Receptor Functions for the Integrin VLA-3: Fibronectin, Collagen, and Laminin Binding Are Differentially Influenced by ARG-GLY-ASP Peptide and by Divalent Cations

Mariano J. Elites, Lisa A. Urry, and Martin E. Hemler
Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115

Abstract. The capability of the integrin VLA-3 to function as a receptor for collagen (Coll), laminin (Lm), and fibronectin (Fn) was addressed using both whole cell adhesion assays and ligand affinity columns. Analysis of VLA-3-mediated cell adhesion was facilitated by the use of a small cell lung carcinoma line (NCI-H69), which expresses VLA-3 but few other integrins. While VLA-3 interaction with Fn was often low or undetectable in cells having both VLA-3 and VLA-5, NCI-H69 cells readily attached to Fn in a VLA-3-dependent manner. Both Arg-Gly-Asp (RGD) peptide inhibition studies, and Fn fragment affinity columns suggested that VLA-3, like VLA-5, may bind to the RGD site in human Fn. However, unlike Fn, both Coll and Lm supported VLA-3-mediated adhesion that was not inhibited by RGD peptide, and was totally unaffected by the presence of VLA-5. In addition, VLA-3-mediated binding to Fn was low in the presence of Ca++, but was increased 6.6-fold with Mg++, and 30-fold in the presence of Mn++. In contrast, binding to Coll was increased only 1.2-fold with Mg++, and 1.7-fold in Mn++, as compared to the level seen with Ca++. Together, these experiments indicate that VLA-3 can bind Coll, Lm, and Fn, and also show that (a) VLA-3 can recognize both RGD-dependent and RGD-independent ligands, and (b) different VLA-3 ligands have distinctly dissimilar divalent cation sensitivities.

Members of the integrin family of cell adhesion receptors are of major importance in mediating cell attachment to extracellular matrix proteins (2, 24, 31, 46). The integrins are membrane-spanning heterodimers, which have been divided into three major subfamilies, each containing a common β subunit associated with multiple α subunits. Adding further complexity to the organization of integrins, at least three examples of α subunits associating with more than one β subunit have recently been described (6, 15, 28, 29, 34, 56). Currently, there are at least 15 different integrin αβ subunit combinations, and considerable effort has been directed towards elucidating their functional characteristics. Among the integrins in the β1 subunit family (VLA proteins), VLA-5 and VLA-4 interact with fibronectin (Fn), VLA-6 binds to laminin (Lm), and VLA-1 and VLA-2 each recognize both collagen (Coll) and Lm (24). The VLA-3 structure is especially versatile having been implicated as a receptor for Fn, Coll, and Lm (19, 57).

VLA-3 was originally described as an antigen (140,000, 120,000, and 30,000 M₁) recognized by the mAb 343, and present on nearly all cultured cell lines except for lymphoid cells (14). On normal tissue, expression was limited to a few cell types, including kidney glomeruli, and the basal cells of epidermis and other epithelia (3, 14, 39, 41). Subsequent biochemical characterization of the α and β subunits (150,000/110,000 M₁, nonreduced) of VLA-3 established that it is a heterodimer belonging to the VLA/integrin family (26, 53). The cDNA sequence for α₁ from hamster (55), and partial sequence from chicken (32) is highly similar (~90%) to the human α₁ sequence (Takada, Y., E. Murphy, E. Pil, C. Chen, M. H. Ginsberg, M. E. Hemler, manuscript submitted for publication), but it is not closely related to any other published integrin α subunit sequence, though clearly resembling α subunits in general.

Evidence for VLA-3-mediated matrix adhesion functions was obtained when cell attachment to Coll and Fn was blocked by a mAb that recognized an αβ subunit complex (57) identical to VLA-3 (54). VLA-3 may also play a role in cell-cell adhesion, since (a) VLA-3 expression has been noted around the periphery of epidermal skin cells (3, 41), (b) immunoelectron microscopy has revealed VLA-3 at sites of intercellular contact (35), and (c) an anti-VLA-3 antibody inhibited homotypic cell-cell adhesion of a keratinocyte cell line (3). Also, VLA-3 has recently been implicated as being one of several integrin receptors for the bacterial protein invasin, which promotes bacterial penetration through mammalian cell membranes (33).

At present there is incomplete and sometimes conflicting evidence concerning possible ligands for VLA-3. VLA-3 was...
initially proposed to be a Fn receptor based on (a) binding to a Fn affinity column (at low salt concentrations), and (b) 80% blocking of HT1080 cell adhesion to Fn by an anti-VLA-3 mAb (57). However, subsequent studies showed only 30% inhibition of HT1080 cell attachment (58), and variable 0–65% inhibition of attachment by keratinocyte cell lines (3). In biochemical studies, purified VLA-3 was shown to bind to Fn-coated plastic (19). However, the original isolation of Fn receptors from MG63 cells (43), and from placenta (44) using Fn affinity columns, yielded exclusively VLA-5 (not VLA-3), despite the large amounts of VLA-3 present in those sources (27, 53). Other laboratories have also reported VLA-5 binding to immobilized Fn, but neither rat (42), chicken (32), nor human (4, 32) VLA-3 was found to bind to Fn affinity columns.

The putative interaction of VLA-3 with Coll has also been supported by conflicting and variable results. Whereas an early study showed that anti-VLA-3 mAbs blocked HT1080 cell attachment to Coll types I and VI by nearly 100% (57), later experiments reported either 50% inhibition (58), or no inhibition of cell adhesion (3) (also see Table I below). In similar fashion, human VLA-3 from HT1080 cells bound to immobilized Coll types I and VI (57), but VLA-3 from melanoma cells did not bind (37), and in a radiolabeled receptor assay, VLA-3 binding to Coll I and IV was either absent or weak (19).

