

# Human Neural Cell Adhesion Molecule L1 and Rat Homologue NILE are Ligands for Integrin $\alpha_v\beta_3$

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**Abstract.** Integrin  $\alpha_v\beta_3$  is distinct in its capacity to recognize the sequence Arg-Gly-Asp (RGD) in many extra-cellular matrix (ECM) components. Here, we demonstrate that in addition to the recognition of ECM components,  $\alpha_v\beta_3$  can interact with the neural cell adhesion molecule L1-CAM; a member of the immunoglobulin superfamily (IgSF). M21 melanoma cells displayed significant  $Ca^{++}$ -dependent adhesion and spreading on immunopurified rat L1 (NILE). This adhesion was found to be dependent on the expression of the  $\alpha_v$ -integrin subunit and could be significantly inhibited by an antibody to the  $\alpha_v\beta_3$  heterodimer. M21 cells also displayed some  $\alpha_v\beta_3$ -dependent adhesion and spreading on immunopurified human L1. Ligation between this ligand and  $\alpha_v\beta_3$  was also observed to promote significant haptotactic cell migration.

To map the site of  $\alpha_v\beta_3$  ligation we used recombinant

L1 fragments comprising the entire extracellular domain of human L1. Significant  $\alpha_v\beta_3$ -dependent adhesion and spreading was evident on a L1 fragment containing Ig-like domains 4, 5, and 6. Importantly, mutation of an RGD sequence present in the sixth Ig-like domain of L1 abrogated M21 cell adhesion. We conclude that  $\alpha_v\beta_3$ -dependent recognition of human L1 is dependent on ligation of this RGD site.

Despite high levels of L1 expression the M21 melanoma cells did not display significant adhesion via a homophilic L1-L1 interaction. These data suggest that M21 melanoma cells recognize and adhere to L1 through a mechanism that is primarily heterophilic and integrin dependent. Finally, we present evidence that melanoma cells can shed and deposit L1 in occluding ECM. In this regard,  $\alpha_v\beta_3$  may recognize L1 in a cell-cell or cell-substrate interaction.

THE neural cell adhesion molecule L1 is a multidomain glycoprotein and a member of the IgSF (Moos et al, 1988). This cell adhesion molecule (CAM)<sup>1</sup> was first described in the mouse, but homologous molecules have now been reported in man, rat (NILE), chick (NgCAM, 8D9, G4), and *Drosophila* (neuroglian) (Grumet et al., 1984; Bock et al., 1985; Lemmon and McLoon, 1986; Mujoo et al., 1986). These L1-related glycoproteins have an extracellular region that consists of six immunoglobulin (Ig)-like domains followed by five fibronectin type III-like repeats. There is a single transmembrane region that is linked to a short, highly conserved cytoplasmic domain (Moos et al, 1988; Sonderegger and Rathjen, 1992). Considerable sequence homology has been found between the mammalian L1-related CAMs. Thus, the deduced identity

between the amino acid sequences of human and mouse L1 is 87% (Reid and Hemperly, 1992), while the amino acid homology between rat and mouse L1 is 96% (Miura et al., 1991).

Reference to L1 as a neural CAM is based on its distribution on post-mitotic neurons of the central and peripheral nervous systems and on pre- or non-myelinating Schwann cells of the peripheral nervous system (Lindner et al., 1983; Rathjen and Schachner, 1984; Martini and Schachner, 1986). L1 has also been described on non-neural cells, including granulocytes, lymphocytes, and epithelial cells of the intestine and urogenital tract (Thor et al., 1987; Kowitz et al., 1992; Kujat et al., 1995). This CAM is further expressed on transformed cells of diverse histological origin, including melanoma, neuroblastoma, embryonal carcinoma, osteogenic sarcoma, squamous lung carcinoma, squamous skin carcinoma, pheochromocytoma, rhabdomyosarcoma, and retinoblastoma cell lines (Mujoo et al., 1986; Linnemann et al., 1989; Reid and Hemperly, 1992). Surprisingly, despite widespread expression of L1 on many highly malignant tumors, little is known of the function of this CAM with respect to tumor progression

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1. *Abbreviations used in this paper:* CAM, cell adhesion molecule; IgSF, Immunoglobulin superfamily; ECM, extracellular matrix; NILE, nerve growth factor-inducible large external glycoprotein; NCAM, neural CAM.

and dissemination. Linnemann et al. (1989) found L1 expression on a metastatic variant of the melanoma cell line K1735, while nonmetastasizing cells were negative for this CAM, suggesting a role for this molecule in tumor progression.

The L1-related glycoproteins have been implicated in a variety of neurological processes, including myelination, neurite fasciculation and outgrowth, and cerebellar cell migration (Lindner et al., 1983; Martini and Schachner, 1986; Lagenaur and Lemmon, 1987). It is generally believed that L1 regulates these processes by virtue of a homophilic interaction i.e., L1-L1. However, it has recently been documented that L1 can also interact with another member of the IgSF referred to as axonin-1/TAG 1 (Kuhn et al., 1991; Felsenfeld et al., 1994). Furthermore, it is conceivable that the large multidomain structure of L1 may facilitate further heterophilic interactions that remain to be identified.

Integrin  $\alpha_v\beta_3$ , also known as the vitronectin receptor, is a transmembrane heterodimer consisting of noncovalently associated  $\alpha_v$  and  $\beta_3$  glycoprotein subunits. The biological consequences of  $\alpha_v\beta_3$  ligation are numerous and include the regulation of cell adhesion, morphology, migration, viability, growth, and differentiation (Wayner et al., 1991; Cheresh, 1992; Clyman et al., 1992; Leavesley et al., 1992). Regulation of these events and capacity for promiscuous ligand binding has placed  $\alpha_v\beta_3$  at the center of many fundamental and important biological processes including angiogenesis, wound healing, development, and neoplastic progression (Cheresh, 1992; Brooks et al., 1994; Montgomery et al., 1994). One of the most important and distinctive features of  $\alpha_v\beta_3$  is its capacity to recognize the sequence Arg-Gly-Asp (RGD) in a large number of extracellular matrix components. Significantly there are two such sequences in the sixth Ig-like domain of rat NILE/L1, while one of these sequences is conserved in man (Miura et al., 1991).

In this study we demonstrate that in addition to homophilic L1-L1 interaction or heterophilic L1-axonin-1/TAG 1 interaction, L1 can also be recognized by the integrin  $\alpha_v\beta_3$ . Furthermore, we show that this novel interaction is dependent on the recognition of a conserved RGD site in the sixth Ig-like domain of human L1. We present further evidence that melanoma cells can deposit L1 in tumor-associated ECM raising the possibility that these tumor cells can modify their environment to promote  $\alpha_v\beta_3$  ligation. Cell-cell or cell-matrix interactions between  $\alpha_v\beta_3$  and L1 may have important ramifications for melanoma progression since  $\alpha_v\beta_3$  expression has been linked to increased tumorigenicity and invasion (Albelda et al., 1991; Felding-Habermann, 1992; Albelda, 1993).

## Materials and Methods

### Reagents and Antibodies

Integrin-specific mAbs used in these studies include LM609 (anti- $\alpha_v\beta_3$ , Cheresh and Spiro, 1987) and P3G2 (anti- $\alpha_v\beta_5$ , Wayner et al., 1991). Antibody P3G2 was kindly provided by Dr. E. A. Wayner (University of Minnesota, Minneapolis, MN). LM609 was produced in one of our laboratories. L1-specific mAb 5G3 was generated in our laboratory (Mujoo et al., 1986) and was purified with Protein-A. Control mAb W6/32 specific for the HLA-class I antigen was purified from ascites in this laboratory. Inhibitory cyclic RGD peptide 66203 (cyclo-RGDfv) and control peptide

69601 (cyclo-RADfv) were synthesized and characterized by Drs. A. Jonczyk, B. Diefenbach, and S. Goodman (E. Merck, FGR) and were kindly made available by Dr. D. A. Cheresh (Scripps Research Institute, La Jolla, CA). EGTA was purchased from Sigma Chem. Co. (St. Louis, MO).

