Isolation of a Novel Integrin Receptor Mediating ARG-GLY-ASP–directed Cell Adhesion To Fibronectin and Type I Collagen from Human Neuroblastoma Cells. Association of a Novel β_1-related Subunit with α_v

Shoukat Dedhar and Virginia Gray

Department of Advanced Therapeutics, Cancer Control Agency of British Columbia, and Department of Pathology, University of British Columbia, Vancouver, British Columbia, V5Z 4E6 Canada

Abstract. We report the isolation from two human neuroblastoma cell lines of an Arg-Gly-Asp–dependent integrin complex capable of binding to vitronectin, fibronectin, and type I collagen. The two neuroblastoma cell lines, SK-N-SH and IMR-32, exhibit specific attachment to fibronectin and type I collagen. SK-N-SH cells exhibit a much stronger attachment to vitronectin than the IMR-32 cells, which attach poorly to this substrate. Affinity chromatography of octylglucoside extracts of ^{125}I surface–labeled cells on GRGDSPK-Sepharose columns resulted in the specific binding and elution with GRGDSP of three radiolabeled polypeptides with relative molecular masses of 135, 115, and 90 kD when analyzed by SDS-PAGE under nonreducing conditions. In the SK-N-SH cells the 135- and 90-kD polypeptides were more abundant whereas in the IMR-32 cells the 135- and 115-kD polypeptides were more highly expressed. Liposomes prepared from fractions containing all three polypeptides bound to vitronectin, fibronectin, and type I collagen, whereas liposomes prepared from the 135- and 115-kD polypeptides bound only to fibronectin and type I collagen. Polyclonal antibodies against the α/β complexes of both the vitronectin receptor and the fibronectin receptor immunoprecipitated all three polypeptides. A monoclonal antibody against β_1 immunoprecipitated only the 135- and the 115-kD polypeptides, whereas a monoclonal antibody against β_3 subunit immunoprecipitated the 135- and 90-kD polypeptides. Although, the 115-kD polypeptide could be recognized by an anti-β_1 antibody, a comparison of peptide maps generated by V8 protease digestion of the 115-kD polypeptide and β_1 subunit immunoprecipitated from GRGDSPK-Sepharose flow-through material indicated that these two polypeptides are distinct. Depletion of the 90-kD polypeptide with an anti-β_3 monoclonal antibody did not effect the ability of the 115- and 135-kD polypeptides to bind to GRGDSPK–Sepharose. These data indicate that the SK-N-SH and IMR-32 neuroblastoma cells express a novel "β_1-like" integrin subunit that can associate with α_v and can bind to RGD. We propose to name this β_1-like subunit βn. The data reported here thus demonstrate that in these two cell lines α_v associates with two β subunits, βn and β_3, forming two heterodimers. The α_v βn complex mediates binding to fibronectin and type I collagen, whereas the α_v β_3 complex mediates binding to vitronectin.

PROTEIN constituents of the extracellular matrix can influence various aspects of neuronal differentiation. Specifically, purified glycoproteins of the extracellular matrix, especially the basement membrane glycoprotein laminin, have been shown to promote neurite outgrowth and survival in vitro (3, 5, 6, 20, 21). Fibronectin as well as collagens type I and IV can also influence neuronal process outgrowth from mammalian and avian neurons (13, 32, 33). Another role for these extracellular matrix molecules may be in axon growth in vivo, since for many neurons axon initiation occurs in regions likely to contain laminin and/or fibronectin (2, 12, 29). In addition, Rettig et al. (28) have demonstrated that culturing of some human neuroblastoma cell lines on extracellular matrix produced by bovine corneal endothelial cells or fibronectin results in the induction of certain antigens accompanied by increased adhesiveness, cell spreading, and morphological changes, indicating that extracellular matrix components can modulate the differentiation phenotype of such cells.

The interaction of cells with the glycoproteins of the extracellular matrix has been shown to be mediated by integral membrane glycoproteins that behave as cell surface receptors for these extracellular matrix components. These receptors belong to the superfamily of integral membrane glycoproteins called integrins (17, 30), which mediate cell–cell or cell–extracellular matrix interactions. The integrins are heterodimeric in structure composed of two (α/β) subunits. The integrin superfamily can be subdivided into families of

© The Rockefeller University Press, 0021-9525/90/06/2185/9 $2.00

receptor structures each having common β subunits noncovalently associated with different α subunits: the fibronectin receptor family, β_1; the leukocyte adhesive receptor family, β_2 (LFA-1, Mac-1, gp 150/95); and the vitronectin/platelet IIb/IIIa family, β_3. The β_1 family of receptors share the β subunit, having a molecular mass of 110 kD; the β_2 family share a β subunit of 95 kD; and the β_3 family share a β subunit of 90 kD (17, 30). The β_1 integrin family includes structures known as VLA proteins, a family of proteins initially identified on surfaces of long-term activated human T lymphocytes (14). These proteins include the human fibronectin receptor and a collagen receptor (14) as well as a laminin receptor (11). Recently, further heterogeneity has been demonstrated in the integrin receptor family in that two examples have been described whereby a single α subunit can be associated with more than one β subunit, namely the α_6 subunit of the VLA integrins (15, 18) and the α subunit of the vitronectin receptor (α_v) (4).

The interactions of fibronectin (25), vitronectin (24), collagen type I (7), fibrinogen, and von Willebrand factor with their respective cell surface receptors have been shown to be mediated via a short amino acid sequence, Arg-Gly-Asp (RGD), initially found to be functional in fibronectin (23), but subsequently found to be present and functional in each of these protein molecules (30). The human receptor for fibronectin can be purified by affinity chromatography on fibronectin–Sepharose columns and specific elution from the column with a soluble Arg-Gly-Asp containing peptide (25). The vitronectin receptor and the platelet IIb/IIIa complex can be purified not only by affinity chromatography on vitronectin and fibrinogen–Sepharose columns, respectively, but also by affinity chromatography on Sepharose coupled to a hepta-peptide Gly-Arg-Gly-Asp-Ser-Pro-Lys (GRGDSPK) (24, 26). The specific receptor for fibronectin (α_5/β_1) cannot be purified on such columns. This is because the affinity of the fibronectin receptor for the GRGDSP peptide as compared to fibronectin has so far been found to be 100- to 1,000-fold (23) lower, whereas the affinities of the protein and peptide ligands for the vitronectin receptor and gp IIb/IIIa differ no more than 10-fold each (30). Thus affinity chromatography of detergent extracts of mammalian cells on GRGDSPK-Sepharose columns leads to the isolation of receptors belonging to the β_3 integrin family but as yet not to the isolation of the β_1 integrins.

To characterize the Arg-Gly-Asp–directed receptors of human neuroblastoma cells, we carried out affinity chromatography of detergent extracts of two human neuroblastoma cell lines, IMR-32 and SK-N-SH, and for comparison, a colon carcinoma cell line, COLO-205, on GRGDSPK-Sepharose columns. We report here the isolation from the two neuroblastoma cell lines, of two RGD-dependent integrin heterodimers, α_v β_3 and α_v βn, the former mediating binding to vitronectin and the latter binding to fibronectin and type I collagen. This report also describes, for the first time, the isolation of a β_1-related integrin subunit (βn), which can associate with the α subunit of the vitronectin receptor, α_v.