Ample support for VLA-3 binding to Lm has been obtained in Lm affinity column experiments (11, 18), and in radiolabeled receptor binding experiments (19). In particular, VLA-3 appears to attach to a globular domain at the end of the Lm long arm (19), perhaps near the carboxyl terminus of the Lm BI subunit. However, in assays involving whole cells, variable results were obtained. Anti-VLA-3 antibodies inhibited Lm attachment by 50–70% for one cell line, but only 10–20% for another (3), and in general anti-VLA-3 mAbs have been ineffective as inhibitors of cell attachment to Lm (11); Table I, below.

Considering that the evidence for VLA-3 binding to various ligands is variable and often not observed, it is surprising that little information is available regarding potential sites of attachment. For example, it had not been determined if VLA-3 attached to Fn at the RGD site, like VLA-5 (not VLA-3), despite the large amounts of VLA-3 in biochemical studies, and in radiolabeled receptor assays, VLA-3 binding to Coll I and IV was either absent or weak (19).

Materials and Methods

Materials and Methods

Cell Lines and Reagents

The small cell lung carcinoma line NCI-H69 was obtained from the American Type Culture Collection (Rockville, MD), and the human melanoma line LOX from Dr. L. B. Chen (Dana-Farber Cancer Institute). All the cell lines listed in Table I were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Collagens type I and IV, human plasma fibronectin, and chymotryptic fragments of fibronectin (i.e., the collagen-binding peptide Fn-45, the cell-binding fragment Fn-120, and the heparin II-binding peptide Fn-40), as well as the hexapeptides GRGESP and GRGDSP, were all purchased from Telios Pharmaceuticals (La Jolla, CA). Laminins from the Engelbreth-Holm-Swarm murine tumor was a gift from H. Kleiman (National Institute of Dental Research, Bethesda, MD). Coupling of polypeptides to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) was carried out according to the manufacturer's instructions, and the amount of ligand incorporated to Sepharose beads was estimated by the difference in absorbance at 280 nm of the supernatant before and after coupling.

mAbs and specific antisera used throughout this study are as follows: 12F1 (anti-α1), TS2/7 (anti-α2), J143 (anti-α3), B-5G10 (anti-α4), A-IAS (anti-β1), and J2-2A2 (control) were obtained as described previously (26, 27). Anti-VLA-2 mAb SEB (60) was from R. Bankert (Roswell Park Memorial Institute, Buffalo, NY); anti-VLA-3 mAb P5B (54, 57), and anti-VLA-5 mAb P1D6 (58) were from E. Wayner (University of Washington, Seattle, WA); GoH3, a mAb against VLA-6 (49) was from A. Sonnenberg (Netherlands Cancer Institute, Amsterdam, Holland); and rat mAb B1Ell (anti-β3) was from C. Damsky (University of California San Francisco, San Francisco, CA). A rabbit antiserum to the COOH-terminal cytoplasmic tail of VLA-6 was a gift from S. Argraves (American Red Cross, Rockville, MD). The anti-α2 mAb LM142 (5) was from D. Cheresh (Scripps Clinic, La Jolla, CA), and the anti-α4 mAbs 13C2 and 23C6 (7) were from M. Horton (Imperial Cancer Research Fund, London, England). An anti-β1 mAb, called mAb 55 (16), was from M. Ginsberg (Scripps Clinic, La Jolla, CA), and rabbit antiserum to the COOH-terminal cytoplasmic tail of β3 was generated as previously described (45).

Flow Cytometry

Cells (NCI-H69 and LOX) were washed with PBS containing 1% BSA and 2% human serum (HS; Gibco Laboratories, Grand Island, NY), and preincubated in the same buffer (PBS/BSA/HS) for 30 min at 4°C. Next, 2–3 × 10⁶ cells aliquots were treated individually with saturating concentrations of mAbs specific for VLA-α (α-α′) and VLA-β subunits (see previous paragraph) in PBS/BSA/HS for 45 min at 4°C. Cells were washed three times with PBS/BSA/HS, subsequently incubated with goat anti-mouse IgG coupled to fluorescein (Cappel Laboratories, Malvern, PA; at a 1:30 dilution of stock) for 45 min at 4°C; washed three times as above, and finally resuspended in PBS/BSA/HS. Fluorescein-labeled cells were analyzed using a FACScan apparatus (a registered trademark of Becton Dickinson & Co., Oxnard, CA).

Cell Attachment and mAb Inhibition Assays

To assess cell attachment to extracellular matrix proteins, 5 × 10⁶ cells were incubated with 5 Cr (0.5 μCi; 1 Ci = 37 GBq) for 6 h at 37°C, washed sequentially with PBS, followed by 1 mM EDTA in PBS, and finally resuspended in serum-free RPMI 1640 supplemented with 1% BSA (RPMI/BSA). Then cells were resuspended in RPMI/BSA and plated in triplicate (5 × 10⁴ cells/well) for 20–30 min at 37°C on 96-well microtiter dishes (0.6 cm diameter per well) that had been previously incubated with Coll type I, Lm, or Fn (1 μg/well). After unbound cells were aspirated and the plates were washed three times with RPMI/BSA, 1 Cr present in 0.1% SDS cell lysates was measured using a gamma counter.

For inhibition experiments, dilutions of inhibitory mAb (1–5 μg/well) were added to ligand-coated microtiter plates before addition of labeled cells, and adhesion assays were conducted exactly as described above.