### Cell Lines

Human melanoma cell line M21 was derived in our laboratory as a subclone from the cell line UCLA-SO-M21 that was kindly provided by Dr. D. L. Morton (University of California, Los Angeles, CA). Variant  $\alpha_v$ -deficient cells (M21-L), were negatively selected from M21 cells by fluorescence-activated cell sorting (FACS) with anti- $\alpha_v$  mAb LM142 (Cheresh and Spiro, 1987). M21-L cells were subsequently transfected with an  $\alpha_v$ -cDNA (Felding-Habermann et al., 1992). M21-L4 cells ( $\alpha_v$ -reconstituted) and M21-L12 cells ( $\alpha_v$ -deficient transfection control) were selected from a panel of transfected subpopulations by FACS with mAb LM142. J558L myeloma cells were originally obtained from the American Tissue Type Collection (ATCC; Rockville, MD) and were stably transfected with the full-length human cDNA encoding for human L1. Transfection was performed using the pJanusin vector (Trauneker et al., 1991; Rader et al., 1993). All cells were maintained in RPMI-1640 supplemented with 10% fetal calf serum.

### Purification of Rat and Human L1

Rat NILE/L1 was purified by immunoaffinity chromatography as previously described (Lemmon et al., 1989). Briefly, neural membranes were prepared from rat brains by separation on sucrose density gradients. The membranes were subsequently extracted with 1% deoxycholate, and the extract purified on a mAb affinity column. The mAb used, 74-5H7, has previously been documented to be specific for mammalian L1 (Lemmon et al., 1989) and was coupled to an Affi-Gel-10 support (Bio-Rad, Richmond, CA). NILE/L1 was eluted from the column with 0.1 M diethylamine (pH 11.5) and was immediately neutralized with Tris-HCl. To ensure removal of any contaminating antibody eluted material was incubated with Affigel-10 beads coated with rabbit anti-mouse immunoglobulin antibodies. Human L1 was purified from M21 cell lysates essentially as described (Wolff et al., 1988). In brief, 4 ml of packed M21 cells were solubilized in a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, containing 0.5% Nonidet P-40 and protease inhibitors). Insoluble material was pelleted by centrifugation at 100,000 g for 1 h and the supernatant passed over an immunoaffinity column consisting of L1-specific mAb 5G3 coupled to an Affi-Gel-10 support (Bio-Rad). After extensive washing, L1 was eluted from the column with 0.1 M diethylamine (pH 11.5) and was immediately neutralized with Tris-HCl. The purity of L1 preparations was judged on the basis of SDS-PAGE and silver staining.

### Construction and Expression of L1 Fusion Proteins

The generation and characterization of L1 fusion proteins used in this study has been described (Zhao and Siu, 1995). In brief, three cDNA fragments coding for Ig-like domains 1, 2, and 3 (Ig 1-3), Ig-like domains 4, 5, and 6 (Ig 4-6) and for all five fibronectin type-III-like repeats (FN 1-5) were prepared and inserted between the EcoRI and BamHI sites of pGEX-3X. The cDNA fragment Ig 1-3 codes for amino acids between positions 24 to 351, the cDNA fragment Ig 4-6 codes for amino acids between positions 352 and 595, and the cDNA fragment FN 1-5 codes for amino acids between positions 596 and 1094 (amino acid numbering according to Hlavin and Lemmon, 1991). In all three cases, GST was fused to the amino terminus of the fusion protein. To produce GST-L1 fusion proteins, transformed *Escherichia coli* strain JM101 was induced by adding 1 mM isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) and the induced bacteria subsequently resuspended in a lysis buffer (50 mM Hepes buffer, 5% glycerol, 2 mM EDTA, 0.1 M DTT, pH 7.9). Fusion proteins were isolated from inclusion bodies, solubilized and refolded as described (Zhao and Siu, 1995). A second cDNA fragment was constructed coding for amino acids inclusive of Ig-like domains 3 through 6 (amino acids between positions 223 and 685). An oligonucleotide primer (sense, 5' ATT GAC AGG AAT TTC CGC CTG CTC TTC CCC ACC 3'; anti-sense, 5' TGT GAC CAC AGT CCT CGA GAC CGG GCT GGG CTC 3') was used to amplify this cDNA and introduced 5' a restriction site for EcoRI and 3' a recognition site for XhoI. After amplification the PCR products were digested with EcoRI and XhoI and ligated into a similarly treated prokaryotic expression vector pGEX-5X-3. The sequence of the resulting plasmid was confirmed. TKX1 cells were transformed with these plasmids and pro-

tein production induced by 0.25 mM IPTG at 32°C. This temperature was used to reduce the formation of inclusion bodies and thereby facilitate purification under non-denaturing conditions. All GST-fusion proteins were subsequently purified by affinity chromatography using a glutathione-Sepharose 4B and were extensively dialyzed against PBS. L1-GST fusion proteins were analyzed by SDS-PAGE and are described by Zhao and Siu (1995).

The RGD site within Ig-like domain 6 in the fragment coding for amino acids inclusive of Ig-like domains 3 through 6 was mutated into a DVD site by inverse PCR mutagenesis (Hemsley et al., 1989). PCR amplification using this cDNA in pGEX-5X-3 as a template was performed in 10 mM Tris-HCl, 50 mM KCl, 0.01% gelatin, 0.05% NP-40, 2 mM MgCl<sub>2</sub>, 0.2 mM deoxynucleotide triphosphate with 2.5 U Pfu and 1 U Taq polymerase, and 1 μM sense and anti-sense 5' oligonucleotide primers. The primers used were: sense 5' CCC AGC ATC ACC TGG GAC GTC GAC GTC GAC GGT CGA GAC CTC GTC 3'; anti-sense, 5' CTG CAA GGA GGG GTC AAA GGA GGC CTG GCA CGT GAA GGT 3'. The sense primer introduced an AatII restriction site. After PCR amplification the resulting product was treated with Klenow fragment of DNA polymerase to remove extra nucleotides possibly added at the 3' end and subsequently blunt ligated. The ligation product was transformed into TKX-1 cells. The newly introduced AatII restriction site facilitated screening for successfully mutated clones. The sequence of these was confirmed.

### Characterization of L1 Expression

Levels of L1 expression on M21 melanoma cells or on J558L myeloma cells transfected with L1 cDNA were determined by FACS analysis with L1-specific mAb 5G3 (Mujoo et al., 1986). Subconfluent cultures were harvested and stained with this mAb at 10 μg/ml. The cells were then treated with an anti-mouse IgG FITC-conjugated antibody and were analyzed with a FACscan flow cytometer (Becton Dickinson, Mountain View, CA). Control cells were treated with nonspecific isotype control mAbs. L1 shed by M21 melanoma cells was further characterized by immunoblot analysis of melanoma-conditioned media. Concentrated cell-free conditioned media were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide) and subsequent immunoblotting with L1-reactive polyclonal antibody 6096. This antibody was kindly provided by Dr. J. Hemperly (Becton Dickinson, Research Triangle Park, NC).