Materials and Methods

Materials

Protein A–Sepharose, cyanogen bromide–activated Sepharose 4B, and type I collagen (calf skin) were purchased from Sigma Chemical Co. (St. Louis, MO). ^{125}I-sodium iodide and ^{14}C-methylated molecular mass standards were purchased from Amersham Corp. (Oakville, Ontario). Chemicals for SDS-PAGE, including molecular mass standards, were purchased from Bio-Rad Laboratories (Richmond, CA). The synthetic peptide Gly-Arg-Gly-Asp-Ser-Pro-Lys (GRGDSPK) was synthesized at the Department of Biochemistry, University of Victoria with the use of an automated peptide synthesizer (Applied Biosystems Inc., Foster City, CA). The synthetic peptide Gly-Arg-Gly-Asp-Ser-Pro was synthesized by the La Jolla Cancer Research Foundation, La Jolla, CA. Rabbit anti-human fibronectin and vitronectin receptor antisera as well as anti-α_v antiserum and purified fibronectin were kind gifts from Drs. Erkki Ruoslahti and Michael Pierschbacher of the La Jolla Cancer Research Foundation, La Jolla, CA. The anti-fibronectin receptor antiserum reacts with β_1 and α_5 integrins (1) and the anti-vitronectin receptor antiserum reacts with α_v and β_3 (31). Monoclonal antibody (AIIB2) against the common β_1 integrin subunit was a kind gift from Dr. Caroline Damsky, University of California, San Francisco. Anti-gp IIIa (β_3) monoclonal antibody was purchased from Dakopatts, Glostrup, Denmark. SK-N-SH, IMR-32, and COLO205 cell lines were obtained from the American Type Culture Collection (Rockville, MD). Octylglucoside (octyl-β-D-glucopyranoside) was purchased from Calbiochem-Behring Corp. (La Jolla, CA). Purified tenascin was a kind gift from Dr. Mario Bourdon, La Jolla Cancer Research Foundation. Purified vitronectin was purchased from Telios Pharmaceuticals, La Jolla, CA.

Cells and Growth Conditions

SK-N-SH, IMR-32, and COLO-205 cell lines were cultured in DME, supplemented with 10% heat-inactivated FBS, glutamine (1%), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Gibco Laboratories, Grand Island, NY). For routine subculturing, cell monolayers were washed with PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.3), and detached with EDTA (1 mM). Total cell numbers were determined by counting on a Coulter Counter (model ZBl; Coulter Electronics, Hialeah, FL).

Cell Attachment Assays

Attachment of cells to various extracellular matrix substrates was carried out in substrate coated 96-well microtiter well plates (Linbro Chemical Co., Hamden, CT). Wells were coated in triplicate with increasing concentrations of each substrate. After overnight incubation at 4°C the unbound substrates were washed away with PBS and the wells blocked with DME containing 2.5 mg/ml BSA for 2 h at 37°C. At this point, the SK-N-SH and IMR-32 cells were harvested from culture, washed three times in serum-free DME, and resuspended in DME containing 2.5 mg/ml BSA at a concentration of 3×10^5 cells/ml. Cells (0.1 ml) were then applied to each well and cell attachment carried out at 37°C for 1 h. Unattached cells were removed and the wells were washed with PBS. Attached cells were fixed with 0.1 ml 3.7% paraformaldehyde solution. After 30 min, the cells were stained overnight with toluidene blue (0.5%) on 3.7% paraformaldehyde. The excess stain was washed away with PBS and cell attachment was quantitated by measuring absorbance at 495 nM using an automated microtiter well plate reader.

Surface Labeling and Preparation of Cell Extracts

Cells were harvested from confluent cultures, and resuspended in PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.2 mM PMSF. The suspended cells were radiolabeled with Na-^{125}I according to Lieben et al. (22). The radiolabeled cells (10^8) were extracted in PBS containing 100 mM octylglucoside, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.2 mM PMSF as described by Pytela et al. (25).

Affinity Chromatography

The RGD-containing peptide affinity matrix was prepared by coupling 50 mg of GRGDSPK peptide to 2 ml of cyanogen bromide–activated Sepharose 4B. The resulting affinity matrix (1 ml) was poured into a column which was then equilibriated with column buffer (PBS containing 50 mM octylglucoside 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM PMSF). The cell extracts were applied to the affinity matrix and allowed to react with the matrix overnight at 4°C. After extensively washing the matrix with column buffer, the RGD-binding polypeptides were eluted slowly with column buffer containing 1 mg/ml of GRGDSP peptide. The last wash fraction and the peptide eluted fractions were then analyzed by SDS-PAGE (7.5%) (22). Molecular mass markers were myosin (200 kD), β-galactosidase (116 kD), phosphorylase b (94 kD), BSA (67 kD), ovalbumin (43 kD), and carbonic anhydrase (30 kD). Bands were visualized by autoradiography.

Liposome Binding Assay

Liposomes prepared from the GRGDSP-eluted affinity chromatography fractions were purified by sucrose density centrifugation (7, 25) and subsequently assayed for binding to extracellular matrix proteins as described previously (7).

Immunoprecipitations

Immunoprecipitations of ^{125}I-radiolabeled cell extracts or column fractions was carried out using the appropriate antibodies and protein A-Sepharose as described previously (9). The antigen-antibody complexes were dissociated by boiling in sample buffer (200 mM Tris-HCl, pH 6.8 containing 3% SDS, 10% glycerol, and 0.001% bromophenol blue). Samples were analyzed by electrophoresis in 7.5% SDS-polyacrylamide gels followed by autoradiography (19).

Peptide Mapping

GRGDSPK-Sepharose affinity fractions and immunoprecipitates of ^{125}I-surface-labeled cell extracts were analyzed by SDS-PAGE under nonreducing conditions. Relevant bands were located on the dried gels by autoradiography, excised, rehydrated, and inserted into slots of a 15% acrylamide SDS-PAGE gel. The slots contained 0.5 μg/ml *Staphylococcus aureus* V8 protease in SDS-PAGE sample buffer containing 1 mM EDTA. The samples were electrophoresed into the stacking gel. Electrophoresis was suspended for 30 min to allow for enzymic digestion. Peptide bands were visualized by autoradiography as described above.

Results

Attachment of SK-N-SH and IMR-32 Human Neuroblastoma Cells to Various Extracellular Matrix Substrates

Attachment of SK-N-SH and IMR-32 cells to vitronectin, fibronectin, type I collagen, and laminin was determined by carrying out a cell attachment assay in protein-coated 96-well microtiter well plates as described in Materials and Methods. Fig. 1 (*A* and *B*) shows that both cell lines attached well to fibronectin and type I collagen, but showed poor attachment to laminin. SK-N-SH cells attached more strongly to vitronectin than did IMR-32 cells, which exhibited very poor attachment to this substrate (Fig. 1 *C*).