To analyze divalent cation sensitivity of VLA-3-mediated adhesion, cells were first washed with 1 mM EDTA in PBS to deplete extracellular levels of preexisting divalent metals. Subsequently, cells were equilibrated separately with 1 mM each of either CaCl₂, MgCl₂, or MnCl₂ in 20 mM Tris, pH 7.4, 135 mM NaCl, 5 mM KCl, 2 mM glutamine, 1.8 mM glucose, and 1% BSA, and plated on ligand-coated dishes to carry out adhesion assays as indicated above.

Specific attachment of cells to extracellular matrix proteins was the average of at least three separate experiments, and was expressed as cells bound per mm² (area of microtiter plate: 28 mm²) ± SD. Background binding of radiolabeled cells to BSA-coated control wells was typically <0.5% of total input counts (or <10 cells/mm²) and was subtracted during calculation of specific attachment.

Affinity Chromatography of Cell Extracts on Immobilized Ligand Columns

LOX melanoma cells were freshly detached from tissue culture plastic
flasks with 0.03% EDTA in PBS, washed twice with PBS, and surface proteins (1-2 x 10^6 cells) were iodinated with 1 μCi of Na^125I (Dupont-NEN Products, Wilmington, DE) and 0.2 mg/ml of lactoperoxidase (Sigma Chemical Co., St. Louis, MO). ^125I-labeled cells were solubilized with 100 mM octylglucoside (Sigma Chemical Co.) and 100 mM octylthioglucoside (Calbiochem-Behring Corp., San Diego, CA) in a buffer containing 0.1 mM MnCl₂, 10 μg/ml aprotinin, 10 mM leupeptin, and 1 mM PMSF (buffer A) for 4 h at 4°C. After centrifugation at 10,000 rpm, supplemented with 0.5 mg/ml BSA as a carrier protein, and loaded onto ligand-Sepharose columns (2 ml of packed gel with 1-5 mg of ligand protein/ml of beads) equilibrated with 25 mM octylglucoside and 25 mM octylthioglucoside in buffer A (buffer B). After the iodinated cell extract was allowed to interact with the immobilized ligand for a minimum of 4 h, the column was washed extensively with buffer B until the radioactivity of the eluate decreased to background levels (20 column volumes). Subsequently, stepwise elution was carried out first with buffer B supplemented with 0.5 M NaCl (20 column volumes), followed by 10 mM EDTA in buffer B (10 column volumes), and finally 4 M urea in buffer B (10 column volumes). Alternatively, ligand-Sepharose columns were eluted with 2 column volumes each of 1 mM GRGDSP followed by 1 mM GRGDSP, both dissolved in buffer B. The affinity supports could be reused a few times after immediate reequilibration in buffer B. Eluted fractions were collected (0.8 ml each), and corresponding aliquots were analyzed for radioactivity using a gamma counter (Packard Instrument Co., Inc., Downers Grove, IL).

Immunoprecipitation with Specific mAbs and Antisera

Selected fractions from the affinity chromatography separations were immunoprecipitated as previously described (26). Briefly, aliquots containing the same amount of radioactivity (cpm) were taken from column fractions and incubated with control mAb (J-2A2) and Staphylococcus aureus Cowan 1 strain (Calbiochem-Behring Corp.) for 2 h at 4°C. After centrifugation of the nonspecifically bound immune complexes, the resulting supernatant was incubated with either 1 μg of specific mAb or, alternatively, 5 μl of specific antisera, followed by addition of a 10% suspension of S. aureus Cowan 1 (50 μl) for 2 h at 4°C. Adsorbed immune complexes were then washed four times with PBS containing 0.5% NP-40, 0.5% deoxycholate, and 0.02% azide, and subsequently suspended in SDS-PAGE sample buffer supplemented with or without 5% 2-mercaptoethanol. After separation of polypeptides by SDS-PAGE (5% polyacrylamide), gels were dried and iodinated with or without 5% 2-mercaptoethanol. After separation of protein bands were detected by exposing the gels to x-ray sensitive film (Kodak) for up to 4 wk. Molecular mass standards were: myosin (200 kD), beta-galactosidase (116 kD), phosphorylase b (92.5 kD), and BSA (67 kD).

Immunofluorescent Staining

Cells growing on Fn- or Coll-type I-coated coverslips (for 4-6 h) were fixed with 37% paraformaldehyde for 20 min, permeabilized with 0.5% NP-40 for 10 min, and stained with either the anti-VLA-3 mAb J143 (14), or the anti-VLA β mAb A-1A5 (25), or polyclonal antibody to the COOH-terminal intracytoplasmic tail of VLA α. Since none of these antibodies are known to block integrin binding to extracellular matrix ligands, they were judged to be suitable for staining receptors involved in focal contacts. Rhodamine-conjugated goat anti-mouse or goat anti-rabbit IgG was used as secondary antibody (1:200-1:300 dilution of stock). Representative fields of cells were photographed through a Planapo 63× oil immersion lens on a Zeiss Axiopt microscope equipped for epifluorescence. Some cells were also photographed using differential interference contrast microscopy to compare the distribution of cells to that of immunofluorescent staining.

Results

VLA-3-mediated Matrix Adhesion Is Not Apparent on Most Adherent Cell Lines

To assess the functional role of VLA-3, the best available anti-VLA-3 blocking reagent (mAb PIB5) was tested for inhibition of cell adhesion to Coll, Fn, and Lm. Typical results, shown in Table I, indicated that for most cell lines analyzed, the anti-VLA-3 mAb PIB5 had little or no inhibitory effect on cell attachment to Coll, Fn, or Lm. In contrast, cell adherence was almost completely abrogated by an anti-VLA β subunit mAb (B1Ell) for all matrix proteins tested. Thus, while β integrins were clearly involved in binding to Coll, Fn, and Lm, VLA-3-mediated matrix adhesion, if any, may have been overshadowed by the contribution of other VLA receptors.