### Adhesion Assays

Adhesion experiments were performed as detailed by Lagenaur and Lemmon (1987) with some modifications. Purified L1 ligands were spotted into the center of the wells of 96-well Titertek plates (ICN Flow, Aurora, OH) in 1 μl of PBS and the ligands subsequently air dried onto the plastic. Unless otherwise stated, an optimal concentration of 75–100 ng/mm<sup>2</sup> was used. After drying, treated and control wells were blocked with 5% BSA for 3 h at 37°C. Unless otherwise stated, cells were then added and allowed to adhere for 90 min at 37°C. The cells were added as a single cell suspension at 1 × 10<sup>5</sup>/well. At this cell number, the cells formed a monolayer over the bottom of each well and in the absence of rotation did not tend to aggregate. The cells were maintained in fibroblast basal medium (FBM; Clonetics, San Diego, CA) supplemented with 1% BSA. To optimize binding this adhesion buffer was also supplemented with 0.4 mM MnCl<sub>2</sub>. At the end of the assay the wells were carefully washed, and non-adherent cells were removed under a constant vacuum. Remaining adherent cells were fixed with 4% paraformaldehyde and enumerated with the aid of an inverted light microscope. Cells were counted per unit area using a 20× high powered objective and an ocular grid. Experimental treatments were performed in triplicate with a minimum of four areas counted per well. For inhibition studies, the cells were pretreated with mAbs (40 μg/ml) or peptides (20 μg/ml) for 30 min before the addition of both cells and inhibitors to preprepared wells. EGTA was added to some of the wells at 5 mM.

### Migration Assay

Migration assays were performed with 24-well Transwell plates (Costar Corp., Cambridge, MA) essentially as described (Leavesley et al., 1992). Each well in a Transwell plate consists of an upper chamber or insert (6.5-mm diam) separated from a lower chamber by a polycarbonate membrane containing 8.0-μm pores. This size of pore allows the directed migration of melanoma cells from the upper to lower chambers. To test for haptotactic migration, L1 ligand was air dried onto the underside of membranes of in-

verted inserts at ~100 ng/mm<sup>2</sup>. M21 cells were subsequently added to upper chambers at 1 × 10<sup>5</sup> cells per chamber in FBM supplemented with 0.5% BSA and 0.4 mM MnCl<sub>2</sub>. After 18 h, directed migration of cells from upper to lower chambers was quantified by enumerating the number of cells both at the bottom of the lower chambers and associated with the undersides of membranes. For inhibition studies, mAbs were added at 40 μg/ml to both the lower and upper chambers.

### Tissue Immunofluorescence

To generate primary tumors, M21 melanoma cells were injected subcutaneously into the flank of female C.B.17 *scid/scid* mice. Each mouse received 2 × 10<sup>6</sup> cells in 100 μl of PBS. After approximately four weeks the primary tumors (>2 mm in diameter) were resected and sectioned for staining with L1-specific mAb 5G3 and with a laminin-specific rabbit polyclonal antibody (Serotec Ltd., Oxford, UK). Before staining, frozen sections were fixed with acetone and preblocked with 5% BSA. Sections were then stained with a mixture of L1-specific mAb 5G3 (40 μg/ml) and anti-laminin antibody (1/350 dilution). Murine mAb 5G3 was detected using a 1/50 dilution of a goat F(ab')<sub>2</sub> anti-mouse Ig-Rhodamine conjugate, while rabbit Ig was detected with a 1/50 dilution of a goat F(ab')<sub>2</sub> anti-mouse Ig-fluorescein conjugate. Both conjugates were absorbed against human proteins. The tissue sections were visualized and photographed using an Olympus BX60F fluorescence microscope equipped with a UPlan Apo 20× objective.

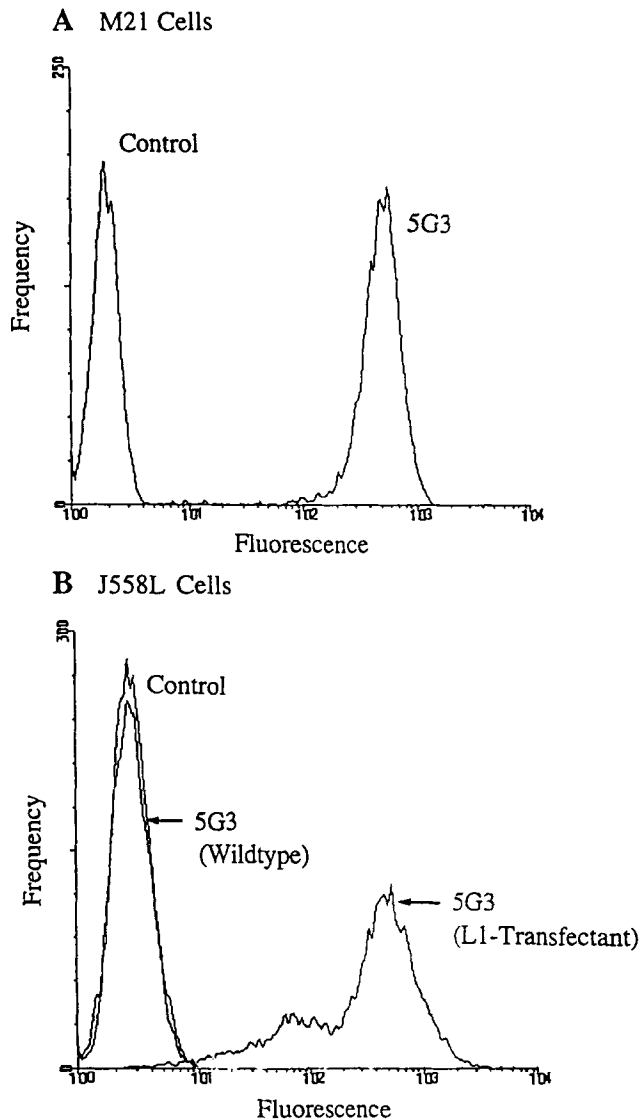
## Results

### Characterization of L1 Expression and Immunopurified L1 Ligands

Expression of L1 on the surface of human M21 cells was assessed with L1-specific mAb 5G3. FACS analysis with this mAb confirmed the presence of significant amounts of L1 on the surface of these cells (Fig. 1 A). L1 immunopurified from M21 cell lysates was mainly evident as a single band of ~215 kD after SDS-PAGE and silver staining (Fig. 2, lane 2). L1 of this molecular mass is consistent with that described for L1 derived from other neuroectodermal tumor cell lines (Mujoo et al., 1986; Linnemann et al., 1989). Minor L1 products were also detected at ~190 and 80 kD. L1 fragments with this molecular mass have also been described and are proposed to represent naturally occurring posttranslational cleavage products (Mujoo et al., 1986; Sadoul et al., 1988). In addition to membrane-associated L1, we also detected significant amounts of L1 in M21 tumor-conditioned media. Western blot analysis with L1-specific antisera showed this shed L1 to be a high molecular mass component (>200 kD) together with lower molecular mass cleavage products (Fig. 2, lane 3). Quantification of this material by immunodot blot analysis and scanning densitometry confirmed the presence of soluble L1 and indicated shedding at the rate of ~250 ng/1 × 10<sup>6</sup> cells over a 72-h period (data not shown).

Rat NILE/L1 was purified from brain tissue. On SDS-PAGE this immunopurified material was evident as three components of ~200, 140, and 80 kD (Fig. 2, lane 1). Detection of immunopurified NILE/L1 at these molecular masses is consistent with previous reports (Kobayashi et al., 1992; Miura et al., 1992). As described for human L1, the lower molecular mass products represent naturally occurring posttranslational cleavage products (Sadoul et al., 1988).

As an experimental system to test for L1-dependent interactions we exploited L1-negative J558L myeloma cells transfected with a cDNA encoding for full length human

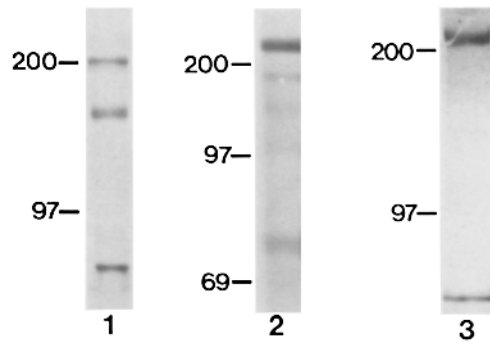


**Figure 1.** Expression of L1 on M21 melanoma cells (A) or J558L myeloma cells transfected with human L1 cDNA (B). Levels of L1 expression were determined by flow cytometric analysis with L1-specific mAb 5G3. Cells were harvested and stained with mAb 5G3 at 10  $\mu\text{g/ml}$ . The cells were then treated with an antimouse IgG FITC-conjugated antibody and analyzed using a FACscan flow cytometer (Becton Dickinson, Mountain View, CA). Control cells were treated with a nonspecific isotype control mAb.