Isolation of Arg-Gly-Asp Receptors From SK-N-SH and IMR-32 Human Neuroblastoma Cells

^{125}I-cell surface-labeled cells were extracted in PBS containing 100 mM octylglucoside, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.2 mM PMSF and the extracts were applied to GRGDSPK-Sepharose columns. After extensively washing the columns, elution was carried out with GRGDSP peptide as described by Pytela et al. (24). Fig. 2 shows the autoradiograph of the eluted fractions after separation on a 7.5% SDS-polyacrylamide gel under nonreducing conditions. As can be seen, elution of the affinity matrix with GRGDSP peptide resulted in the elution of three radioactive polypeptides of relative molecular masses 135, 115, and 90 kD from both the SK-N-SH (Fig. 2, *top*) and IMR-32 (Fig. 2, *bottom*) cells. The 115-kD polypeptide eluted from the SK-N-SH cells is present in a much smaller amount than the 135- and 90-kD polypeptides. In the IMR-32 cells, however, the 90-kD band is the least abundant of the three. The 135- and 90-kD proteins correspond in terms of molecular mass to the α and β subunits of the vitronectin receptor isolated from MG-63 osteosarcoma cells by Pytela et al. (25). Also shown in Fig. 2, *bottom* lane 7 is an immunoprecipitate of an extract of ^{125}I surface-labeled IMR-32 cells with an anti-fibronectin

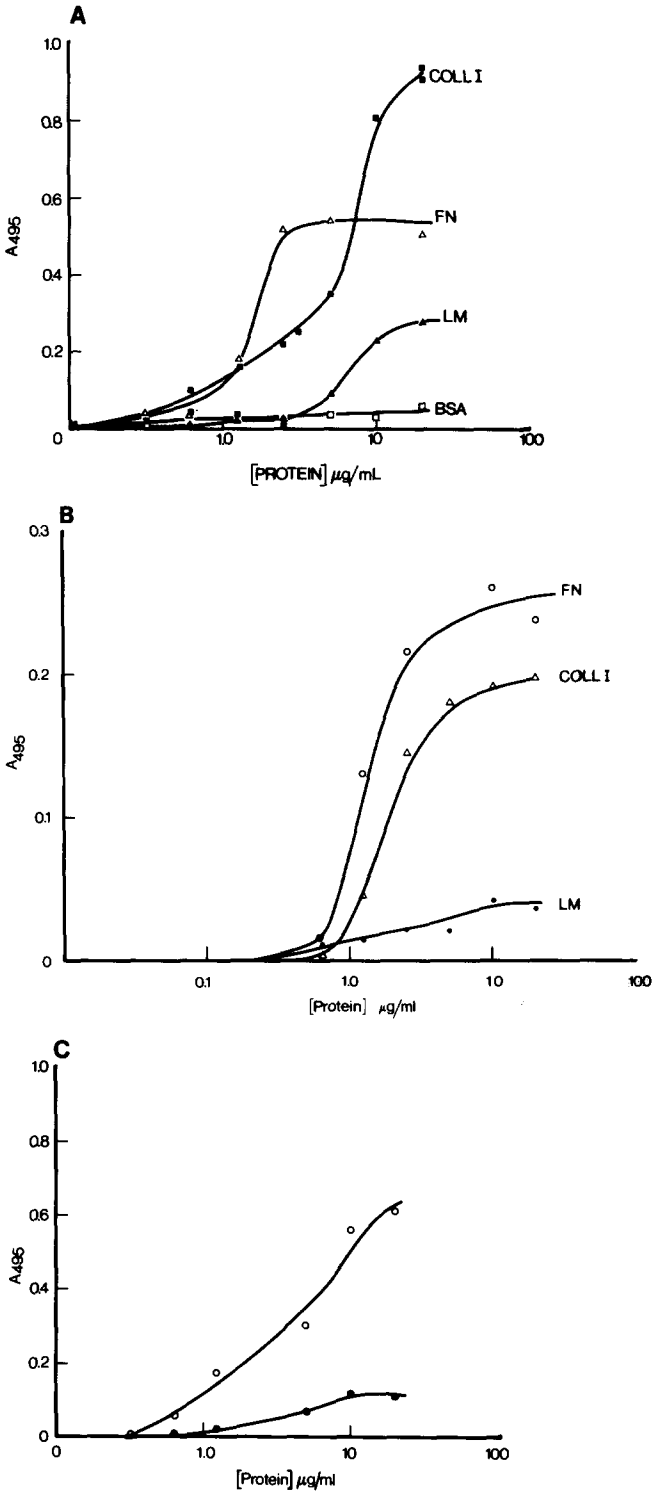

Figure 1. Attachment of SK-N-SH and IMR-32 cells to fibronectin (*FN*), type I collagen (*COLL*), laminin (*LM*) and vitronectin. Attachment assays were carried out as described in Materials and Methods. Cells (2×10^4) were plated into wells coated with increasing concentrations of the indicated ligand in the presence of 2.5 mg/ml BSA. The plates were incubated at 37°C for 1 h in an atmosphere of 7% CO_2. Nonattached cells were washed away with PBS, and the attached cells were fixed with 3% paraformaldehyde and stained with 0.5% toliudene blue in 3.7% paraformaldehyde. Cell attachment was measured as a function of absorbance of destained cells at 495 nM. (*A*) Attachment of SK-N-SH cells; (*B*) attachment of IMR-32 cells; (*C*) attachment of SK-N-SH cells. (○) and IMR-32 (●) to vitronectin.