To simplify analysis of VLA-3 function, a VLA-3-positive cell line was sought which expressed few other VLA proteins. As shown in Fig. 1 A, the small cell lung carcinoma line NCI-H69 expressed a very unusual VLA protein phenotype consisting of a moderate amount of VLA-3 and VLA-6, but no VLA-1, VLA-2, VLA-4, or VLA-5. Also, there was no detectable α (assessed using three different antibodies), β₁, or β₃ on NCI-H69 cells (not shown). Thus, in the ab-

| Cell line | Collagen type I | Fibronectin | Laminin |
|-----------|----------------|-------------|---------|
|           | PIB5 | B1Ell | PIB5 | B1Ell |
| 76N       | 120 ± 11 | 2 ± 0.2 | 99 ± 10 | 16 ± 1.5 |
| CCL-228   | 110 ± 8 | 1 ± 0.1 | 97 ± 8 | 12 ± 1 |
| EJ        | 107 ± 10 | 7 ± 0.5 | 96 ± 5 | 5 ± 0.3 |
| J82       | 122 ± 12 | 2 ± 0.2 | 108 ± 9 | 5 ± 0.4 |
| LOX       | 97 ± 5  | 5 ± 0.5  | 105 ± 6 | 4 ± 0.3 |
| MRC-5     | 104 ± 7 | 4 ± 0.4 | 103 ± 9 | 10 ± 0.9 |
| RD        | 97 ± 8  | 5 ± 0.4  | 95 ± 6  | 3 ± 0.2 |
| SK-N-SH   | 98 ± 6  | 4 ± 0.2  | 99 ± 10 | 9 ± 0.6 |
| ZR-T      | 102 ± 10 | 2 ± 0.2  | 115 ± 9 | 26 ± 2 |
| HT1080    | 112 ± 10 | 3 ± 0.2  | 98 ± 6  | 5 ± 0.4 |

The numbers shown represent cell attachment as a percentage of that observed in the presence of the control mAb J-2A2. Other mAb used were PIB5 (anti-VLA-3) and B1Ell (anti-VLA-3) for all matrix proteins tested.Attachment assays were carried out as described in Materials and Methods, and (–) indicates not done. The level of uninhibited binding ranged from 500 to 1,500 cells/mm² for the various cell lines, and background adhesion to BSA-coated controls was typically <10 cells/mm².
Figure 1. Cell surface expression of VLA proteins. Flow cytometry analysis was performed on small cell lung carcinoma cells NCI-H69 (A), and LOX melanoma cells (B). Saturating concentrations of the following mAbs were used for staining (see Materials and Methods): J-2A2 (control), TS2/7 (anti-VLA-1), SE8 (anti-VLA-2), J143 (anti-VLA-3), B-5G10 (anti-VLA-4), P1D6 (anti-VLA-5), GoH3 (anti-VLA-6), and A-1A5 (anti-VLA-β).

In contrast, the melanoma cell line LOX expressed a VLA protein phenotype that is representative of many adherent cell lines (Fig. 1 B). The VLA-3, VLA-5, and VLA-6 proteins were expressed at comparably high levels, and VLA-2 was present at a moderate level, whereas VLA-1 (not shown) and VLA-4 were present in lower amounts.

VLA-3 and NCI-H69 Cells Mediates Adhesion to Matrix Proteins

To optimize adherence of NCI-H69 cells to Coll, Lm, and Fn, the effect of different divalent cations was tested (Fig. 2). While NCI-H69 cells bound efficiently to Lm and Fn, adhesion to Coll was weaker but statistically significant (Fig. 2). Attachment to matrix proteins of NCI-H69 cells, which had been previously depleted of preexisting cations (see Materials and Methods), was best supported by 1 mM Mn++, followed by 1 mM Mg++, and then 1 mM Ca++. Titration of three different divalent cations over a 10-fold range revealed that maximal attachment of NCI-H69 cells to matrix proteins was achieved in the range of 0.5–1.0 mM for each cation (results not shown).

To then determine which integrins might be mediating NCI-H69 cell adhesion to the various ligands, inhibition studies were carried out. Attachment of NCI-H69 cells to Fn (Fig. 3 A), Lm (Fig. 3 B), and Coll (Fig. 3 C) was inhibited by the anti-VLA-3 mAb P1B5 by nearly 70, 40, and 66%, respectively. The anti-VLA-6 mAb GoH3 also inhibited binding to Lm (by 53%), but did not inhibit adhesion to Coll or to Fn. Control antibodies, including anti-VLA-2 and anti-VLA-5 did not inhibit (not shown), consistent with the absence of those receptors from NCI-H69 cells. Anti-VLA-β1 subunit mAb BIE11 inhibited attachment to all three ligands by 90–100%, indicating that NCI-H69 cell adhesion was largely due to β1 integrins. As mentioned above, other integrin subunits such as αv, β3, and β5, which may contribute to fibronectin adhesion on some cells, were undetectable on NCI-H69 cells.

The same mAb reagents used for Fig. 3, A–C were also tested for inhibitory effects on the melanoma cell line LOX (Fig. 3, D–F). The mAb P1B5 (anti-VLA-3) did not inhibit adhesion to any of the ligands tested (as also indicated in Table I). Instead, Fn attachment was blocked nearly 85% by anti-VLA-5 (Fig. 3 D), Lm binding was diminished 56% by anti-VLA-2 and 77% by anti-VLA-6 (Fig. 3 E), and Coll attachment was reduced 76% by anti-VLA-2 (Fig. 3 F). In addition, anti-β1 inhibition was 95% or greater for all ligands, thus indicating that LOX cell adhesion was mostly due to β1 integrins. These results suggest that for LOX cells, VLA proteins other than VLA-3 are the major receptors for Fn, Lm, and Coll.