L1. To confirm expression of L1 by transfected J558L myeloma cells, flow cytometric analysis was performed with L1-specific mAb 5G3. The transfected J558L myeloma cells were found to express high levels of L1 in an amount comparable to that found on M21 cells (Fig. 1 B). Wild-type J558L myeloma cells were confirmed to be negative for L1 expression (Fig. 1 B).

#### **M21 Cell Adhesion to NILE/L1 Is Calcium-dependent and Does Not Involve Homophilic L1-L1 Interaction**

Immunopurified NILE/L1 was directly coated onto the bottom of 96-well plates and used for cell adhesion experiments. M21 cells adhered to the purified NILE/L1 in a



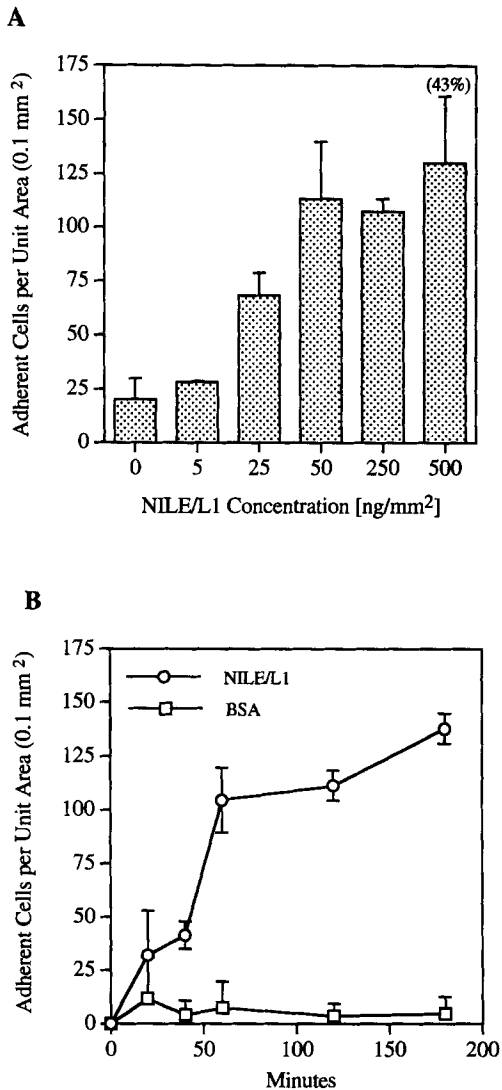
**Figure 2.** Characterization of L1 immunopurified from rat brain (lane 1) or from M21 cell lysate (lane 2). The immunopurified ligands were subject to 7% SDS-polyacrylamide gel electrophoresis and subsequent silver staining. L1 present in M21 conditioned media (lane 3) was detected by Western blotting with a L1-reactive polyclonal antibody.

concentration dependent fashion (Fig. 3 A). Adhesion was first evident after 20 min and was optimal after 1 h (Fig. 3 B). Taking into account the number of cells seeded per unit area, between 35–45% of the cells seeded adhered to the NILE/L1 (Figs. 3 A and 4 A). Of the adherent cells, ~50% showed evidence of cell spreading after 120 min (Fig. 4 B).

To further characterize the adhesion observed, it was determined whether the interaction with NILE/L1 was  $\text{Ca}^{++}$ -dependent. Chelation of extracellular calcium with EGTA (5 mM) completely abrogated the M21 cell adhesion (Figs. 4 C and 5). Likewise, no significant adhesion was observed when  $\text{Ca}^{++}$ -free HBSS was used as an adhesion buffer (not shown). However, short term maintenance in the absence of  $\text{Ca}^{++}$  did not affect M21 cell viability. This cation dependence argues against a role for homophilic L1-L1 interaction in the adhesion observed since this type of interaction was documented to be  $\text{Ca}^{++}$ -independent (Sonderegger and Rathjen, 1992).

To determine whether this lack of homophilic L1 interaction could be attributed to the purified ligand or the M21 cells themselves we looked for evidence of  $\text{Ca}^{++}$ -independent adhesion by myeloma cells transfected with full-length human L1 cDNA. Contrary to M21 cells, these transfected cells did show  $\text{Ca}^{++}$ -independent adhesion to the same purified NILE/L1 glycoprotein (Fig. 5). Furthermore this adhesion was absolutely dependent upon L1-L1 interaction since untransfected, L1-negative myeloma cells showed no evidence of adhesion (Fig. 5). It is interesting to note that while M21 cells were observed to spread on the purified NILE/L1, the L1(+) myeloma cells showed no evidence of cell spreading even after 3 h (Fig. 4 D). Significantly, heat denaturation of the L1 ligand (100°C, 10 min) abolished L1(+) myeloma cell attachment (data not shown). This finding suggests that the L1 will only support the homophilic ligation observed when in a native conformation. Importantly, our immunopurified NILE/L1 ligand appears to be in a native or close to native conformation that can support both homophilic L1-L1 interaction and attachment of M21 cells via a heterophilic mechanism.

Together these data indicate that melanoma cell-associated L1 does not significantly contribute to cell adhesion,



**Figure 3.** Adhesion of M21 cells to immunopurified NILE/L1. (A) M21 cells were allowed to adhere to NILE/L1 at concentrations ranging from 5–500 ng/mm<sup>2</sup>. (B) M21 cells were seeded onto NILE/L1 at 250 ng/mm<sup>2</sup> and allowed to adhere for various time points. Adherent cells were counted per unit area with a 20 $\times$  high powered objective. Experimental treatments were performed in triplicate with a minimum of four areas counted per well. The bracketed number indicates mean % adhesion taking into account the number of cells originally seeded per unit area. Error bars represent  $\pm 1$  SD.

despite high levels of expression (Fig. 1 A). Thus, these melanoma cells appear to recognize and adhere to L1 exclusively through a heterophilic interaction that is Ca<sup>++</sup>-dependent.

#### **M21 Cell Adhesion to L1/NILE Is Dependent on Integrin $\alpha_v\beta_3$**

Heterophilic interaction between members of the integrin family and members of the IgSF have been documented (Staunton et al., 1990; Ager and Humphries, 1991). Recently, it has been reported that  $\alpha_v\beta_3$  can recognize PE-CAM (Piali et al., 1995). We therefore determined whether an  $\alpha_v$  integrin may also be involved in melanoma cell adhesion