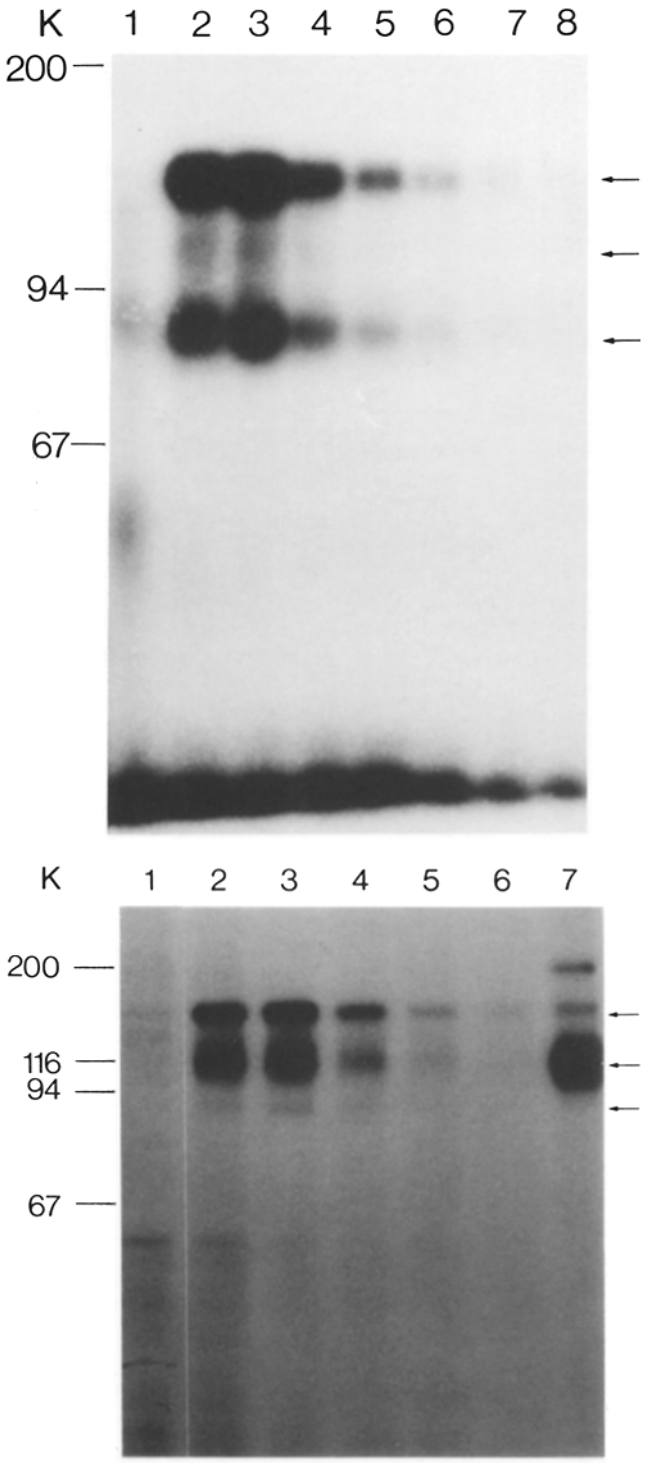

Figure 2. Affinity chromatography of ^{125}I surface-labeled SK-N-SH (*top*) and IMR-32 (*bottom*) octyglucoside cell extracts on GRGDSPK-Sepharose. Cells were surface labeled, extracted, and affinity chromatography carried out as described in Materials and Methods. In both *top* and *bottom*: lanes *1*, wash fraction; lanes *2–6*, GRGDSP (1 mg/ml) eluted fraction numbers 2–6. (*Bottom*) Lane *7*, immunoprecipitate of ^{125}I surface-labeled IMR-32 cells with polyclonal anti-fibronectin receptor antibody. Molecular mass markers indicated one myosin (200 kD), β-galactosidase (116 kD), phosphorylase b (94 kD), and BSA (67 kD). Arrows indicate the positions of the 135/115/90-kD polypeptides.

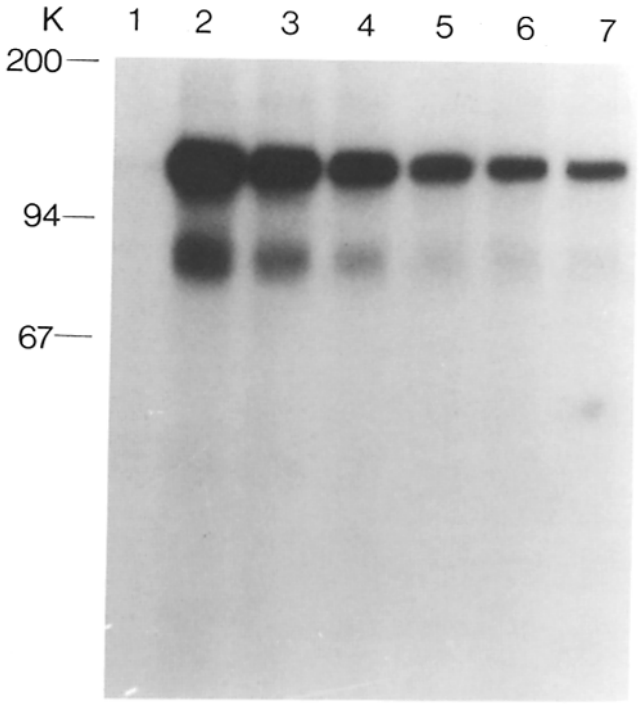

Figure 3. Affinity chromatography of ^{125}I surface-labeled COLO-205 octylglucoside cell extract on GRGDSPK-Sepharose. Affinity chromatography was carried out as described for Fig. 2. Lane *1*, wash fraction; lanes *2–6*, GRGDSP (1 mg/ml) eluted fraction numbers 2–6.

receptor antibody. This antibody immunoprecipitates the β_1 subunit and all α subunits associated with it. This lane is included to demonstrate that the 115-kD band from the RGD column migrates at the same position as the β_1 subunit from the same cells.

Affinity chromatography, in a similar manner, of extracts of a colon carcinoma cell line COLO-205, resulted in the elution of only two polypeptides of relative molecular mass 140 and 90 kD (Fig. 3), corresponding to the 135/90-kD polypeptides eluted from the neuroblastoma cell lines. The 115-kD Arg-Gly-Asp–recognizing polypeptide therefore appears to be present only in the two neuroblastoma cell lines.

Ligand Specificity of Arg-Gly-Asp Receptors Isolated from SK-N-SH, IMR-32, and COLO-205 Cells

To determine the ligand specificity of the Arg-Gly-Asp–recognizing cell surface polypeptides eluted from the GRGDSPK-Sepharose columns, the fractions containing the three radiolabeled polypeptides were pooled and incorporated into liposomes as described previously (7). Liposomes were also prepared from the wash fractions. The Liposomes were purified by sucrose density centrifugation (7, 25) and assayed by binding to protein coated microtiter well plates.

Fig. 4 *A* shows that the liposomes prepared from the fractions containing the 135/115/90-kD polypeptides of the IMR-32 cells bound to vitronectin, but surprisingly, bound also to type I collagen and to fibronectin. These liposomes did not bind to any significant extent to BSA or to tenascin. Liposomes prepared from the wash fractions (Fig. 4 *B*), which did not contain these three polypeptides, did not show

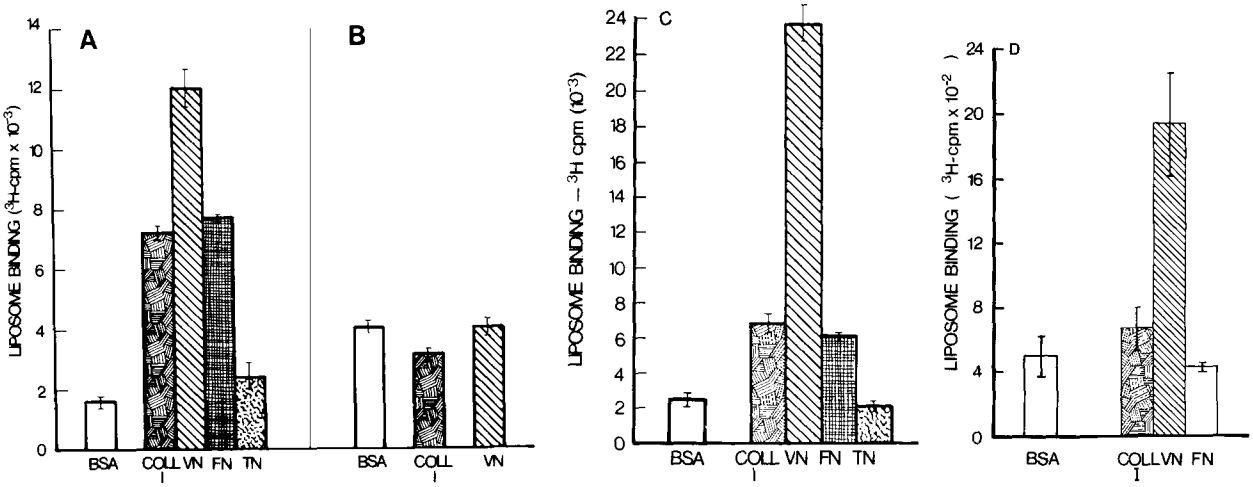

Figure 4. Specificity of binding of liposomes prepared from GRGDSP-eluted fractions of IMR-32 and SK-N-SH cells. Liposomes were prepared from either the wash fractions or peptide-eluted fractions. Liposomes were prepared by mixing [^{3}H]phosphatidylcholine with the appropriate protein containing fractions. Detergent was removed by dialysis against PBS for 24 h at 4°C, resulting in the formation of liposomes, which were purified through a sucrose density gradient (25), diluted in PBS containing 2.5 mg/ml BSA, and allowed to bind for 5 h at 4°C to microtiter plate wells previously coated with the indicated ligands. The supernatants were removed and the wells were washed twice with PBS. Bound liposomes were dissolved in 1% SDS and quantitated by liquid scintillation counting. (*A*) GRGDSP-eluted fractions from IMR-32 cells; (*B*) wash fractions; (*C*) GRGDSP-eluted fractions from SK-N-SH cells; (*D*) GRGDSP-eluted fractions from COLO-205 cells.

specific binding to vitronectin or type I collagen as compared to BSA.

