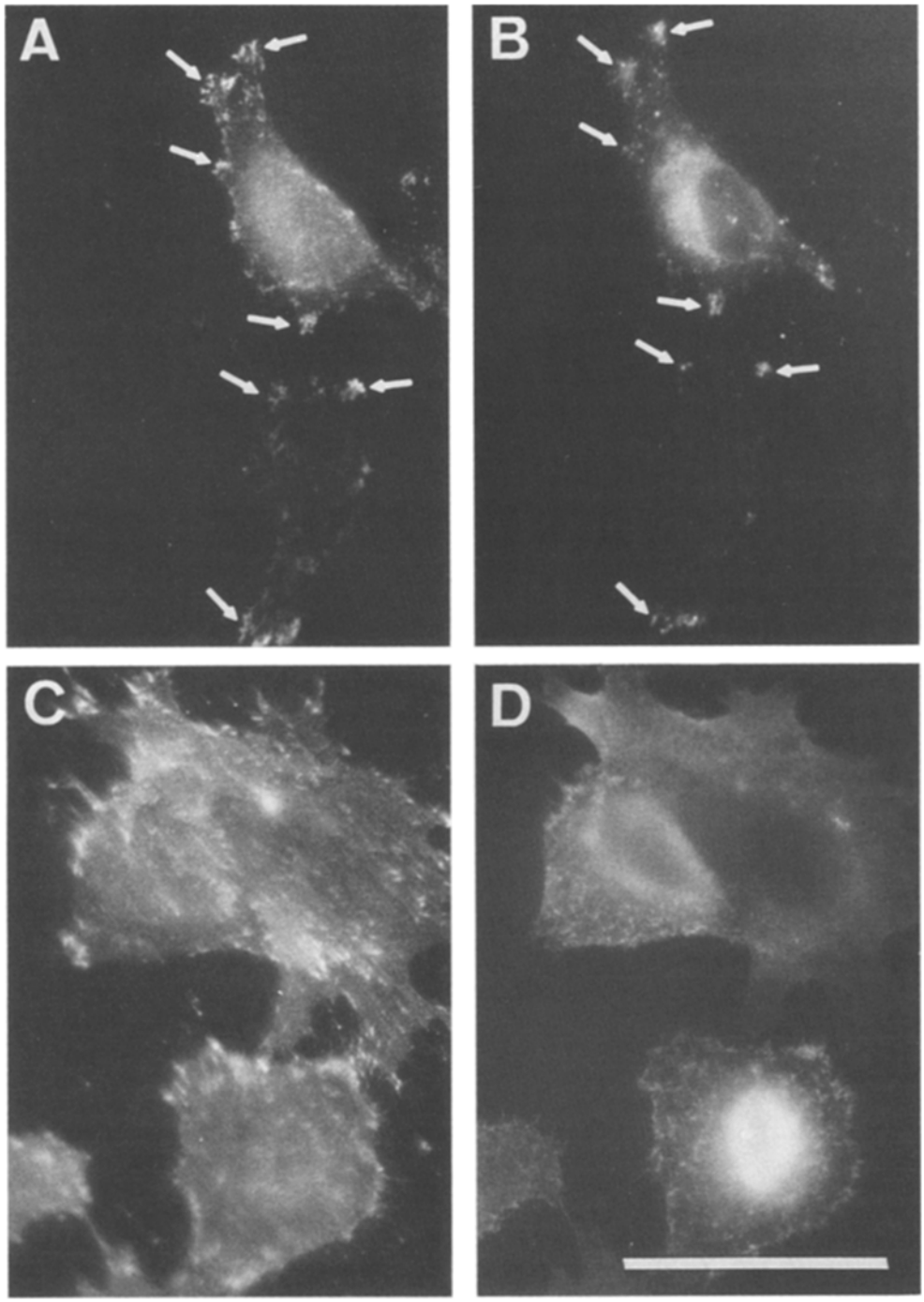

Figure 2. The M21 cell receptor for vitronectin and GD2 colocalizes on the cell surface and in focal adhesion plaques. M21 melanoma cells attached to vitronectin-coated coverslips were subjected to double-label immunofluorescence as described in Materials and Methods. The primary antibodies included rabbit anti-vitronectin receptor (*A–D*) and the murine anti-GD2 mAb 14.18 (*A* and *B*) or anti-chondroitin sulfate proteoglycan, mAb 9.2.27 (*C* and *D*). Rhodamine-labeled anti-rabbit (*A* and *C*) and fluorescein-labeled anti-mouse (*B* and *D*) were used as secondary antibody. (*Arrows*) Areas of colocalization. Bar, 25 μm.

(*large arrowhead*) was observed on both the basal and apical surfaces (Fig. 3, *D–F*) as well as on a cross section of a microprocess making direct contact with the vitronectin substrate (Fig. 3 *E*). The small arrowheads (Fig. 3 *E*) depict noncolocalized GD2 that most probably resulted from this ganglioside's excess expression on the cell surface.