Differential Effects of RGD on VLA-3-mediated Adhesion to Matrix Proteins

To determine whether cell adhesion mediated by VLA-3 is RGD-dependent, peptide inhibition studies were conducted. Attachment of NCI-H69 cells to Fn (Fig. 3 A) was inhibited 51% by the peptide Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) in this experiment, and 60–70% in other experiments (not shown). In contrast, the control peptide Gly-Arg-Gly-Glu-Ser-Pro (GRGESP) had no effect on NCI-H69 cell adhesion (Fig. 3 A). Because VLA-3 appears to be the major integrin responsible for Fn binding, the GRGDSP peptide is probably...
Figure 3. Inhibition of cell attachment to matrix proteins. Adhesion of NCI-H69 cells to microtiter plates coated with a 5 μg/well of Fn (A), Lm (B), or Coll type I (C) was tested in the presence of inhibitors. In a parallel experiment, adhesion of LOX cells to plastic plates coated with 1 μg/well of Fn (D), Lm (E), or Coll type I (F) was analyzed. The following mAbs were tested for inhibition of cell binding (concentration: 1–5 μg/well): J-2A2 (control), 5E8 (anti-VLA-2), PIB5 (anti-VLA-3), P1D6 (anti-VLA-5), GoH3 (anti-VLA-6), and B1Ell (anti-VLA β). The peptides GRGESP and GRGDSP were present at 0.2 mg/ml.

Consistent with the suggestion that VLA-3 may mediate attachment to the RGD site on Fn, NCI-H69 cells bound well to the Fn-120 fragment of Fn, but not to other Fn fragments that do not contain the RGD sequence (not shown).

Contrary to Fn, NCI-H69 cell attachment to Coll and Lm was not inhibited by the GRGDSP peptide. Furthermore, when GRGDSP peptide was added together with the anti-VLA-3 mAb, it did not increase the level of inhibition (Fig. 3, B and C). However, a combination of anti-VLA-3 and anti-VLA-6 antibodies inhibited Lm attachment by 67%, an effect greater than either alone. In contrast, anti-VLA-6 plus anti-VLA-3 mAbs did not increase inhibition of binding to Coll (or Fn) beyond that seen for anti-VLA-3 alone. Taken together, the results suggest that VLA-3 may bind to the RGD site on FN, but may use a different recognition site for Coll and Lm.

**Differential Effects of Divalent Cations on VLA-3-dependent Adhesion Functions**

The experiments shown in Fig. 3 were carried out in the presence of 1 mM Mn++, to optimize NCI-H69 cell attachment. To further assess the specific effects of divalent cations on VLA-3 functions, cell adhesion assays were performed using different divalent cations, after depletion of preexisting cations with EDTA (see Materials and Methods). As shown in Fig. 4, attachment of NCI-H69 cells could be (a) three- to fourfold greater on Fn than on Coll (when Mn++ was present), (b) about the same on both Fn and Coll (when Mg++ was present), or (c) substantially less on Fn than on Coll (in the presence of Ca++). Stated another way, adhesion to Coll was relatively unchanged whether Ca++, Mg++, or Mn++ was present, whereas attachment to Fn was elevated with Mn++, moderate with Mg++, and almost absent with Ca++. Adhesion to Lm (not shown) was also influenced by divalent cations. However, because both VLA-6
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Figure 4. Effect of divalent cations on NCI-H69 cell adhesion. Microtiter plates were coated with increasing concentrations of Fn and Coll type I, and attachment of NCI-H69 cells was examined in the presence of 1 mM concentrations of either MnCl₂, MgCl₂, or CaCl₂ after NCI-H69 cells were previously depleted of preexisting cations by washing with 1 mM EDTA in PBS (see legend to Fig. 2 and Materials and Methods).

Figure 5. Affinity chromatography of LOX cells on Fn-Sepharose. Octylglucoside extracts of LOX cells containing MnCl₂ were fractionated on a column of immobilized Fn (see Materials and Methods). Aliquots of column eluates were subjected to immunoprecipitation with mAbs, followed by SDS-PAGE analysis. The mAbs were: 12F1 (lanes a, e, and i), J143 (lanes b, f, and j), P1D6 (lanes c, g, and k), and 6-1A5 (lanes d, h, and l).
could also be specifically eluted from the Fn column with GRGDSP peptide, in agreement with previously published results (43). In contrast, attempts to elute VLA-3 using the GRGDSP peptide in this experiment were inconclusive (not shown).

To localize the VLA-3 binding site on human plasma Fn, binding of LOX cell extracts to intact Fn and to the 120-kD chymotryptic fragment of Fn (which includes the RGD cell attachment sequence) was compared. Fig. 6A shows the relative amounts of VLA-3 from LOX cells that were unbound, or bound and subsequently eluted with EDTA. VLA-3 bound partially to both intact Fn (Fig. 6A, compare lanes a and b) and to the 120-kD fragment (compare lanes d and e), with a little less binding to the 120-kD fragment (compare lanes b and e). In contrast, VLA-5 bound quantitatively to both intact and 120-kD fragment of Fn (Fig. 6A, lanes c and f). Note that 100% of the VLA-3 but only 10% of the total VLA-5 yield is shown. In a control experiment, there was no binding of either VLA-3, or VLA-5 to the 40-kD Hep II fragment of Fn, which contains the sequence EILDVPST (not shown). In summary, although VLA-3 binding was substantially weaker, both VLA-3 and VLA-5 appeared to interact with the 120-kD cell binding fragment of Fn.