Liposomes containing the 135/115/90-kD polypeptides from SK-N-SH neuroblastoma cells similarly bound specifically and strongly to vitronectin but also bound to type I collagen and fibronectin (Fig. 4 *C*). Liposomes prepared from the GRGDSP-eluted fractions from COLO-205 cells and containing only the 140- and 90-kD polypeptides showed specific binding only to vitronectin and did not bind to type I collagen or to fibronectin (Fig. 4 *D*).

Identity of the 135/115/90-kD Arg-Gly-Asp–recognizing Cell Surface Polypeptides of Neuroblastoma Cells

The relative molecular masses of the 135- and 90-kD polypeptides correspond to those of the α and β subunits of the vitronectin receptor (25, 27) when analyzed by SDS-PAGE under nonreducing conditions. The 115-kD polypeptide however has a relative molecular mass corresponding to the β subunit of the fibronectin receptor family of integrins (β_1) (30) (Fig. 2, *bottom*). The β_1 subunit has not as yet been shown to bind to short synthetic peptides containing the Arg-Gly-Asp sequence, but rather requires a much longer RGD-containing polypeptide (120-kD fibronectin fragment) (27, 30). To identify these polypeptides therefore, IMR-32 fractions containing them were pooled and immunoprecipitated with anti-fibronectin receptor polyclonal antibody, anti-β_1 monoclonal antibody, anti-β_3 monoclonal antibody, and anti-α_v polyclonal antibody. As shown in Fig. 5, anti-fibronectin receptor antibody immunoprecipitated all three polypeptides, anti-β_1 monoclonal antibody immunoprecipitated the 135- and 115-kD polypeptides only, the anti-β_3 monoclonal antibody immunoprecipitated the 135- and the 90-kD polypeptides only, and the anti-α_v monoclonal antibody immunoprecipitated all three polypeptides. None of the three polypeptides were immunoprecipitated with monoclonal antibodies against the VLA α subunits α_5 or α_3. Thus, on the basis of these results one can identify the 135-kD polypeptides as α_v, the 90-kD polypeptide as β_3, and the 115-kD polypeptide as β_1.

To determine whether the 115-kD polypeptide could bind to RGD in the absence of the vitronectin receptor α and β subunits (α_v, β_3), we depleted the complex of the α_v and β_3 subunits by immunoprecipitating with an anti-β_3 monoclonal antibody. The supernatant from this reaction was used for carrying out GRGDSP affinity chromatography.

As shown in Fig. 6 (lane *1*), the immunoprecipitate with anti-β_3 antibody contains 135- and 90-kD polypeptides. However, interestingly, the 115-kD polypeptide was still able to bind to the RGD column (Fig. 6, lane *3*).

To determine the ligand specificity of the α_v/β_1-like integrin, $\alpha_v\,\beta_3$ was depleted from the RGD-eluted fractions by repeated immunoprecipitation with an anti-β_3 monoclonal antibody, and liposomes were prepared from an anti-vitronectin receptor immunoprecipitate of the β_3-depleted supernatant. As shown in Fig. 7, this preparation contained only the 135- (α_v) and 115-kD (β_1-like) polypeptides. β_3 (90 kD) could not be detected in this preparation. Similar results were obtained if the β_3-depleted supernatant was immunoprecipitated with anti-β_1 monoclonal (AIIB2) (data not shown). Liposomes prepared from the anti-β_3 immunoprecipitate, containing α_v and β_3, bound specifically to vitronectin only (Table I), whereas liposomes prepared from the anti-vitronectin receptor immunoprecipitate of the supernatant, which contained α_v (135K) and the β_1-like (115 kD) polypeptides, bound only to fibronectin and collagen type I (Table I).

These results indicate that the three polypeptides isolated from IMR-32 and SK-N-SH cells by RGD affinity chromatography represent two integrin heterodimers, i.e., α_v (135

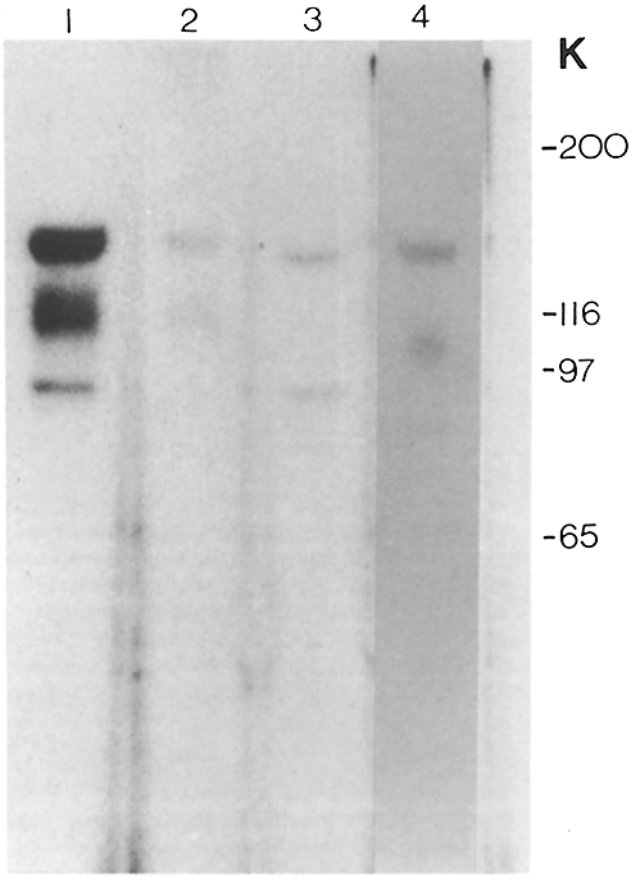

Figure 5. Immunoprecipitation of integrins from GRGDSP-eluted fractions of Arg-Gly-Asp affinity chromatography. Eluted fractions 1–3 were pooled and used for immunoprecipitation reactions, which were carried out using protein A-Sepharose as described previously (8). Immunoprecipitates were analyzed by SDS-PAGE under nonreducing conditions followed by autoradiography. Lane *1*, fractions 1–3 immunoprecipitated with rabbit anti-human fibronectin receptor antibody; lane *2*, fractions 1–3 immunoprecipitated with anti-β_1 monoclonal antibody; lane *3*, fractions 1–3 immunoprecipitated with anti-human β_3 monoclonal antibody; lane *4*, fractions 1–3 immunoprecipitated with rabbit anti-human α_v. Molecular mass markers used were similar to those used in Fig. 2.

kD)/β_3 (90 kD) and α_v (135 kD)/β_1-like (115 kD). In addition, although the 115-kD polypeptide exhibits epitopes recognized by the anti-β_1 antibodies, its behavior in terms of binding to RGD and association with α_v suggested that it may be different from authentic β_1. To thus further characterize this subunit, we immunoprecipitated the flow-through from the GRGDSPK-Sepharose column with an anti-β_1 monoclonal antibody. This flow-through contained a substantial amount of β_1 subunit, as shown in Fig. 8. This immunoprecipitate, and the three RGD-eluted polypeptides were analyzed by SDS-PAGE, the β_1 band from the immunoprecipitate and the 115-kD β_1-like band from the RGD-eluted material were excised from the gel, digested with V8 protease, and the resulting peptides were analyzed by SDS-PAGE as described in Materials and Methods.