Divalent Cation-dependent Colocalization of GD2 with the Vitronectin Receptor on the Surface of M21 Melanoma Cells

The existence of divalent cation-dependent complexes between GD2 and the VNr on the surface of M21 melanoma cells was investigated by indirect transmission immunoelectron microscopy. For this purpose, M21 cells grown in suspension were exposed to 10 mM EDTA, reconstituted in HBSS with or without 1 mM divalent cations, fixed, and allowed to react with monoclonal murine anti-GD2 antibody and rabbit anti-VNr antibody, followed by species-specific secondary antibodies labeled with gold particles distinguished by their size. As shown in Fig. 4, in the absence of divalent cations (*A*, *C*, and *E*) neither HLA (*A*, *short arrows*) or GD2 (*C*, *E*, *small arrowheads*) colocalized with VNr (*long arrows*). In contrast, in the presence of divalent cations (Fig. 4, *B*, *D*, and *F*) GD2 and VNr showed colocalization

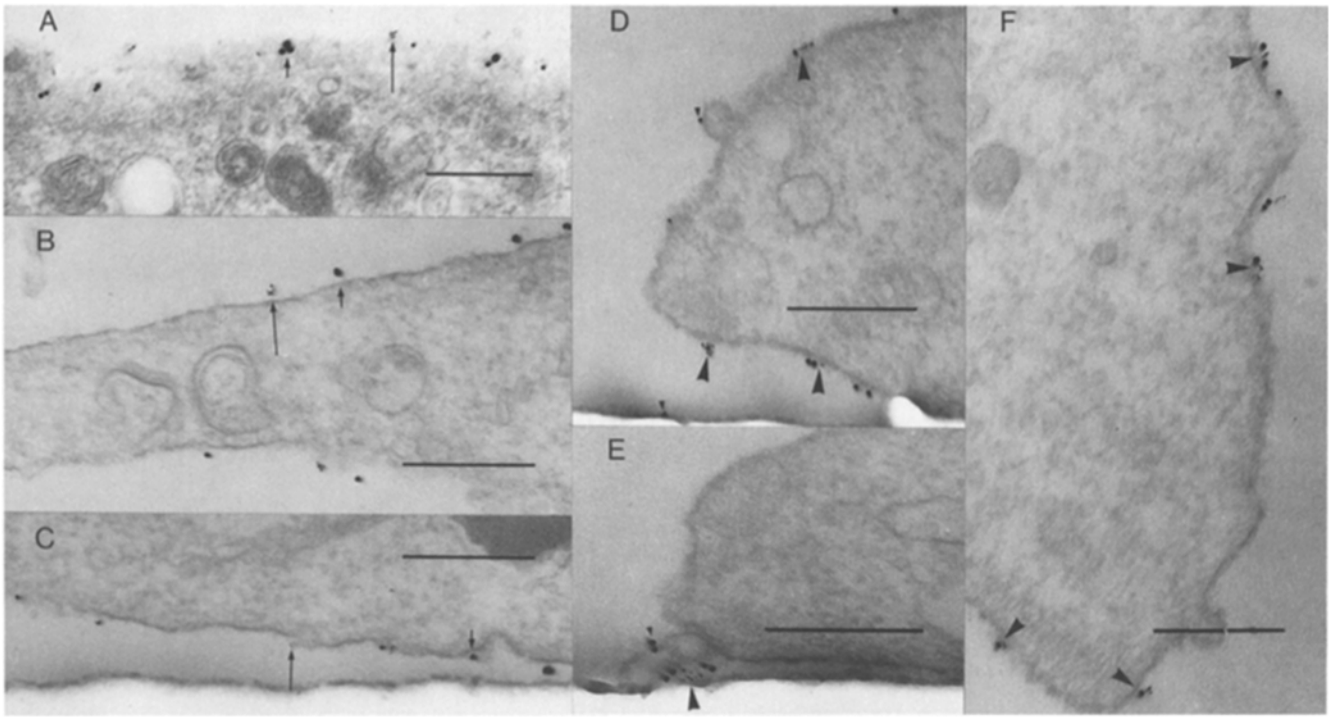

Figure 3. Colocalization of the cell receptor for vitronectin and GD2 by transmission immunoelectron microscopy on substrate-attached M21 cells. M21 melanoma cells attached to vitronectin-coated microtiter wells were subjected to double-label transmission immunoelectron microscopy as described in Materials and Methods. The primary antibodies included rabbit anti-vitronectin receptor (*A* and *F*) and murine mAb 14.18 (anti-GD2) (*D–F*) or mAb W6/32 (anti-HLA) (*A–C*). Small gold (7–9 nm) coupled to anti-rabbit Ig and large gold (20–30 nm) coupled to anti-mouse Ig were used as secondary antibody. *Small* and *large arrows* correspond to the noncolocalized HLA and receptor, respectively, whereas *small* and *large arrowheads* correspond to either free GD2 or GD2/receptor complexes, respectively. Bars, 0.5 μm.

(*D, F, large arrowhead*) whereas HLA and VNr did not (*B, large* and *small arrows*, respectively). The specificity of the colocalization is apparent since M21 cells stained with either anti-VNr antibody and an irrelevant mouse antibody or mAb 14.18 used in conjuction with normal rabbit serum failed to show clustering of small and large gold particles (data not shown).

To determine the relative percentage of colocalization of these antigens, quantitation of the gold particle distribution was performed on both adherent and suspension cells stained with antibodies directed to GD2 or HLA, and VNr as described in Figs. 3 and 4. As shown in Table I, 73 ± 6% of the anti-VNr and anti-GD2 label is detected as colocalized, whereas only 16 ± 3% colocalization could be observed when adherent cells were stained with anti-VNr and anti-HLA (W6/32). Similar results could be observed in cells grown in suspension. Antibodies directed to VNr and GD2 showed 83 ± 4% colocalization when cells were stained in the presence of calcium. However, cells that were stained in the absence of calcium only showed 21 ± 8% colocalized gold particles.