VLA-3 Binding to Fn Is RGD Dependent

Affinity chromatography of LOX cells over Fn-Sepharose resulted in quantitative depletion of VLA-5 from the extract, while substantial amounts of VLA-3 remained in the unbound fraction (Fig. 5, lanes b and c). To assess the binding capacity of the remaining VLA-3 protein, a second fractionation step was carried out on a Fn-120-Sepharose column using a VLA-5-depleted LOX cell extract (Fig. 6B). In the absence of VLA-5, VLA-3 bound specifically to the Fn-120 column, since most VLA-3, but not VLA-2, was conspicuously absent from the flowthrough (Fig. 6B, lanes i and m). Elution of VLA-3 from the Fn-120 column could then be accomplished by treatment with EDTA (Fig. 6B, lane j). As expected, VLA-5 could not be detected in either unbound or bound fractions (Fig. 6B, lanes i and m). The amount of VLA-3 in the EDTA eluate (lane j) was approximately equal to the total VLA protein that could be immunoprecipitated (lane n), thus suggesting that VLA-3 was the only VLA heterodimer in this fraction.

To evaluate the putative role of RGD and VLA-3 binding to Fn (or to its 120-kD fragment), VLA-5-depleted extracts of LOX cells were fractionated on Fn-Sepharose (Fig. 7). Consistent with the results described above, VLA-3 remained bound to the affinity column and was not eluted by washing with the control peptide GRGESP (Fig. 7, lane h). However, VLA-3 was readily eluted upon incubation with GRGD (Fig. 7, lane m). In a separate experiment, RGD-dependent elution of VLA-3 from Fn columns was also obtained using extracts from the cell line NCI-H69 (not shown), which expresses VLA-3, but not VLA-5 (see Fig. 1A). Therefore, for at least two different cell lines VLA-3 may interact with the RGD site in Fn. This interaction was best observed when VLA-5 was first depleted (e.g., as from LOX cells), or was entirely absent (e.g., as from NCI-H69 cells).
VLA-3 Binding to Coll and Lm Is RGD-independent

To examine the possibility that the RGD sequence may also be required for the interaction of VLA-3 with Coll and Lm, affinity chromatography separations were carried out. VLA-3 bound to columns of immobilized Coll type I (Fig. 8 A), Lm (Fig. 8 B), and Coll type IV (not shown). In each case partial elution was obtained with 0.5 M NaCl (Fig. 8, lanes c and l), and complete elution with EDTA (lanes h and q). However, no elution of VLA-3 was obtained using RGD peptide (not shown). VLA-2 also bound to the Coll I (Fig. 8 A, lane g) and Lm (not shown) columns, but unlike VLA-3, this integrin was not eluted with 0.5 M NaCl. Only a trace of VLA-6 was eluted from the Lm column with EDTA (Fig. 8 B, lane s), and no binding of VLA-5 was observed for either the Coll or Lm columns (Fig. 8, lanes d, i, m, and r). In conclusion, VLA-3 binding to Coll and Lm, unlike that to Fn, appears to be largely RGD independent.

Localization of VLA-3 and Other Integrins in Attached Cells

To compare the cell surface distribution of VLA-3 and VLA-5 receptors on cells adhering to matrix proteins, indirect immunofluorescence studies were performed. A group of cells from the bladder carcinoma line EJ spread on Fn-coated coverslips (Fig. 9 a), and formed elaborate cell processes, which stained intensely for VLA-3. Staining for VLA-3 was distributed diffusely over the cell surface and not localized into cell contacts (Fig. 9 a). Interestingly, a few cells (Fig. 9 a, asterisk), or cell processes (arrow) within the same field (Fig. 9 b) were completely VLA-3-negative, thus indicating that VLA-3-positive staining was not an artifact. The surface distribution of VLA-5 receptors on EJ cells attached to Fn (Fig. 9 c) was clearly different from that of VLA-3. Staining for VLA-5 (Fig. 9 c) appeared to be intercellular (arrow), and occurred in some regions as bridgelike processes between cells (arrowhead).

To examine putative differences between cell surface distribution of VLA-3 on normal cells relative to transformed lines, immunofluorescence studies on the primary fibroblast cell line MRC-5 were also conducted. When MRC-5 fibroblasts were plated on Fn and stained for VLA-5, many focal contacts were clearly observable (Fig. 9 d). In contrast, diffuse staining of VLA-3, without localization to focal contacts, was seen for MRC-5 fibroblasts whether plated on Coll (Fig. 9 e), or on Fn (not shown). As a control, MRC-5 fibroblasts did form focal contacts on Coll as seen by staining of VLA-β1 subunit (Fig. 9 f). VLA-1 and VLA-2 receptors appeared to account for the localization of the β subunit into focal contacts (not shown). In conclusion, the results above suggest that VLA-3 surface receptors are not involved in the formation of focal adhesion contacts during cell spreading on matrix proteins.

VLA-3 and Cell–Cell Aggregation

To determine if there was any involvement of VLA-3 in the homotypic aggregation of NCI-H69 cells (the cells spontaneously form large clumps), inhibition experiments were attempted. After NCI-H69 cell aggregates were disrupted by vigorous pipetting or by transient EDTA treatment, anti-VLA-3, anti-VLA-6, or anti-β1 mAbs were added and found.
not to inhibit reaggregation. Thus, although VLA-3 is likely to be a cell–cell adhesion receptor in other contexts (see Introduction), we have found no evidence that VLA-3, or any other VLA protein is involved in the particular cell–cell adhesion responsible for homotypic NCI-H69 cell aggregation.