As can be seen from Fig. 9 *A* the peptides generated from these two polypeptides are quite different, indicating that the 115-kD polypeptide that binds to RGD is different from the β_1 integrin subunit. Fig. 9 *B* shows V8 protease digests of

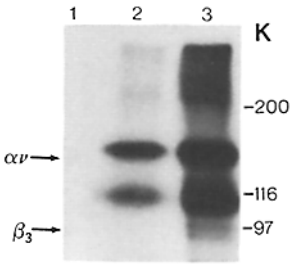

Figure 6. Ability of βn to bind to GRGDSPK-Sepharose in the absence of β_3. The three polypeptides eluted from a GRGDGPK-Sepharose column to which an octylglucoside extract of ^{125}I surface-labeled IMR-32 cells had been applied, was immunoprecipitated with an anti-β_3 monoclonal antibody. The supernatant from this reaction was then applied to another GRGDSPK-Sepharose column and eluted with GRGDSP. Lane *1*, immunoprecipitate with anti-β_3 monoclonal antibody; lane *2*, flow through of the second GRGDSP-Sepharose column to which the supernatant of the anti-β_3 immunoprecipitate had been applied; lane *3*, GRGDSP-eluted material from this second GRGDSPK-Sepharose column.

α_v isolated by immunoprecipitation of ^{125}I surface-labeled IMR-32 cell extracts and the α_v isolated from these cells by affinity chromatography, both of which give very similar peptide maps, although some minor differences are apparent. It is likely that the peptide mapping procedure used here tends to accentuate differences. However, the differences in the peptides generated from β_1 and βn are more obvious (Fig. 9 *A*), indicating that these two proteins are likely to be structurally distinct. A V8 protease digest of β_3 is also shown indicating that it is completely different from α_v, β_1 and the β_1-like polypeptides. To distinguish the β_1-like subunit from β_1 we have named the former βn.

Discussion

In this report we demonstrate the isolation from human neuroblastoma cells of three cell surface polypeptides capable of binding to the cell attachment promotion tripeptide, Arg-

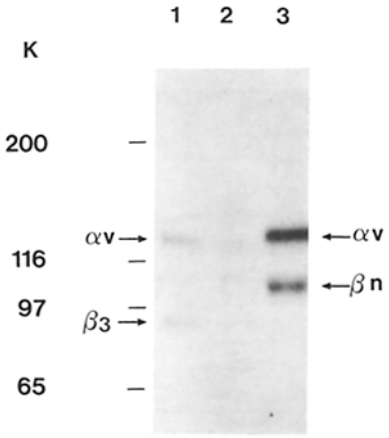

Figure 7. Purification of α_v β_3 and α_v βn from GRGDSP-eluted fractions. Fractions containing the 135 (α_v), 115 (βn), and 90-kD (β_3) polypeptides were pooled and depleted of α_v β_3 by two rounds of immunoprecipitation with an anti-β_3 monoclonal antibody. The supernatant was then immunoprecipitated with anti-vitronectin receptor antibody that immunoprecipitated α_v βn. Lanes *1* and *2*, anti-β_3 immunoprecipitates; lane *3*, anti-vitronectin receptor immunoprecipitate of β_3-depleted supernatant. These immunoprecipitates containing purified α_v β_3 (lane *1*) and α_v βn (lane *2*) were used to prepare liposomes described in Table I.

Table I. Specificity of Binding of Liposomes Containing the 135/90-kD (α_v/β_3) and the 135/115-kD (α_v/β_1-like) Polypeptides

Polypeptides	Liposome binding			
	BSA	Vitronectin	Fibronectin	Type I collagen
	[^{3}H]Phosphatidylcholine-cpm			
135/90 kD (α_v/β_3)	1,643 ± 103	5,889 ± 43	1,513 ± 113	1,828 ± 212
135/115 kD (α_v/β_1-like)	1,561 ± 152	1,411 ± 203	7,121 ± 82	7,561 ± 314

Liposomes were prepared from the anti-β_3 immunoprecipitate and anti-vitronectin immunoprecipitate of the supernatant from the eluted fractions from the RGD column containing the three polypeptides. As shown in Fig. 7 the anti-β_3 immunoprecipitate contained the 135- (α_v) and the 90-kD (β_3) polypeptides, and the anti-vitronectin receptor immunoprecipitate of the supernatant contained the 135- (α_v) and the 115-kD (β_1-like) polypeptides. Liposomes were prepared and the binding assay carried out as described for Fig. 4. The data represent mean and standard errors of four data points.

Gly-Asp. The neuroblastoma cell lines used, SK-N-SH and IMR-32, both attach to Arg-Gly-Asp-containing extracellular matrix proteins fibronectin, type I collagen, and vitronectin although the IMR-32 cells attached very poorly to vitronectin. We have demonstrated that these three cell surface polypeptides with relative molecular masses of 135, 115, and 90 kD bind to a GRGDSPK-sepharose matrix and can be specifically eluted with GRGDSP peptide in solution when analyzed under nonreducing conditions. The 90- and 135-kD polypeptides have been identified as β_3 and α_v integrin subunits, respectively, and appear to be identical to the human vitronectin receptor (24). The 115-kD polypeptide is recognized by polyclonal as well as monoclonal anti-β_1 antibodies and is coprecipitated with α_v. Western blot analysis has demonstrated that the polyclonal fibronectin receptor antibody used here recognizes β_1 and α_5 integrin subunits (1) (data not shown). However in immunoprecipitation experiments this antibody preparation does appear to have

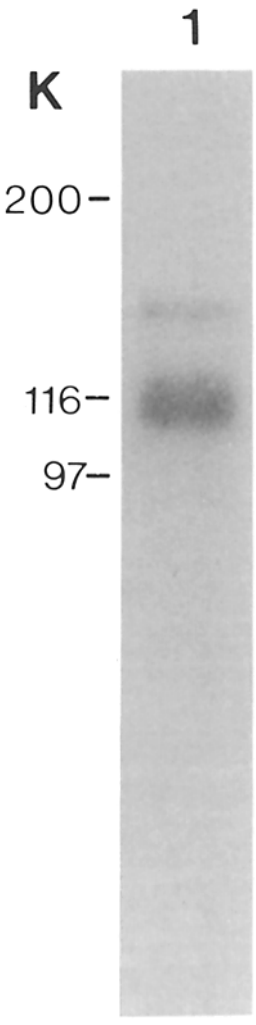

Figure 8. Identification of β_1 integrin subunit in the flow-through of a GRGDSPK-Sepharose column. The flow-through from a GRGDSPK-Sepharose column, to which an ocytyglucoside extract of ^{125}I surface-labeled IMR-32 cells had been applied, was immunoprecipitated with an anti-β_1 monoclonal antibody. The immunoprecipitated material was analyzed by SDS-PAGE under nonreducing conditions, followed by autoradiography.