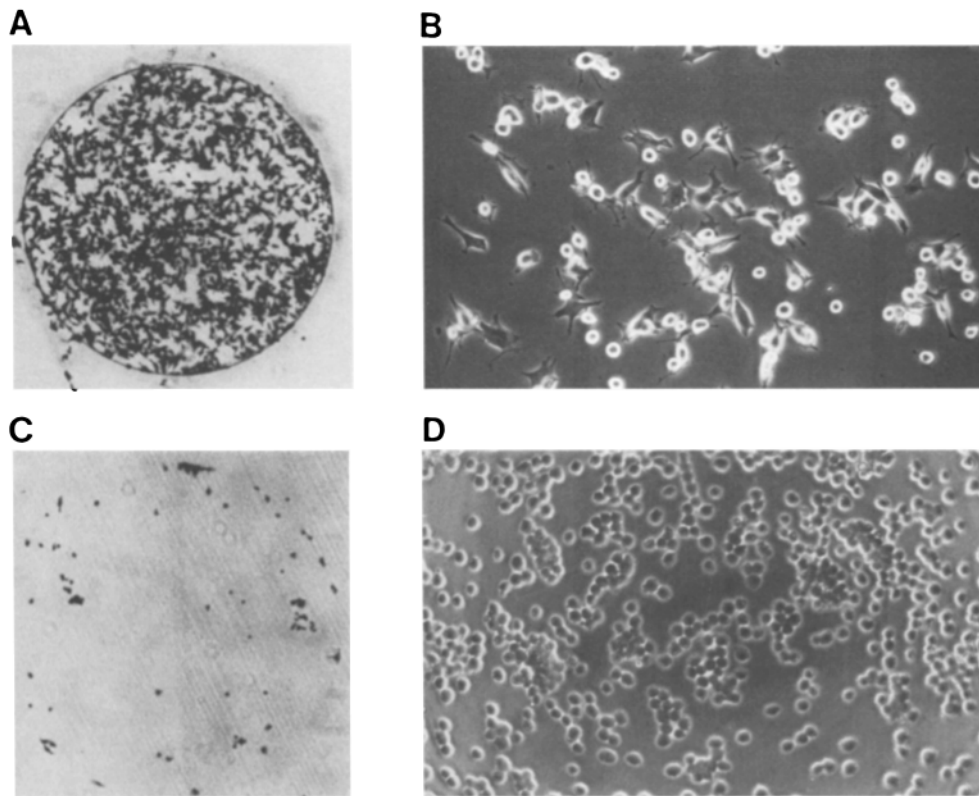
to purified NILE/L1. In this regard, it was previously documented that M21 cells express significant levels of  $\alpha_v\beta_3$  and some  $\alpha_v\beta_5$  (Felding-Habermann et al., 1992). To address this issue we exploited M21 cell variants either expressing or lacking the  $\alpha_v$ -integrin subunit (Felding-Habermann et al., 1992). Thus, adhesion experiments were performed with wild-type  $\alpha_v(+)$  M21 cells, selected  $\alpha_v$ -negative M21-L cells,  $\alpha_v(+)$  M21-L4 cells (M21-L cells transfected with the  $\alpha_v$ -subunit cDNA), and  $\alpha_v$ -negative M21-L12 cells (M21-L mock transfectants). Importantly, only the  $\alpha_v(+)$  M21 and M21-L4 cells showed significant adhesion to rat NILE/L1 (Fig. 6 A). These results demonstrate that M21 cells can use an  $\alpha_v$ -integrin to bind to NILE/L1.

To define the  $\alpha_v$  integrin involved, adhesion experiments were performed with function blocking mAbs to  $\alpha_v\beta_3$  (LM609) and to  $\alpha_v\beta_5$  (P3G2). Confirming a role for  $\alpha_v\beta_3$ , adhesion was almost completely abrogated by mAb LM609 (Fig. 6 B). In contrast, neither mAb P3G2 or a control binding mAb to HLA class I antigen (W6/32) significantly affected M21 adhesion. Further supporting a role for  $\alpha_v\beta_3$ , adhesion could also be abrogated by cyclic RGD peptide 66203 (cyclo-RGDfv) which can specifically inhibit  $\alpha_v\beta_3$  ligation (Fig. 6 B). In contrast, control peptide 69601 (cyclo-RADfv) did not significantly affect adhesion. Significantly, while heat denaturation of the NILE/L1 ligand prevented homophilic interaction with the L1(+) myeloma cells, it did not abrogate M21 cell attachment via  $\alpha_v\beta_3$  (data not shown). This would suggest that this integrin can recognize both native and denatured forms of the molecule.

#### **Optimal Adhesion and Spreading on Human L1 Requires $\alpha_v\beta_3$ Ligation to an RGD Sequence in Its Sixth Immunoglobulin-like Domain**

Rat L1/NILE has two RGD sequences, and one of these sequences is conserved in man (Hlavin and Lemmon, 1991). To determine whether  $\alpha_v\beta_3$  can still recognize human L1 we looked for evidence of  $\alpha_v\beta_3$ -dependent adhesion and spreading on L1 immunopurified from M21 cell lysates. As observed with rat L1, optimal adhesion to the human L1 required expression of the  $\alpha_v$ -subunit (Fig. 7 A) while adhesion of wild-type M21 cells could be partially abrogated with mAb LM609 (Fig. 7 B). However, it is important to note that the contribution of  $\alpha_v\beta_3$  to adhesion to human L1 was less than that to rat L1/NILE. There may be several explanations for this finding. First, as stated, human L1 only has one RGD site as compared to two in rat L1/NILE. Secondly, it is clear that  $\alpha_v\beta_3$  is not the only receptor responsible for the recognition of human L1. Indeed, we have preliminary evidence that a  $\beta_1$ -integrin may also be responsible for adhesion to human L1 and to a lesser extent to rat L1/NILE (data not shown). It is important to note, however, that while LM609 only partially prevented adhesion it did have a greater impact on M21 cell spreading. Thus, inhibition of cell spreading in the presence of LM609 was greater than 60% (not shown).

To define the  $\alpha_v\beta_3$  binding site within human L1, we performed a series of adhesion experiments with recombinant L1-fragments. Three fragments covering the entire extra-cellular domain of human L1 were tested and con-

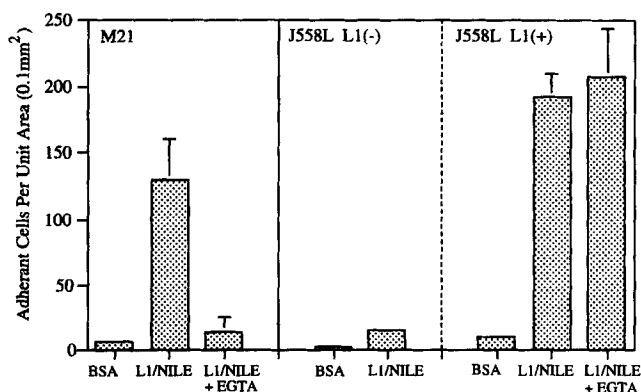


**Figure 4.** Photomicrographs of M21 cells seeded onto immunopurified NILE/L1 in the presence (C) or absence (A and B) of 5 mM EGTA. (D) Morphology of L1(+) J588L transfectants seeded onto NILE/L1. Adherent cells were photographed at 4 $\times$  (A and C) or at 20 $\times$  (B and D). Note that adherent M21 cells are largely confined to the circular area spotted with immunopurified NILE/L1 (A), and that adhesion is abrogated by the presence of EGTA (C). Note that the M21 cells show significant cell spreading, while the adherent J588L cells remain rounded even after 180 min (D).

sisted of Ig-like domains 1 through 3 (Ig 1-3), Ig-like domains 4 through 6 (Ig 4-6), and the fibronectin-like domains 1-5 (FN 1-5). Of these fragments, the fragment Ig 4-6 (amino acids 352-595) was most effective in supporting M21 cell adhesion (Fig. 8 A). Significantly, adhesion to this fragment was absent in  $\alpha_v$ -negative cells (Fig. 8 B) and consistent with  $\alpha_v\beta_3$  ligation, attachment by wild-type M21 cells could be abrogated by mAb LM609 (Fig. 8 C). A second longer L1 fragment inclusive of Ig-like domains 3 through 6 (Ig 3-6) was also tested in adhesion assays and

was also found to support significant M21 cell adhesion (Fig. 8 D). Adhesion to this fragment was also inhibited by mAb LM609 (data not shown). This fragment was isolated under conditions that did not require refolding of the protein (refer to Materials and Methods).