Vitronectin Receptor Isolated from M21 Cells Contains Ca^{2+} and Disialoganglioside GD2

To determine whether a functional VNr isolated from M21 cells contained associated Ca^{2+} and GD2, octylglucoside extracts of M21 cells that were surface labeled with ^{125}I and metabolically labeled with $^{45}Ca^{2+}$ were subjected to affinity chromatography on GRGDSPK-Sepharose. Elution was with a buffer containing either GRGESP or GRGDSP and consecutive fractions of the eluate were analyzed for radioactivity or protein profile by SDS-PAGE autoradiography (Fig. 5 *inset*) and GD2 by ELISA. As shown in Fig. 5, both the ^{125}I-surface-labeled VNr and $^{45}Ca^{2+}$ appeared as sharp peaks (fractions *4–7, right*) that were only eluted from the column after GRGDSP was applied. In addition, GD2 also eluted from this matrix, although slightly ahead of the peaks of radioactivity (Fig. 5, fractions *3–7, right*). In experiments with metabolically labeled ^{3}H-GD2 and nonlabeled VNr, interactions of the ganglioside with the peptide column were not observed unless receptor and cations were present (data not shown). In addition, neither receptor nor GD2 could be eluted from this matrix in the presence of the control peptide GRGESP (Fig. 5, fractions *1–10, left*). Thin layer chromatography results also confirmed the presence of intact GD2 in the VNr-enriched fractions and ruled out the possibility that this reactivity was due to the reaction of anti-GD2 mAb 14.18 with a carbohydrate on this glycoprotein receptor (data not shown).

To determine whether GD2 was present in other glycoprotein fractions from M21 cells, octylglucoside extracts were subjected to affinity chromatography on the RGD peptide column, WGA, and Con A–Sepharose and examined for GD2 content by ELISA. As shown in Fig. 6, the GD2 content, determined by ELISA and expressed as OD 492 nm per μg of protein, was ~100-fold greater for material eluted from the peptide column than for several glycoproteins that specifically eluted from the WGA-Sepharose column (which does