**Discussion**

**Multiple Ligand Binding Functions for VLA-3**

We have used both biological and biochemical assays to establish that VLA-3 can function as a receptor for Coll, Lm, and Fn. Previous studies had suggested that VLA-3 might bind these ligands (3, 18, 19, 57, 58), but the results were variable and a number of experiments failed to support this conclusion (see Introduction and Table I). A key aspect of our study has been to use the small cell lung carcinoma line NCI-H69, which lacks the receptors VLA-1, VLA-2, VLA-4, and VLA-5, to demonstrate VLA-3-mediated cell attachment. While VLA-3 on NCI-H69 cells mediated adhesion to Lm, Fn, and to a lesser extent to Coll, parallel experiments failed to show a similar role of VLA-3 on the melanoma cell line LOX. Instead, Coll binding was mediated by VLA-2, Lm by VLA-2 and VLA-6, and Fn by VLA-5. Thus, the role of VLA-3 in the attachment of many adherent cell lines (such as LOX and others listed in Table I) to purified matrix ligands is not obvious because other integrins, with overlapping functions, may play more dominant roles.

Affinity chromatography on immobilized Fn provided independent confirmation of the results obtained with cell adhesion assays. When VLA-5 was first depleted from LOX cell extracts, or was absent to begin with (as in NCI-H69 cells), nearly 100% of VLA-3 was observed to adhere to Fn. However, VLA-3 binding to Fn affinity columns in the presence of VLA-5 was relatively weak. This result may explain why VLA-3 has often not been detected in previous Fn affinity column experiments (4, 32, 42–44).

VLA-3 binding to Fn-Sepharose has been previously reported at low salt concentrations (57), but was not seen with physiological buffers (32), thus suggesting that VLA-3 binding requires low, nonphysiological salt levels. However, in this study we have shown that it is not physiological salt levels that prevent VLA-3 binding (e.g., in Fig. 6 B VLA-3 bound quite well in the presence of 0.15 M NaCl), but rather the absence of VLA-5 seems to be the most critical factor (as mentioned above).

At high ionic strength conditions (0.5 M NaCl), VLA-3, but not VLA-5, was preferentially eluted from Fn columns. Also at high ionic strength conditions (0.5 M NaCl), VLA-3, but not VLA-2 could be selectively eluted from Coll and Lm affinity supports. Together these results suggest that VLA-3 may have a lower avidity for its ligands compared to VLA-5 binding to Fn, or VLA-2 binding to Coll or Lm. Alternatively, the salt labile binding of VLA-3 to its ligands might indicate more of an electrostatic type of interaction.

Interestingly, although VLA-6 is regarded as a Lm receptor of major functional importance (50, 51), it has not been
Figure 9. Immunofluorescent localization of VLA-3 compared to other integrins. (a) Bladder carcinoma (EJ) cells plated on Fn and stained for VLA-3 as described in Materials and Methods. (b) DIC image of the same field as in a; cells marked by arrowheads and by an asterisk are the same in both fields. (c) EJ cells plated on Fn and stained for VLA-5. The brought staining around the area of the nucleus is also observed with unrelated polyclonal antisera, and thus appears to be nonspecific. (d) MRC-5 fibroblast cells plated on Fn and stained for VLA-5. (e) MRC-5 fibroblasts plated on Coll and stained for VLA-3. (f) MCR-5 fibroblasts plated on Coll and stained for the VLA β1 subunit.
observed to bind well to Lm affinity columns in this or other studies (11, 18, 38). Thus, in vitro biochemical assays alone may sometimes not reflect the functional contribution of an integrin within the context of an intact cell.

Although the current studies clearly establish multiple binding functions of VLA-3, we must also consider that the purified preparations of Coll, Lm, and Fn may not be the most physiologically relevant ligand(s) for VLA-3. For example, several other forms of Lm have recently been discovered, including S-laminin (30), heart laminin (40), and merosin (10). Also, there are many Coll types which have not been tested for VLA-3 binding. In addition, it was recently found that keratinocytes could secrete extracellular matrix mixtures that support both VLA-3-mediated attachment and localization to focal adhesions, better than any of the purified VLA-3 ligands (3). Finally, although VLA-3 does not appear to have a role in NCI-H69 cell–cell aggregation, emerging evidence from other studies suggests a potential role for VLA-3 in cell-cell adhesion (see Introduction). In this regard, it remains to be seen if VLA-3 might resemble another integrin, VLA-4, which has recently been shown to have both matrix and cellular ligands (12).

**VLA-3-mediated Binding Functions Are Both RGD Dependent and RGD Independent**

It appears that VLA-3 may recognize the RGD site in Fn because (a) RGD peptide caused specific elution of VLA-3 (from two different cell lines) from Fn and Fn-120 affinity columns, and (b) RGD peptide inhibited VLA-3-dependent binding to Fn in cell adhesion assays. These results clearly show that VLA-3 can interact with RGD-containing peptide, and they strongly suggest that VLA-3 is recognizing the RGD sequence in Fn. Also supporting VLA-3 binding to RGD in Fn, we found that VLA-3 directly binds to the RGD-containing 120-kd Fn fragment, and we found that VLA-3 binding to Fn was much more pronounced in the absence of VLA-5, another integrin that recognizes the RGD site.

Because RGD peptide plus anti-VLA-3 mAb inhibited a little more effectively than either reagent alone, we cannot rule out that VLA-3 might recognize an additional “non-RGD” site in fibronectin, or that RGD peptide might also be inhibiting a small contribution from one or more other integrins which also binds to Fn in an RGD-dependent manner. In this regard, the integrins VLA-5, VNR, GPIIb/IIIa (47), αβ1 (1, 56), and αβ6 (6) also have the ability to recognize RGD sites in their respective ligands, but, as mentioned above, there is no evidence for expression of these other integrins on NCI-H69 cells.