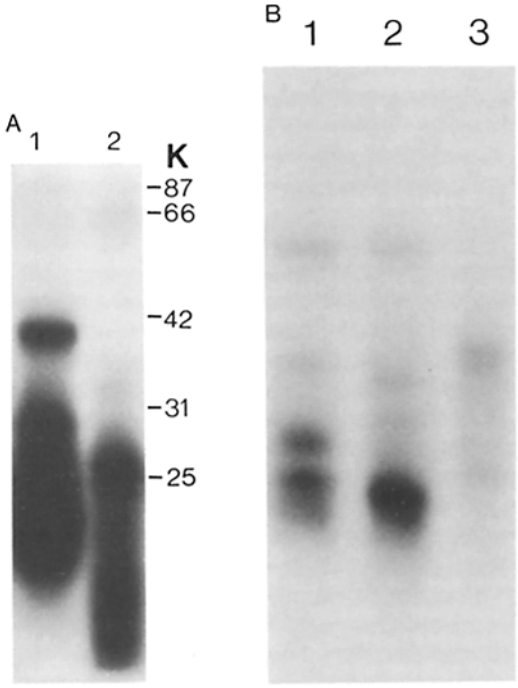

Figure 9. Peptide maps of *Staphylococcus aureus* V8 protease digests. (*A*) The β_1 band immunoprecipitated from the flow-through material of a GRGDSPK-Sepharose column to which an octylglucoside extract of ^{125}I surface-labeled IMR-32 cells had been applied (see Fig. 7) and the 115-kD βn band that bound to the same GRGDSPK-Sepharose column, were located on the gels by autoradiography excised from the gel, digested with *Staphylococcus aureus* V8 protease, and the resulting peptides analyzed on a 15% SDS-polyacrylamide gel as described in Materials and Methods. Lane *1*, V8 digest of β_1 immunoprecipitated from the flow-through of the GRGDSPK-Sepharose column; lane *2*, V8 protease digest of βn which bound to the GRGDSPK-sepharose column. (*B*) *Staphylococcus aureus* V8 protease digests of lane *1*, α_v immunoprecipitated from ^{125}I surface-labeled cell extract of IMR-32 cells; lane *2*, 135-kD polypeptide (α_v) isolated by RGD affinity chromatography; and lane *3*, 90-kD polypeptide (β_3).

vitronectin receptor cross-reacting antibodies in it (data not shown).

These results are consistent with the interpretation that the α_v integrin subunit in IMR-32 and SK-N-SH cells can associate with β_3 and also with a β_1-like subunit (βn). The ability of the β_1-like (115 kD) polypeptide to bind to RGD and associate with α_v suggests that it may be different from β_1. Indeed a comparison of the peptide maps of this and authentic β_1 demonstrated these two polypeptides to be distinct. This 115-kD RGD-binding subunit (βn) might be restricted in its expression to the neuroblastoma cell lines since this polypeptide could not be isolated from a colon carcinoma cell line, COLO-205, and has not been previously reported to be present on other nonneuroblastoma cell lines, e.g., MG-63 (24). It is interesting to note that the IMR-32 cells, which attach very poorly to vitronectin, also express very low levels of the 90-kD (β_3) polypeptide.

Liposomes prepared from fractions containing all three polypeptides bound to vitronectin as expected, but surprisingly, also bound to fibronectin and type I collagen. Liposomes prepared from GRGDSP-eluted fractions after affinity chromatography of COLO-205 cells extracts showed significant binding to vitronectin only. These fractions consisted only of the 135- and 90-kD polypeptides corresponding to α_v and β_3 integrin subunits, but lacked βn present in the SK-N-SH and IMR-32 neuroblastoma fractions. These results suggest that the binding of the neuroblastoma liposomes to fibronectin and type I collagen may be mediated by the 115-kD polypeptide. This interpretation is also supported by the finding that liposomes prepared from α_v βn, after depletion of β_3, mediated binding to fibronectin and type I collagen only.

Although distinct receptor complexes showing exclusive binding specificities for fibronectin, type I collagen, and vitronectin have been isolated from human placenta as well as other cell lines (7, 24, 25, 27), receptor complexes binding to several different proteins in an RGD-dependent manner are also expressed by some cell types. The glycoprotein IIb/IIIa present on platelets as well as an endothelial cells binds to at least four different proteins in an RGD-dependent manner (26), as does the chicken integrin CSAT antigen, which binds to fibronectin, vitronectin, laminin, and collagen (16). Human cells also express a β_1 integrin complex (VLA-3), which also has a broad ligand specificity since it can bind to fibronectin, collagen, and laminin (14).

The data presented here show that α_v can associate with more than one β subunit in the same cell. In the neuroblastoma cells examined here, α_v is found to associate with β_3 and this complex binds exclusively to vitronectin, as has been described previously by others. However in these cells, it is also found to be associated with a novel subunit βn, which is immunologically related to the β_1 integrin subunit but appears to be structurally distinct based on peptide maps. This α_v/βn integrin mediates binding to fibronectin and type I collagen.

Recently, two other examples of association of alternate β subunits with α_v have been described (4, 10). Thus α_v associates with β_x in carcinoma cells (4), and an alternate, phosphorylated β subunit (β_s) has been shown to associate with α_v in MG-63 osteosarcoma cells (10). Although β_x and β_s may be closely related or be identical since their relative molecular masses are similar, it is unlikely that βn is related to β_x. The relative molecular masses of βn and β_x are quite different when analyzed by SDS-PAGE under nonreducing conditions, 116 kD for βn versus 97 kD for β_x. Furthermore, whereas βn cross-reacts with anti-fibronectin receptor antibodies as well as anti-β_1 monoclonal antibody (AIIB2), β_x is not recognized by anti-β_1 antibodies (4). Thus, immunologically at least, βn is more closely related to β_1 than it is to β_x. However, a direct comparison of βn, β_x, and β_1 will be required to determine the relationship between these integrin subunits.

In conclusion, we have isolated from two human neuroblastoma cell lines a novel RGD-dependent integrin complex consisting of two heterodimers, α_v/β_3 and α_v/βn, the former mediating binding to vitronectin, and the latter to fibronectin and type I collagen. This complex contains a novel β_1-like integrin subunit, which we have called βn. Future studies will concentrate on elucidating the functional role of this complex on neuroblastoma cells.

We would like to thank Linda Wood for preparing the manuscript.

S. Dedhar acknowledges the support of the National Cancer Institute of Canada and the British Columbia Health Care Research Foundation.

Received for publication 16 November 1989 and in revised form 19 January 1990.