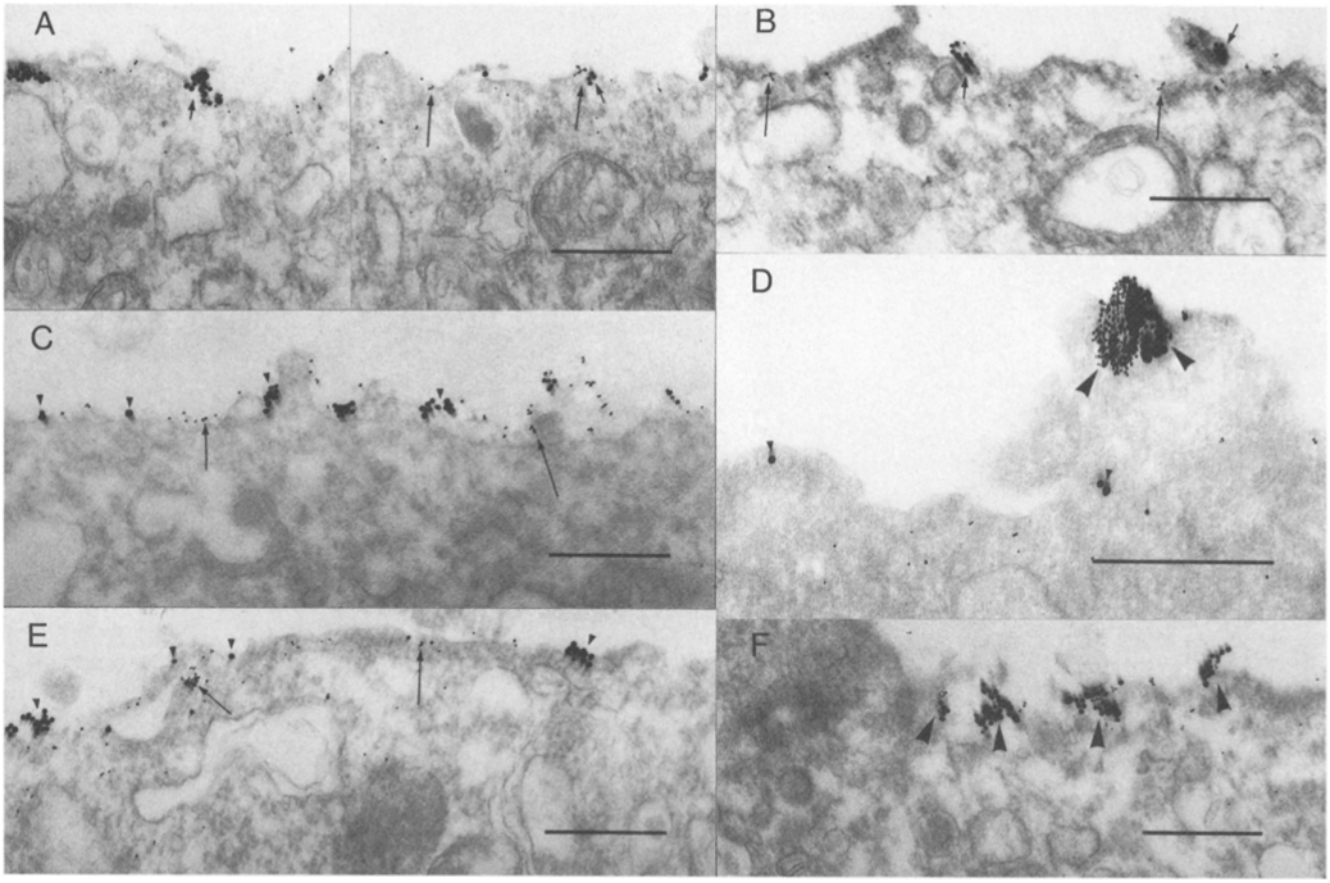

Figure 4. Divalent cation-dependent colocalization of GD2 with the vitronectin receptor on the surface of M21 cells. M21 cells grown in suspension culture were exposed to 10 mM EDTA in PBS, treated with HBSS in the absence (*A*, *C*, and *E*) or presence of 1 mM Ca^{2+} and MG^{2+} (*B*, *D*, and *F*) and fixed with 3% paraformaldehyde. The cells were then allowed to react with murine mAb W6/32 (anti-HLA), (*A* and *B*) mAb 14.18 (anti-GD2) (*C–F*), and rabbit anti-VNr (*A–F*), washed, and stained with anti-rabbit Ig coupled to small gold (7–9 nm) and anti-mouse Ig coupled to large gold (20–30 nm). After further fixation, the cells were pelleted, embedded, and sections were cut and examined as described in Materials and Methods. (*A* and *B*) *Short arrows* denote HLA, and *long arrows* denote VNr. (*C–F*) *Long arrows* denote free receptor, *small arrowheads* denote free GD2, and *large arrowheads* denote GD2/receptor complexes. Bars, 0.5 μm.

not bind this receptor) with the monosaccharide *N*-acetyl glucosamine, as indicated by Coomassie Blue staining of fractions separated by SDS-PAGE (data not shown). However, material eluted specifically from a Con A–Sepharose column contained VNr among other proteins, as well as some GD2. As shown in Fig. 6, GD2 was enriched ~10-fold in fractions eluted from the RGD-containing affinity matrix when compared with the glycoproteins eluted from Con A-Sepharose in the presence of α-methyl-D-mannoside.

Divalent Cations and GD2 Are Functionally Involved in Arg-Gly-Asp Recognition

To ascertain whether divalent cations and/or GD2 were required for Arg-Gly-Asp recognition, the VNr was isolated from M21 human melanoma cells by affinity chromatography on the RGD-containing peptide column, and the eluted receptor was allowed to bind to lentil lectin Sepharose, washed with 10 mM EDTA to remove associated divalent cations, and then specifically eluted with α-methyl-D-mannoside. The elution profile of the lentil lectin Sepharose column appears in Fig. 7. VNr isolated from M21 cells that were metabolically labeled with $^{45}Ca^{2+}$ bound to the column and a peak of radioactivity could be eluted with 10 mM EDTA, demonstrating the removal of a major portion of the Ca^{2+} from this receptor complex. A significant amount of $^{45}Ca^{2+}$ was also observed after elution with α-methyl-D-mannoside that suggests the possibility of another, more tightly asso-

*Table I. Colocalization of GD2 and VNr on the Surface of M21 Cells**

Cells	Colocalized immunogold particles
	%
Adherent	
GD2/VNr	73 ± 6
HLA/VNr	16 ± 3
Suspension	
GD2/VNr (+ Ca^{2+})	83 ± 4
GD2/VNr (− Ca^{2+})	21 ± 8

* Adherent or suspension grown M21 cells were stained with immunogold as described in legends to Figs. 3 and 4, respectively. Individual gold particles were counted and determined to be colocalized when large and small gold particles were separated by less than the diameter of one large gold particle (30 nm). Each value is the mean ± SD of three to four determinations. Each determination represents the percentage of colocalized gold particles on 100 μm of linear cell membrane from a total of 10–20 M21 cells and included from 200–1,500 individual particles.