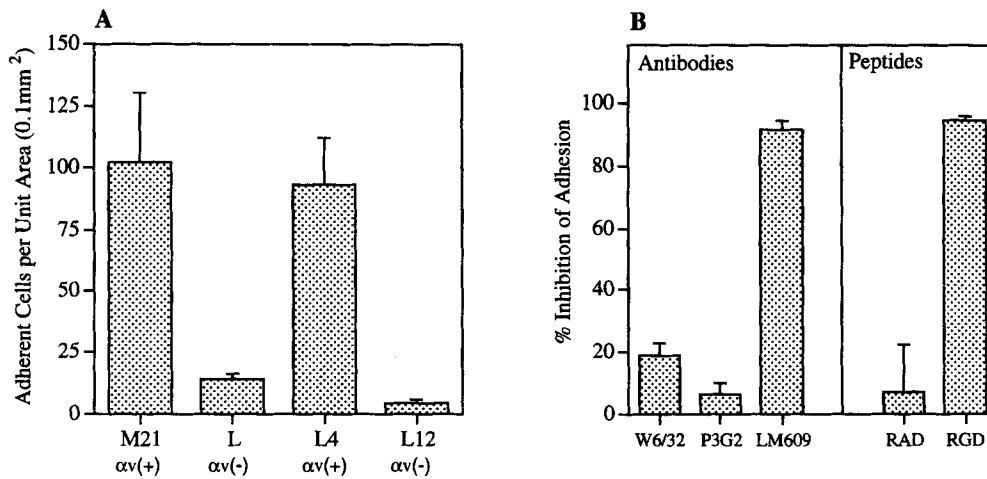
Attachment to two fragments that both include the sixth Ig like domain of human L1 (Ig 3-6, Ig 4-6) is consistent with the presence of an RGD site in the sixth Ig-like domain (Hlavin and Lemmon, 1991). To confirm the importance of this RGD site we generated a further Ig 3-6 fragment with a disrupted RGD sequence (refer to Materials and Methods). Consistent with RGD-dependent adhesion by  $\alpha_v\beta_3$  there was an almost complete loss of attachment to the mutated fragment (Fig. 8 D).



**Figure 5.** M21 cells adhere to immunopurified NILE/L1 via a heterophilic, calcium-dependent mechanism. M21 cells, J588L cells, or J588L L1 transfectants were allowed to adhere to NILE/L1 for 120 min in the presence or absence of EGTA (5 mM). Adherent cells were counted per unit area using a 20 $\times$  high powered objective. Experimental treatments were performed in triplicate with a minimum of four areas counted per well. Error bars represent  $\pm 1$  SD.

### Human L1 Supports Haptotactic Melanoma Migration via Integrin $\alpha_v\beta_3$

It is well documented that  $\alpha_v\beta_3$  ligation can promote cell migration (Cheresh 1992; Leavesley et al., 1992). Therefore, we determined whether ligation of human L1 by  $\alpha_v\beta_3$ , could induce cell motility or migration. This was assessed using Transwell plates essentially as described (Leavesley et al., 1992). Two important findings are evident from these studies. First, human L1 can support haptotactic migration by melanoma cells (Fig. 9) and second,  $\alpha_v\beta_3$  appears to play a major role in the induction of this migration. Thus, mAb LM609 reduced migration to the immunopurified human L1 by  $\sim 60\%$  (Fig. 9). However, the same antibody reduced adhesion by only  $\sim 35\%$  (Fig. 7). Together these data suggest that  $\alpha_v\beta_3$  recognition of human L1 may be more important for the induction of cell spreading and motility than adhesion.



**Figure 6.** Adhesion of M21 cells to immunopurified NILE/L1 is dependent on ligation of integrin  $\alpha_v\beta_3$ . (A) M21  $\alpha v$ -variants were seeded onto NILE/L1 and allowed to adhere for 120 min. (B) M21 cells were seeded onto NILE/L1 and allowed to adhere in the presence or absence of specific antagonists. Cells were pretreated with mAbs (40  $\mu\text{g/ml}$ ) or peptides (20  $\mu\text{g/ml}$ ) for 30 min before the addition of both cells and inhibitors to prepared wells. Adherent cells were counted per unit area with a 20 $\times$  high powered objective.

Experimental treatments will be performed in triplicate with a minimum of four areas counted per well. Integrin-specific mAbs used in these studies included LM609 (anti- $\alpha_v\beta_3$ ), and P3G2 (anti- $\alpha_v\beta_5$ ). Control mAb W6/32 is specific for HLA-class I antigens. Peptides used included inhibitory cyclic RGD peptide 66203 (cyclo-RGDfv) and control RAD peptide 69601 (cyclo-RADfv). For inhibition studies results are expressed as percent inhibition relative to adhesion by untreated control cells. Error bars represent  $\pm 1$  SD.

### L1-CAM Shed by M21 Melanoma Cells Can Be Detected in Tumor-associated Extracellular Matrix

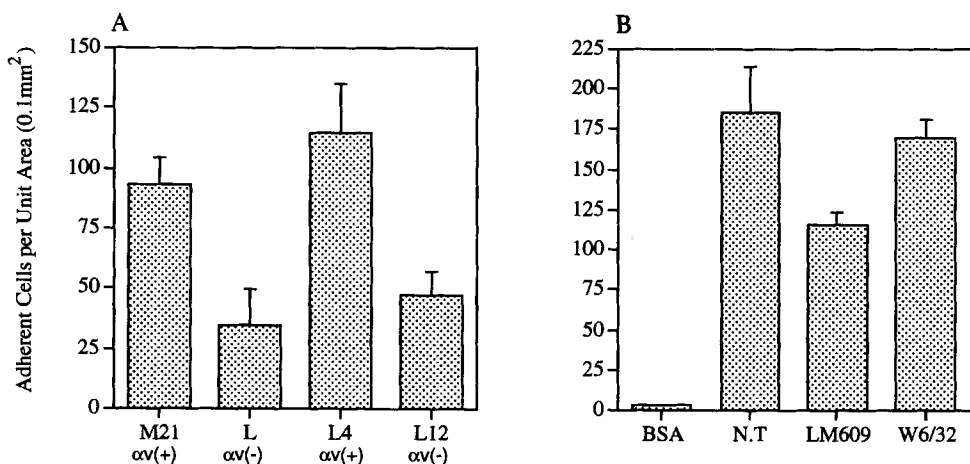
It is documented that L1 is shed by neuroectodermal tumors (Mujoo et al., 1986), and that shed L1 can associate with elements of the ECM (Martini and Schachner, 1986; Poltorak et al., 1990). Ligation between  $\alpha_v\beta_3$  and L1, could therefore occur between  $\alpha_v\beta_3$  (+) cells and L1 deposited in the ECM. In this study we demonstrate that M21 cells also shed high molecular mass L1 fragments (Fig. 2, lane 3). Given these observations we wished to determine whether this shed L1 could bind to tumor-associated ECM. To address this question we looked for evidence of L1 deposition in the stroma of M21 cell tumors grown in *scid/scid* mice. Interestingly, staining of M21 tumor sections revealed good colocalization between human L1 and strands of intra-tumor laminin (Fig. 10). This is an important finding since it suggests that by shedding L1, mel-

noma cells may be able to modify their extracellular environment to promote  $\alpha_v\beta_3$  ligation.

### Discussion

In this manuscript we describe a novel interaction between L1, a member of the IgSF, and the integrin  $\alpha_v\beta_3$ . We demonstrate that this interaction is dependent on  $\alpha_v\beta_3$  binding a single RGD sequence in the sixth Ig-like domain of human L1. We further show that despite high levels of L1 expression, M21 melanoma cells do not express significant homophilic L1-L1 adhesion.