References

1. Argraves, W. S., S. Suzuki, H. Arai, K. Thompson, M. D. Pierschbacher, and E. Ruoslahti. 1987. Amino acid sequence of the human fibronectin receptor. *J. Cell Biol.* 105:1183–1190.
2. Bronner-Fraser, M. 1986. Distribution of the putative cell surface receptor for fibronectin and laminin in the avian embryo. *J. Cell Biol.* 103:1061–1071.
3. Calof, A. L., and L. F. Reichardt. 1985. Response of purified chick motoneurons to myotube conditioned medium: laminin is essential for the substratum binding neurite-outgrowth promoting activity. *Neurosci. Lett.* 59:183–189.
4. Cheresh, D., J. W. Smith, H. M. Cooper, and V. Quaranta. 1989. A novel vitronectin receptor integrin ($\alpha_v\beta_x$) is responsible for distinct adhesive properties of carcinoma cells. *Cell.* 57:59–69.
5. Davis, G. E., M. Manthorpe, E. Engvall, and S. Varon. 1985. Isolation and characterization of rat schwannoma neurite-promoting factor: evidence that the factor contains laminin. *J. Neurosci.* 5:2662–2671.
6. Davis, G. E., S. N. Blaker, E. Engvall, S. Varon, M. Manthorpe, and F. Gage. 1987. Human amnion membrane serves as a substratum for growing axons in vitro and in vivo. *Science (Wash. DC).* 236:1106–1109.
7. Dedhar, S., E. Ruoslahti, and M. D. Pierschbacher. 1987. A cell surface receptor complex for type I collagen recognizes the Arg-Gly-Asp sequence. *J. Cell Biol.* 104:585–593.
8. Dedhar, S., W. S. Argraves, S. Suzuki, E. Ruoslahti, and M. D. Pierschbacher. 1987. Human osteosarcoma cells resistant to detachment by an Arg-Gly-Asp-containing peptide overproduce the fibronectin receptor. *J. Cell Biol.* 105:1175–1182.
9. Dedhar, S., C. Haqq, and V. Gray. 1989. Specific overproduction of VLA-1 integrin in two human neuroblastoma cell lines selected for resistance to detachment by an Arg-Gly-Asp containing synthetic peptide. *J. Biol. Chem.* 264:4832–4836.
10. Freed, E., J. Gailit, P. van der Geer, E. Ruoslahti, and T. Hunter. 1989. A novel integrin β subunit is associated with the vitronectin receptor α subunit (α_v) in a human osteosarcoma cell line and is a substrate for protein kinase C. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:2955–2965.
11. Gehlsen, K. R., L. Dillner, E. Engvall, and E. Ruoslahti. 1988. The human laminin receptor is a member of the integrin family of cell adhesion receptors. *Science (Wash. DC).* 241:1228–1229.
12. Halfter, W., and S. Diers. 1986. Axonal pathofinding in organ-cultured embryonic avian retinae. *Dev. Biol.* 114:296–310.
13. Hall, D. E., K. M. Neugebauer, and L. F. Reichardt. 1987. Embryonic neural retinal cell response to extracellular matrix proteins: developmental changes and effects of CSAT antibody. *J. Cell Biol.* 104:623–634.
14. Hemler, M. 1988. Adhesive protein receptors on hematopoietic cell. *Immunol. Today.* 9:109–113.
15. Hemler, M. E., C. Crouse, and A. Sonnenberg. 1989. Association of the VLA α^6 subunit with a novel protein. A possible alternative to the common VLA β_1 subunit on certain cell lines. *J. Biol. Chem.* 264:6529–6535.
16. Horwitz, A., K. Duggan, R. Greggs, C. Decker, and C. Buck. 1985. The

cell substrate attachment (CSAT) antigen has properties of a receptor for laminin and fibronectin. *J. Cell Biol.* 101:2134–2144.
17. Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell.* 48:549–554.
18. Kajiji, S., R. N. Tamura, and V. Quaranta. 1989. A novel integrin ($\alpha_E\beta_4$) from human epithelial cells suggests a fourth family of integrin adhesion receptors. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:673–680.
19. Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage, T4. *Nature (Lond.).* 227:680–685.
20. Larder, A. D., D. K. Fijii, and L. F. Reichardt. 1985. Laminin is associated with the neurite outgrowth-promoting factors found in conditioned media. *Proc. Natl. Acad. Sci. USA.* 82:2183–2187.
21. Larder, A. D., D. K. Fijii, and L. F. Reichardt. 1985. Purification of a factor that promotes neurite outgrowth: isolation of laminin and associated molecules. *J. Cell Biol.* 101:893–913.
22. Lebien, T. E., D. R. Bove, J. C. Bradley, and J. H. Kersey. 1982. Antibody affinity may influence antigenic modulation of the common acute lymphoblastic leukemia antigen in vitro. *J. Immunol.* 129:2287–2292.
23. Pierschbacher, M. D., and E. Ruoslahti. 1984. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature (Lond.).* 309:30–33.
24. Pytela, R., M. D. Pierschbacher, and E. Ruoslahti. 1985. A 125/115 Kd cell surface receptor specific for vitronectin interacts with the Arg-Gly-Asp adhesion sequence derived from fibronectin. *Proc. Natl. Acad. Sci. USA.* 82:5766–5770.
25. Pytela, R., M. D. Pierschbacher, and E. Ruoslahti. 1985. Identification and isolation of a 140 Kd cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell.* 40:191–198.
26. Pytela, R., M. D. Pierschbacher, M. H. Ginsberg, E. F. Plow, and E. Ruoslahti. 1986. Platelet membrane glycoprotein IIb/IIIa: member of a family of Arg-Gly-Asp-specific adhesion receptors. *Science (Wash. DC).* 231:1559–1562.
27. Pytela, R., M. D. Pierschbacher, S. Argraves, S. Suzuki, and E. Ruoslahti. 1987. Arginine-glycine-aspartic acid adhesion receptors. *Methods Enzymol.* 144:475–489.
28. Rettig, W. J., V. V. V. S. Murty, M. J. Mattes, R. S. K. Chaganti, and L. Old. 1986. Extracellular matrix-modulated expression of human cell surface glycoproteins A42 and J143. Intrinsic and extrinsic signals determine antigenic phenotype. *J. Exp. Med.* 164:1581–1599.
29. Rogers, S. L., K. J. Edson, P. C. Letourneau, and S. C. McLoon. 1986. Distribution of laminin in the developing peripheral nervous system of the chick. *Dev. Biol.* 113:429–435.
30. Ruoslahti, E., and M. D. Pierschbacher. 1987. New perspectives in cell adhesion: RGD and integrins. *Science, (Wash. DC).* 238:491–497.
31. Suzuki, S., W. S. Argraves, H. Arai, L. R. Languino, M. D. Pierschbacher, and E. Ruoslahti. 1987. Amino acid sequence of the vitronectin receptor: subunit and comparative expression of adhesion receptor mRNAs. *J. Biol. Chem.* 262:14080–14085.
32. Tomaselli, K. J., L. F. Reichardt, and J. L. Bixby. 1986. Distinct molecular interactions mediate neuronal process outgrowth on non-neuronal cell surfaces and extracellular matrices. *J. Cell Biol.* 103:2659–2672.
33. Tomaselli, K. J., C. H. Damsky, and L. F. Reichardt. 1987. Interactions of a neuronal cell line (PC12) with laminin, collagen IV, and fibronectin: identification of integrin-related glycoproteins involved in attachment and process outgrowth. *J. Cell Biol.* 105:2347–2358.