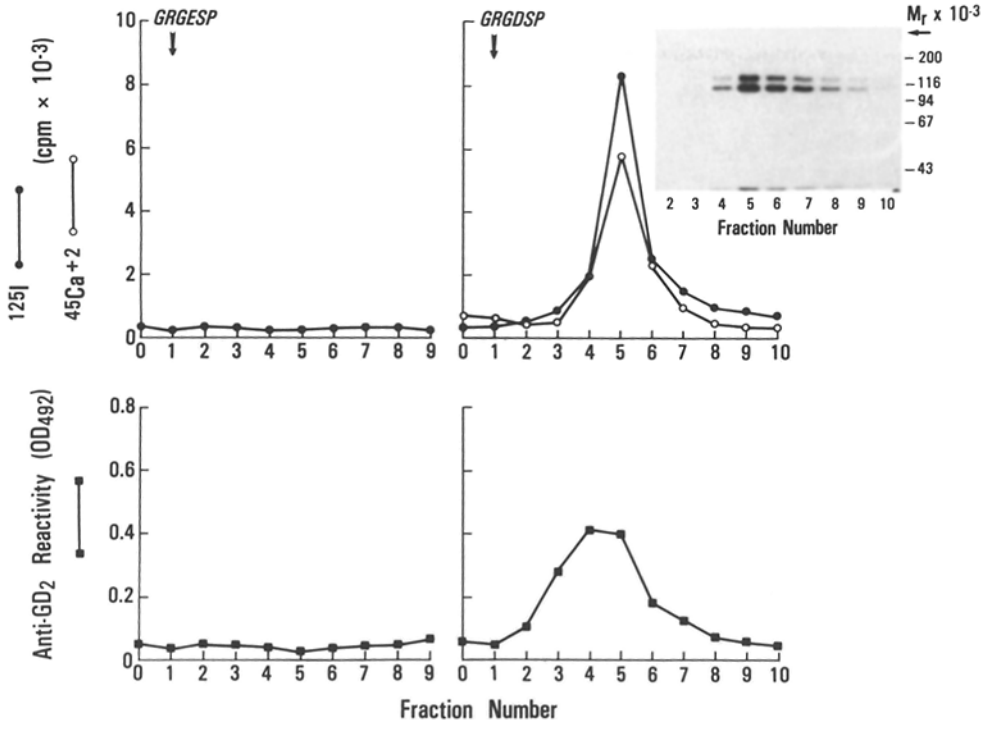

Figure 5. Affinity chromatography of the M21 human melanoma vitronectin receptor complex. M21 human melanoma cells (10^8) were either surface labeled with ^{125}I (●) or metabolically labeled with $^{45}CaCl_2$ (○), combined with unlabeled cells (4-ml packed pellet), and extracted as described in Materials and Methods. The extract was chromatographed on a column of Sepharose 4B (bed volume 5 ml) containing a covalently bound, cell attachment-promoting peptide GRGDSPK. The column was washed with 5 ml of 50 mM octylglucoside followed by 50 ml of 25 mM octylthioglucoside containing GRGDSP (1 mg/ml), after which the column was specifically eluted with the synthetic peptides GRGESP or GRGDSP (*arrows*) in 25 mM octylthioglucoside. Fractions of 1.25 ml were collected and cpm were determined in a beta or gamma counter of 50-ml aliquots of each fraction. Aliquots (15 μl) were analyzed by SDS-PAGE (10% acrylamide) under reducing conditions and examined by autoradiography. The *horizontal arrow* marks the top of the separating gel. The relative GD2 (■) content of each fraction was determined by ELISA using mAb 14.18 as a primary antibody as described in Materials and Methods.

ciated Ca^{2+}-binding site not affected by 10 mM EDTA, although it is possible that some of the $^{45}Ca^{2+}$ could have been eluted from the lentil lectin itself. In contrast, VNr from ^{125}I surface-labeled M21 cells showed a minimal elution peak of radioactivity in the presence of EDTA when subjected to the same lectin column chromatography. However, most of the ^{125}I-labeled material could be eluted with α-methyl-D-mannoside.

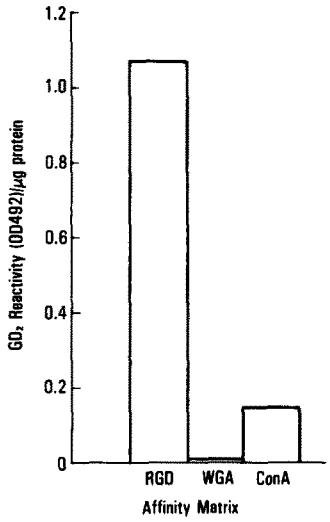

Figure 6. GD2 expression in various M21 cell glycoprotein fractions. Glycoprotein fractions from M21 melanoma cells were prepared by affinity chromatography on Sepharose-coupled matrices containing GRGDSPK, Con A, or WGA, as described in Materials and Methods. Eluted material (1 μg of total protein) from each column was adsorbed to individual wells of a polystyrene microtiter plate. The relative GD2 content was determined by ELISA with mAb 14.18 as the primary antibody and expressed as the relative absorbance at OD 492 above background (i.e., in the absence of a reactive primary antibody). Each bar represents the mean of four replicates analyses.

The possibility was examined that the association of GD2 with the VNr complex is calcium dependent. To this end, GD2 content was determined by ELISA in VNr-enriched fractions eluted from lentil lectin Sepharose. As shown in Table II, GD2 and VNr coeluted from this column only when it was washed with PBS containing 1 mM $CaCl_2$. However, GD2 was completely eliminated from the complex when it was subjected to elution with 10 mM EDTA, yet this treatment had a minimal effect on the quantity of receptor detected with the anti-VNr antibody. In addition, each of these VNr-enriched fractions were examined by SDS-PAGE and shown to be identical to the material isolated originally (Fig. 1) since it contained both the 130 and 105-kD subunits (data not shown).

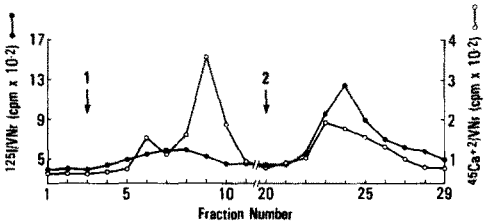

Figure 7. Lentil lectin affinity chromatography of vitronectin receptor complex. Vitronectin receptor, radiolabeled with ^{125}I (●) or $^{45}Ca^{2+}$ (○), obtained as above, was chromatographed on a lentil lectin Sepharose column (bed volume 1 ml). Fractions (0.25 ml) were collected during elution in octylglucoside containing either 10 mM EDTA (*arrow 1*) or 200 mM α-methyl-D-mannoside (*arrow 2*) and the cpm of each fraction were determined in a beta or gamma counter.

Table II. Effects of EDTA on the GD2 Content of the Vitronectin Receptor Complex

Antibody	Reactivity (OD 492) after elution from lentil lectin Sepharose*	
	1 mM $CaCl_2$	10 mM EDTA
Anti-VNr	2.114‡	1.782
14.18 (anti-GD2)	0.495	0.006
W6/32 (anti-HLA)	0.002	0.004

* Vitronectin receptor complex was allowed to bind to lentil lectin Sepharose, washed with PBS containing either 1 mM $CaCl_2$ or 10 mM EDTA, and then eluted with 200 mM α-methyl-D-mannoside as described in Materials and Methods. Antigenic reactivity was tested by ELISA using various primary antibodies.
‡ Average of duplicates.