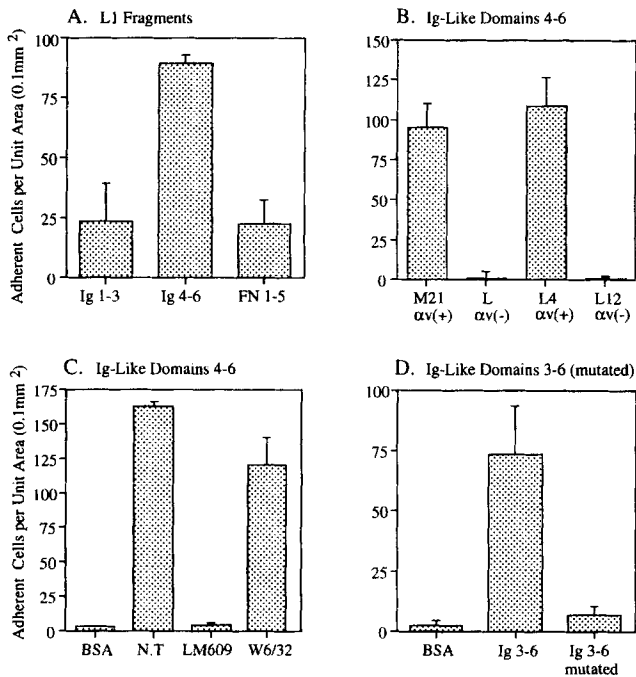
Although L1 is described as a neural CAM, its expression is not confined to cells of the nervous system. Indeed, this CAM has been described on cells as diverse as lymphocytes and intestinal epithelial cells (Thor et al., 1987; Kowitz et al., 1992). L1 has also been detected on trans-



**Figure 7.** Adhesion of M21 cells to immunopurified human L1 is partially dependent on ligation of integrin  $\alpha_v\beta_3$ . (A) M21  $\alpha v$ -variants were seeded onto L1 and allowed to adhere for 90 min. (B) M21 cells were seeded onto L1 and allowed to adhere for 90 min to in the presence or absence of specific mAbs. The cells were pretreated with the mAbs (40  $\mu\text{g/ml}$ ) for 30 min before the addition of both cells and inhibitors to prepared wells. Adherent cells were counted per unit area with a

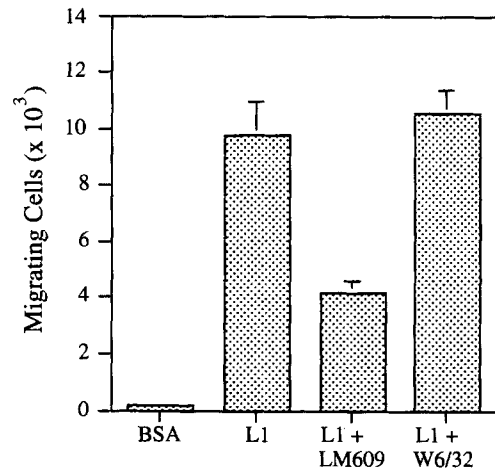
20 $\times$  high powered objective. Experimental treatments were performed in triplicate with a minimum of four areas counted per well. mAbs used include LM609 (anti- $\alpha_v\beta_3$ ), and control mAb W6/32 (anti-HLA-class I). Error bars represent  $\pm 1$  SD. N.T. is ligand alone with no treatment.





**Figure 8.** Adhesion of M21 cells to recombinant L1 fragments. (A) M21 cells were seeded onto L1 fragments consisting of Ig-like domains 1 through 3 (Ig 1-3), Ig-like domains 4 through 6 (Ig 4-6), and fibronectin-like repeats 1 through 5 (FN 1-5) and were allowed to adhere for 90 min. (B) M21  $\alpha_v$ -variants seeded onto Ig-like domains 4 through 6 (Ig 4-6). (C) M21 cells were seeded onto Ig-like domains 4 through 6 (Ig 4-6) in the presence or absence of mAb LM609 (anti- $\alpha_v\beta_3$ ), or control mAb W6/32 and were allowed to adhere for 90 min. The cells were pretreated with the mAbs (40  $\mu\text{g/ml}$ ) for 30 min before the addition of both cells and inhibitors to preprepared wells. (D) M21 cells were seeded onto a recombinant fragment inclusive of Ig-like domains 3 through 6 with a mutated RGD sequence in the sixth Ig-like domain. Adherent cells were counted per unit area with a 20 $\times$  high powered objective. Experimental treatments will be performed in triplicate with a minimum of four areas counted per well. Error bars represent  $\pm 1$  SD. N.T. is ligand alone with no treatment.

formed cell lines of different histological origin (Mujoo et al., 1986; Linnemann et al., 1989; Reid and Hemperly, 1992). The  $\alpha_v\beta_3$  heterodimer has been reported on endothelial cells, fibroblasts, smooth muscle cells, cells of the immune system, and some tumor cell lines (Cheresh, 1992; Brooks et al., 1994). Such expression suggests that L1- $\alpha_v\beta_3$  pairing may occur between multiple cell types including cells of the immune and nervous systems and the vasculature. In this regard, it is interesting that Hubbe et al. (1993) have shown that L1 can promote the adhesion of murine leukocytes to endothelial cells and demonstrated that this interaction is calcium dependent and required an undefined heterophilic ligand. While L1- $\alpha_v\beta_3$  interaction may contribute to heterotypic or homotypic cell-cell adhesion, it may be that this interaction is more important for the generation of secondary responses relating to cell motility, survival and growth. Thus, in addition to promoting cell adhesion,  $\alpha_v\beta_3$  is documented to promote cell migration (Leavesley et al., 1992), suppress apoptosis (Montgomery et al., 1994), and stimulate mitogenesis (Sanders et al., 1992).

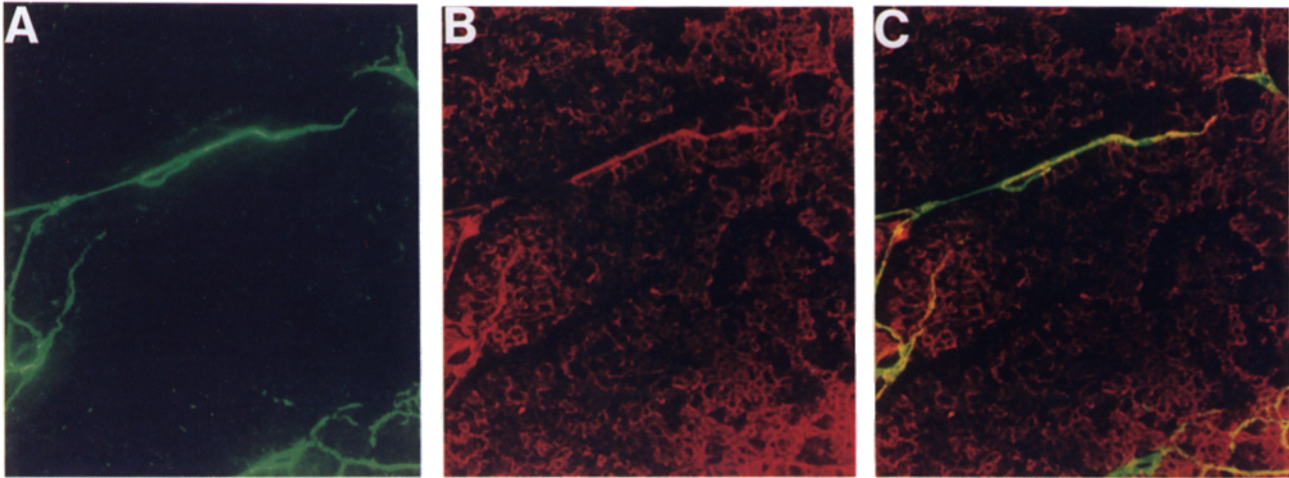


**Figure 9.** Haptotactic migration of M21 cell to human L1. L1 was coated onto the underside of membranes of Transwell inserts at  $\sim 100$  ng/mm<sup>2</sup>. M21 cells were subsequently added to upper chambers at  $1 \times 10^5$  cells per chamber and after 18 h the directed migration of cells from upper to lower chambers quantified by enumerating the number of cells both at the bottom of the lower chambers and associated with the undersides of membranes. mAb LM609 (anti- $\alpha_v\beta_3$ ) and control mAb W6/32 were added at 40  $\mu\text{g/ml}$  to both the lower and upper chambers. Treatments were performed in duplicate. Error bars represent  $\pm 1$  SD.