In contrast to Fn, VLA-3 recognized other ligands in the RGD-independent manner. In experiments reported herein, the peptide GRGDSP did not inhibit or disrupt VLA-3 attachment to Coll, or to Lm in either cell adhesion, or affinity column assays. Consistent with our result, others have found that VLA-3 binds to mouse Lm at a site near the globular end of the long arm (19), in a region devoid of any RGD sequences (48). Other studies have also shown that RGD peptides did not inhibit adhesion of either purified VLA-3 (18) or whole cells (9) to Lm. Thus, among integrins known to interact with multiple ligands (21, 24, 36), VLA-3 appears to be unique in having both RGD-dependent and -independent ligands, implying that VLA-3 may have two functionally distinct ligand binding sites.

**Differential Sensitivity of VLA-3 Adhesion Functions to Divalent Cations**

VLA-3 binding to different matrix ligands is differentially influenced by various divalent cations. For VLA-3-dependent adhesion to Fn, Mn++ caused a pronounced stimulation, and Ca++ was markedly inhibitory relative to Mg++, whereas for Coll, the different cations gave essentially similar results. Table II summarizes the essential data showing critical differences between VLA-3 ligands. Although the effect of divalent cations on VLA-3 binding to Lm was not determined, we would predict that Lm would resemble Coll, assuming that the two RGD-independent ligands would also share similar metal requirements.

Several other integrin receptors are also known to be selectively influenced by the presence of various divalent metals. For example, binding of VLA-5 to an RGD peptide and to a Fn fragment has been shown to be strongly upregulated by Mn++ (17). In contrast, for other RGD-dependent integrins, including VNR and GPIIb/IIIa, the presence of Mn++ did not have a stimulatory effect, compared to Mg++ (17). Strong inhibitory effects with Ca++ have been reported in several RGD-independent interactions, such as VLA-2 binding to Coll (52), and VLA-6 binding to Lm (50).

While all known integrin functions are divalent cation dependent, there is little precedent for a single integrin having distinct ligand binding functions that are differentially regulated by divalent metals. The marked differences in cation sensitivity for VLA-3 binding to Fn, Coll, and/or Lm suggests that adhesion to separate ligands could be independently regulated in vivo, e.g., by localized changes in calcium levels. A shift in divalent cation concentration would potentially effect VLA-3 quite differently than other VLA proteins, such as VLA-2, VLA-5, and VLA-6. For example, an increase in Ca++ levels, relative to Mg++, might be expected to differentially downregulate the Coll and Lm binding functions of VLA-2 and VLA-6, respectively, but not of VLA-3. On the other hand, a rise in Ca++ concentration would presumably cause downregulation of Fn binding by VLA-3, but not by VLA-5.

The positive correlation between RGD dependence and sensitivity to divalent metals (see Table II) strongly supports the concept of two functionally distinct ligand binding sites in VLA-3. Furthermore, a direct role is suggested for divalent cations, particularly Mn++, in supporting RGD binding by VLA-3. A model is envisioned whereby one particular divalent cation site may participate in the interaction of VLA-3 with RGD, perhaps with RGD directly involved in metal coordination (order of preference: Mn++ >> Mg++ >> Ca++). In contrast, RGD-independent interactions of VLA-3, such as Coll (and maybe also Lm), may involve a

| Table II. Summary of Differences between VLA-3 Recognition of Fn, Lm, and Collagen |
|-----------------------------|-----------------|-----------------|-----------------|-----------------|
| VLA-3 ligand | Inhibition by RGD peptide | Inhibition by Ca++ | Stimulation by Mn++ |
|----------------|-----------------|-----------------|-----------------|
| Coll-IV | No | Low (15%) | Low (1.4-fold) |
| Lm | No | ? | ? |
| Fn | Yes | High (88%) | High (four-fold) |

Inhibition and stimulation by divalent cations are calculated relative to binding observed in the presence of Mg++.
different ligand binding site, which is relatively insensitive to the nature of the ligand present. In this regard, human VLA-3 has at least three potential divalent cation sites in its $\alpha$ subunit, as revealed by sequence analysis of human (Takada, Y., E. Murphy, P. Pil, C. Chen, M. H. Ginsberg, M. E. Hemler, manuscript submitted for publication) and hamster (55 $\alpha^3$ DNA.

A Unique Role for VLA-3 Compared to Other Integrins?

Despite the apparent overlap in functions between VLA-3 and other integrins (VLA-1, VLA-2, VLA-5, and VLA-6), VLA-3 is likely to play a unique role in cell adhesion phenomena. In addition to its broader ligand specificity, and its unusual ligand-dependent sensitivity to divalent cations, VLA-3 has lower apparent avidity for its ligands, and fails to rapidly localize into focal adhesions as compared to other integrins. Thus, for example, whereas increased VLA-5 expression may contribute to more deposition of Fn matrix, VLA-3 has at least three potential divalent cation sites in its $\alpha$ subunit. Together, these findings support the notion that VLA-3 possesses functions and expression that differ from other integrins.

In summary, this report indicates that VLA-3 adhesion functions are remarkably versatile. VLA-3 interacts with multiple matrix ligands by apparently both RGD-dependent and RGD-independent mechanisms. Furthermore, different VLA-3 binding activities show markedly disparate sensitivities to the nature of the cation present. In this regard, human VLA-3 has at least three potential divalent cation sites in its $\alpha$ subunit, as revealed by sequence analysis of human (Takada, Y., E. Murphy, P. Pil, C. Chen, M. H. Ginsberg, M. E. Hemler, manuscript submitted for publication) and hamster (55 $\alpha^3$ DNA.

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