The functional requirement of VNr for divalent cations was assessed by determining the capacity of the receptor devoid of cations and GD2 to attach to the GRGDSPK-matrix in comparison with that of the intact receptor complex. As shown in Fig. 8, the intact receptor complex actively bound to the adhesion matrix, since 70% of the total radiolabeled material bound and could be specifically eluted with a soluble RGD-containing peptide (Fig. 8, fractions *10–16*). This 70% binding activity represents the total receptor activity that can be recovered from the GRGDSPK-Sepharose affinity matrix in the presence of soluble peptide. In contrast, after depletion of divalent cations and GD2, >80% of VNr appeared in the flow through and column wash with only 19% eluting with soluble RGD. However, there was some weak interaction of the adhesion matrix with the calcium-depleted receptor, since it was somewhat retarded by the column appearing as a rather broad peak comprised by fractions 1–9 (Fig. 8).

The divalent cation requirement for VNr receptor recognition of RGD could also be demonstrated by the capacity of

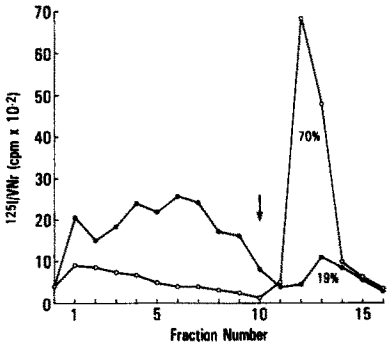

Figure 8. Effects of divalent cation removal on vitronectin receptor function. Vitronectin receptor isolated from ^{125}I-surface-labeled M21 cells was initially allowed to bind to lentil lectin Sepharose and then treated with either EDTA in PBS to deplete the receptor of divalent cations and ganglioside or PBS containing 1 mM Ca^{2+} and Mg^{2+}. Elution followed with α-methyl-D-mannoside. The receptor-containing fraction (15,000 cpm) was then rechromatographed on a column of GRGDSPK-Sepharose 4B (bed volume 1.2 ml). Radioactivity in the vitronectin-enriched fractions (0.25 ml) treated with EDTA (●) or without EDTA (○) were determined in a gamma counter. Fractions 1–9 contain flow through. The specifically bound receptor was eluted with 1 mg/ml of GRGDSP (*arrow*).

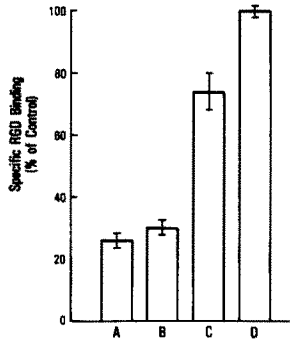

Figure 9. M21 vitronectin receptor function after reconstitution with Ca^{2+} and/or GD2. Vitronectin receptor isolated from ^{125}I-surface-labeled M21 cells was subjected to lentil lectin affinity chromatography, treated with EDTA, and eluted with α-methyl-D-mannoside. The eluted receptor glycoprotein in 1 ml of 50 mM octylglucoside was reconstituted with either 5 μM GD2 (*B*), 1 mM Ca^{2+} and Mg^{2+} (*C*), both (*D*), or neither of the above (*A*) for 1 h at 37°C followed by 1 h at 4°C. Each reconstituted fraction was then allowed to bind to a GRGDSPK-Sepharose column (1.2 ml bed volume) and specific RGD-binding material was eluted with 1 mg/ml GRGDSP. Fractions (0.25 ml) were collected after the addition of the peptide solution (*arrow*) and the cpm of each fraction was determined in a gamma counter. The total cpm of receptor eluted with RGD was compared with the total material eluted before removal of cations or GD2 (control) and each bar represents the RGD-eluted material after reconstitution as a percent of control and is the mean ± SEM of three separate experiments.

EDTA (5 mM) to completely elute the receptor from the RGD-affinity matrix (data not shown).

Reconstitution of Vitronectin Receptor Function in the Presence of Divalent Cations and GD2 and other Lipids

Reconstitution experiments were performed to determine the functional significance of divalent cations and GD2 in the VNr adhesion complex. Thus, VNr was depleted of divalent cations and GD2 by EDTA treatment (as above) and then reconstituted with either divalent cations (Ca^{2+} and/or Mg^{2+}, 1 mM), gangliosides or phospholipid (5 μM), both, or neither of these components. Each reconstituted receptor was allowed to bind to the GRGDSPK-Sepharose column and was then subjected to elution with the soluble RGD-containing peptide. As shown in Fig. 9, the mean of three separate experiments demonstrated that little or no receptor activity could be demonstrated in the absence of divalent cations and GD2. The addition of GD2 in the absence of cations also provided minimal receptor function (30 ± 3% SEM), whereas addition of divalent cations alone resulted in moderate receptor function (75 ± 6% SEM). However, complete recovery of receptor activity (99 ± 2% SEM) was observed only when GD2 was added together with divalent cations. These results indicate that VNr has a functional requirement for divalent cations and that the presence of GD2 serves to augment its function.