The interaction between L1 and  $\alpha_v\beta_3$  may have significant ramifications for the progression of malignant melanoma and other L1(+) tumors. In this regard, expression of  $\alpha_v\beta_3$  has been directly linked to melanoma progression (Albeda et al., 1991; Felding-Habermann, 1992; Albelda, 1993; Montgomery et al., 1994). Significantly, the same  $\alpha_v$ -negative M21 variants used in this study, have been shown to be significantly less tumorigenic in the skin of nude mice than their  $\alpha_v$ (+) counterparts (Felding-Habermann, 1992). We have previously reported that the capacity of  $\alpha_v\beta_3$  to promote neoplastic progression may be linked to its ability to promote tumor cell survival and growth (Montgomery et al., 1994). Interestingly, L1 expression has also been linked to melanoma progression. Thus, Linnemann et al. (1989) describe L1 expression on a metastatic variant of the melanoma cell line K1735, while non-metastasizing K1735 cells were found to be negative.

In addition to promoting interaction between cells it is also conceivable that ligation may occur between  $\alpha_v\beta_3$ (+) cells and L1 deposited in the ECM. In this respect, L1 may have a dual function both as a CAM and a substrate adhesion molecule (SAM). In support of this concept we present evidence that M21 cells shed high molecular mass L1 fragments and demonstrate L1 immunoreactivity in the stroma of M21 tumors. In this regard, a number of studies have described incorporation of shed L1 into the occluding ECM. In the adrenal medulla, for example, L1 immunoreactivity has been found in the ECM adjacent to chromaffin cells (Poltorak et al., 1990). Martini and Schachner (1986) have reported L1 expression in basement membranes associated with murine Schwann cells and in association with collagen fibrils of the endoneurium. Given these observations, it is conceivable that by shedding L1 tumors can modify their immediate extracellular environ-





**Figure 10.** Colocalization of shed L1 and laminin in the stroma of a M21 tumor. A primary tumor generated in an immunodeficient mouse was resected, sectioned and stained for both laminin (A) and L1 (B). L1 was detected using human L1-specific mAb 5G3. The laminin was detected using a laminin-specific rabbit polyclonal antibody (Serotec, UK). Murine mAb 5G3 was labeled using a human absorbed goat F(ab')<sub>2</sub> anti-mouse Ig-Rhodamine conjugate, while the rabbit Ig was labeled using a human absorbed goat F(ab')<sub>2</sub> anti-mouse Ig-fluorescein conjugate. Tumor sections were visualized and photographed using an Olympus BX60F fluorescent microscope and a UPlan Apo 20× objective. To further demonstrate colocalization, fluorescein and rhodamine fluorescence was merged (C). Colocalization is evident as yellow colorization. Note that laminin is evident as strands within the tumor stroma (A), while L1 is evident both on the M21 cells and in association with the strands of laminin (B and C).

ment to promote  $\alpha_v\beta_3$  ligation. While we demonstrate good colocalization between L1 and intra-tumor laminin we cannot claim on the basis of these data alone that direct ligation occurs between these ligands. However, it is interesting that an L1-related molecule in the chick (Ng-CAM) has been shown to bind to the short arms of laminin (Grumet et al., 1993a). It is also of interest that L1 has been shown to interact with matrix chondroitin sulfate proteoglycans (Grumet et al., 1993b; Friedlander et al., 1994).

Molecular cloning and sequencing of human and rat L1 has demonstrated the presence of a single RGD sequence that is conserved between both species (Hlavin and Lemmon, 1991; Miura et al., 1991). Our findings suggest that conservation of this sequence may reflect its functional significance as a binding site for  $\alpha_v\beta_3$ . Accepting that this RGD site is important for integrin recognition, one would expect it to be accessible in the native molecule. To test this we performed a structural analysis based on the documented sequence of the sixth Ig-like domain of human L1 (Hlavin and Lemmon, 1991). By analogy to known structures of other C2 type Ig-like domains (Williams and Barclay, 1988; Rao et al., 1993), the RGD sequence in Ig-like domain 6 of L1 may correspond to the C'-E loop (data not shown). Since this region of the L1 molecule has a high density of charged residues it is expected that this loop will be exposed to the external aqueous environment and thus be available for integrin interaction. The findings of our structural analysis are similar to those recently reported for the interaction between the integrin VLA-4 and Ig-like molecule VCAM-1, in which the integrin was shown to recognize an exposed C-D loop region (Wang et al., 1995).

Denaturation experiments suggest that  $\alpha_v\beta_3$  can recognize L1 in either a native or denatured state (data not shown). This is consistent with the fact that  $\alpha_v\beta_3$  can also recognize either native or denatured vitronectin (unpub-

lished observation). This is an important finding since it suggests  $\alpha_v\beta_3$  may be able to recognize forms of L1 that have been denatured as a result of proteolysis. In this regard, L1 cleavage products have been described both in the ECM and tightly associated with the cell membrane (Sadoul et al., 1988). Recognition of denatured L1 may be particularly important in the context of melanoma, since these transformed cells generally express increased proteolytic activity.

Given the presence of two RGD sites in rat NILE/L1 we cannot exclude recognition of both by this integrin. Indeed such an interaction may explain why we observed greater  $\alpha_v\beta_3$ -dependent adhesion on rat L1 than on its human homologue. It is important to emphasize that a component of M21 cell binding to L1 could not be abrogated with antagonists of  $\alpha_v\beta_3$ . Thus, it is probable that other heterophilic ligands are capable of recognizing L1. In this regard, we have some preliminary evidence that a  $\beta_1$ -integrin may also be involved. However, the  $\beta_1$ -heterodimer involved remains to be defined. Since RGD-dependent adhesion is not exclusively a property of  $\alpha_v\beta_3$  we do not exclude the possibility that other integrins may also display RGD dependent adhesion to L1. The extent and nature of integrin involvement may ultimately vary according to cell type.

It is important to note that while transfected L1(+) myeloma cells displayed significant homophilic adhesion on immunopurified L1/NILE, such an interaction was not observed with the M21 melanoma cells. This was the case despite the fact that both cell lines expressed high levels of L1. A reduction in L1-L1 adhesion in transformed cells may reduce cohesion in the primary tumor and thus promote metastasis. In this regard, an interesting parallel may be drawn between our findings and those reported for NCAM. Thus, NCAM is likewise expressed on many highly malignant tumors and most significantly, due to ei-

ther alternative mRNA splicing or posttranslational modifications, tumor-associated NCAM has a reduced capacity for adhesive homophilic interactions (Moolenaar et al., 1992). The mechanism(s) regulating the adhesive potential of L1-L1 interactions remains to be determined. However, it is interesting to note that melanoma cells express an alternative form of L1 that has a four amino acid deletion (RSLE) within the cytoplasmic region (Kobayashi et al., 1991). In contrast, this sequence is present in the L1 expressed by the transfected J558L myeloma cells that did express significant homophilic L1-L1 adhesion. While a homophilic L1-L1 interaction did not appear to support significant melanoma adhesion we do not exclude the possibility that this interaction may still occur at a level that can promote signal transduction.

Documented interactions between integrins and members of the IgSF include those between VCAM-1 and VLA-4, between ICAM-1/2 and LFA-1 (Staunton et al., 1990; Ager and Humphries, 1991), and that recently reported between  $\alpha_v\beta_3$  and PE-CAM (Piali et al., 1995). Here we have described an additional novel pairing between  $\alpha_v\beta_3$  and the neural cell adhesion molecule L1. The consequences of  $\alpha_v\beta_3$  ligation are diverse and include the regulation of cell adhesion, migration, survival, growth, and differentiation. Identification of L1 as a heterophilic ligand for  $\alpha_v\beta_3$  suggests a role for L1 that may go beyond those currently described during neurological development.

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