To determine whether components other than GD2 and calcium can augment VNr receptor activity, reconstitution experiments were performed in the presence of Mg^{2+}, other gangliosides, and phospholipids. Mg^{2+}, unlike Ca^{2+}, was incapable of restoring receptor activity in the presence or absence of ganglioside indicating that the cation requirement of VNr is somewhat restricted. In the presence of Ca^{2+}, gangliosides other than GD2 had some receptor-augmenting activity relative to receptor reconstituted with cations alone. The gangliosides examined in these experiments were GM3, GM1, GD1a, and GT1, which in the presence of Ca^{2+} were shown to augment VNr binding to RGD by 20–40% over that

observed with Ca^{2+} alone. In contrast, phosphatidylcholine or phosphatidylserine had no significant stimulatory effect on receptor activity, suggesting that the oligosaccharide portion of the ganglioside may be required for augmenting receptor function. In addition, thin layer chromatography analysis of VNr-containing fractions demonstrated the preserve of GD3 as well as GD2 (data not shown). Taken together, these data suggest that a number of gangliosides have the capacity to interact with the M21 cell VNr.

Discussion

The role of gangliosides in cell adhesion has been under investigation since initial reports suggested that their exogenous addition to cultured cells inhibits cell attachment (28, 36, 53). These same studies implied that gangliosides could serve as receptors for adhesive substrates such as fibronectin. However, recent observations implicate a number of cell surface glycoproteins as receptors for extracellular matrix adhesive proteins (43). Based on these observations, reevaluation of the role of gangliosides in the cell attachment process has been necessary. For example, it seemed possible that gangliosides being anionic, might provide a more generalized electrostatic attraction between cells and substrate. Gangliosides may also interact with an independent site on the substrate adhesive protein (47, 52). Alternatively, gangliosides may serve to modulate or potentiate the activity of specific cell surface glycoprotein receptors that are capable of interacting with a given substrate. The data presented in this report support this latter hypothesis by providing direct biochemical, immunochemical, ultrastructural, and functional evidence that the disialoganglioside GD2 on the surface of human melanoma cells forms a calcium-dependent complex with a glycoprotein membrane receptor for vitronectin.

The M21 human melanoma cells used in this study serve as an ideal model system to investigate the role of gangliosides in cell attachment for the following reasons. First, a monoclonal antibody (mAb 14.18) is available for the analysis of GD2 (10), the major ganglioside of M21 cells (11). This monoclonal antibody partially inhibits the binding of M21 cells to a number of RGD-containing substrates (8). Secondly, these cells attach to fibronectin, vitronectin, and RGD-peptide substrates (8). As demonstrated here, one of the receptors mediating such attachment has properties similar to a vitronectin receptor described in other types of cells (40, 49). The vitronectin receptor expressed by the M21 cells is composed of two polypeptides of 130 and 105 kD that associate posttranslationally in a noncovalent manner and share some structural and antigenic properties with the IIb/IIIa glycoprotein adhesion receptor on platelets (9). We chose this receptor for our study because it is abundant in the M21 cells and can be readily isolated (9) using affinity chromatography on RGD-containing peptides (42).

The first indication of the complexing between GD2 and the vitronectin receptor was their colocalization at the cell surface and in adhesion plaques as demonstrated by immunofluorescence and immunoelectron microscopic analysis with specific antibodies. The presence of the ganglioside and the receptor in focal adhesion plaques suggests that they are specifically involved in the adhesion of the M21 cells. In contrast to the receptor, another melanoma-associated glycoprotein, i.e., a chondroitin sulfate proteoglycan, was not found in the focal adhesions by SDS-PAGE analysis or by direct immunofluorescence (11).

The colocalization of GD2 with the vitronectin receptor was dependent on divalent cations and could be shown to be specific for the receptor. Immunoelectron microscopy indicated that the vitronectin receptor on the surface of M21 melanoma cells was associated with GD2 only in the presence of calcium. In contrast, in cells that were treated with EDTA, the receptor and GD2 immune reactivities were distributed independently of one another. The specificity of the GD2-vitronectin receptor colocalization was apparent since an anti-HLA antibody, which also binds to the surface of M21 cells, did not significantly localize with the vitronectin receptor under any circumstances. Other controls showed that the colocalization of GD2 with the receptor was not due to cross-reactivity of any of the antibodies.

The colocalization studies suggested that the vitronectin receptor and at least some of the cell surface GD2 exist as a complex at the cell surface and that the association between these two molecules is dependent on divalent cations. That this is the case is supported by direct biochemical evidence for the presence of such a complex. The complexing between the vitronectin receptor and GD2 was evident from the presence of GD2 in GRGDSPK-Sepharose affinity matrix fractions containing the vitronectin receptor, as detected by reaction with the anti-GD2 mAb 14.18 and by thin layer chromatography. GD2 was enriched 100-fold in such receptor fractions isolated from M21 cell extracts compared with GD2 in glycoprotein fractions obtained from similar extracts by chromatography on WGA. In fractions eluted from Con A columns, the enrichment of GD2 was only 10-fold on a ganglioside to protein basis when compared with the isolated receptor fractions reflecting the fact that, although the M21 vitronectin receptor binds to Con A, it is present in the Con A–bound fraction along with other glycoproteins. These data indicate a molecular interaction between GD2 and the vitronectin glycoprotein receptor. It is interesting to note that this receptor complex was isolated from cells that had grown for several days in suspension culture, indicating that the formation of such a complex did not depend on active cell–substratum interactions.

It is likely that gangliosides other than GD2 can associate with the VNr. In this regard, thin layer chromatography analysis of RGD-eluted VNr fractions revealed the presence of GD3 in addition to GD2 (data not shown). The fact that GD2 and GD3 are the major gangliosides of M21 cells and both associate with the VNr suggests the possibility that gangliosides on other cell types show similar associations.

Additional biochemical studies demonstrated that the vitronectin receptor–GD2 complex expressed on M21 cells binds Ca^{2+} and that binding of the divalent cation is necessary for the existence of the complex. We found that after these cells had been intrinsically labeled with $^{45}Ca^{2+}$, the radiolabel coeluted with the receptor from the GRGDSPK-Sepharose affinity matrix. GD2 also copurified with the vitronectin receptor although, upon elution of the adhesion matrix with soluble RGD-peptide, the ganglioside typically eluted slightly ahead of the receptor. We attribute this to dissociation of the receptor–ganglioside complex upon elution. The elution of the ganglioside ahead of the receptor is consistent with the results reported by Heath et al. (25), demon-

strating that when gangliosides were loaded onto a Sepharose column together with rabbit immunoglobulin, they eluted just before the protein. These authors attributed the differences to the formation of ganglioside micelles that would elute rapidly due to their large size. Additionally, retardation of the receptor may occur because of the competition of the matrix and the eluting peptide for the receptor. Regardless of the slight difference in the elution profiles of the receptor and GD2, their elution by the GRGDSP peptide was clearly specific, since a control peptide failed to elute either molecule.

To investigate the role of divalent cations within the vitronectin receptor GD2 complex, we removed the divalent cation from the receptor bound to lentil lectin Sepharose by washing with EDTA. The receptor eluted from such a column was found to have lost most of its ability to bind to the GRGDSPK-Sepharose matrix. An analysis of the GD2 content of the vitronectin receptor depleted of cations in this manner showed that GD2 was also lost. Reconstitution of the receptor with calcium and a variety of gangliosides demonstrated that a significant part of the receptor activity could be regained simply by addition of calcium (75% of original RGD-binding activity) whereas Mg^{2+} alone had no effect. However, receptor activity was increased when exogenous ganglioside was added together with the calcium, while the ganglioside alone failed to promote significant activity. Phospholipid could not substitute for the effects of ganglioside, suggesting that the potentiation effect by gangliosides may involve their oligosaccharide moiety. These results suggest that calcium and not magnesium is required for receptor activity, and that gangliosides can serve to optimize this activity. However, at present we cannot rule out the possibility other divalent cations can substitute for Ca^{2+}. Recent primary sequence analysis of the alpha chain of the human VNr has, in fact, revealed multiple calcium-binding sites (Suzuki, S., S. Argraves, H. Arai, L. Languino, M. D. Pierschbacher, and E. Ruoslahti, manuscript submitted for publication), suggesting a possible site on the protein involved in complex formation. Based on initial reconstitution experiments, gangliosides other than GD2 could potentiate receptor activity. VNr reconstituted with Ca^{2+} and either GM3, GM1, GD1a, or GT1, showed between 20–40% increased receptor activity. Additional experiments are required, however, to determine whether a particular ganglioside has an optimal effect.

Our result may aid in explaining the previous observation that monoclonal antibodies directed to GD2 or GD3 could partially inhibit M21 melanoma cell attachment to immobilized vitronectin or the synthetic peptide GRGDSPC (12). Since our results show that the vitronectin receptor is complexed with GD2 at the cell surface, the anti-ganglioside antibody may sterically inhibit the binding of the receptor to its ligand. Alternatively, the antibody could interfere with the potentiating activity of the ganglioside on the receptor function. Our results also shed light on the findings of several investigators (28, 36, 53) who have reported that exogenous addition of gangliosides to cultured cells caused a loss of their attachment to a fibronectin substrate. These investigators suggested that gangliosides may serve as the actual cellular adhesion receptors. However, since the fibronectin and vitronectin receptors are related proteins (43, 49), and since anti-GD2 also inhibit the attachment of M21 cells to fibronectin substrate (12), the fibronectin receptor is probably also associated with gangliosides. That exogenous ganglioside addition could inhibit cell attachment may be explained by an electrostatic perturbation of the functional receptor–ganglioside complex on the cell surface or by an actual chelation of the divalent cations from the membrane receptors.

The possibility that gangliosides on the cell surface may interact and modulate receptor function without acting directly as receptors has also been suggested for other receptors. Such receptors include the platelet-derived growth factor (3) and epidermal growth factor (4) receptors.

The role of the ganglioside in the adhesion receptor complexes may be to orient the divalent cation required for the receptor to bind to its ligand. This hypothesis is supported by the fact that gangliosides are known to interact with divalent cations through their terminal sialic acid residues (1, 2). In fact, (45) demonstrated that physiological levels of Ca^{2+} led to cross-linking and condensing of ganglioside headgroups in model membranes through complexing of carboxyl groups of sialic acid. It is possible that in the presence of divalent cations, laterally mobile carbohydrate-bearing components such as gangliosides may show a tendency to cluster about glycoproteins containing one or several carboxyl groups. Thus, depending on their ganglioside composition, cells with identical RGD-dependent receptors may vary in their affinity for a given substrate, thereby allowing firm attachment of certain cells or migration and invasion as observed for embryonic and metastatic cells (33). This hypothesis may also help to explain the observed variation in ganglioside composition associated with cellular differentiation (15, 23) and oncogenic transformation (7, 13, 14, 22, 44). It is clear that our results suggest that cell-substratum interactions involve a process whereby individual receptors function within an asymmetric domain of the lipid bilayer as receptor complexes containing glycoproteins, divalent cations, and gangliosides.

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Note Added in Proof: The receptor termed VNr in this study has been shown to also recognize fibrinogen and von Willebrand factor and is expressed on human endothelial cell (Cheresh, D. A., 1987, *Proc. Natl. Acad. Sci. USA*, In press). Human endothelial cells synthesize and express an Arg-Gly-Asp-directed adhesion receptor involved in attachment to fibrinogen and von Willebrand factor.

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