A Single Immunoglobulin-like Domain of the Human Neural Cell Adhesion Molecule L1 Supports Adhesion by Multiple Vascular and Platelet Integrins

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Abstract. The neural cell adhesion molecule L1 has been shown to function as a homophilic ligand in a variety of dynamic neurological processes. Here we demonstrate that the sixth immunoglobulin-like domain of human L1 (L1-Ig6) can function as a heterophilic ligand for multiple members of the integrin superfamily including αvβ3, αvβ1, α5β1, and αⅦbβ3. The interaction between L1-Ig6 and αⅦbβ3 was found to support the rapid attachment of activated human platelets, whereas a corresponding interaction with αvβ3 and αvβ1 supported the adhesion of umbilical vein endothelial cells. Mutation of the single Arg-Gly-Asp (RGD) motif in human L1-Ig6 effectively abrogated binding by the aforementioned integrins. A L1 peptide containing this RGD motif and corresponding flanking amino acids (PSITWRGDGRDLQEL) effectively blocked L1 integrin interactions and, as an immobilized ligand, supported adhesion via αvβ3, αvβ1, α5β1, and αⅦbβ3. Whereas β3 integrin binding to L1-Ig6 was evident in the presence of either Ca²⁺, Mg²⁺, or Mn²⁺, a corresponding interaction with the β1 integrins was only observed in the presence of Mn²⁺. Furthermore, such Mn²⁺-dependent binding by α5β1 and αvβ1 was significantly inhibited by exogenous Ca²⁺. Our findings suggest that physiological levels of calcium will impose a hierarchy of integrin binding to L1 such that αvβ3 or active αⅦbβ3 > αvβ1 > α5β1. Given that L1 can interact with multiple vascular or platelet integrins it is significant that we also present evidence for de novo L1 expression on blood vessels associated with certain neoplastic or inflammatory diseases. Together these findings suggest an expanded and novel role for L1 in vascular and thrombogenic processes.

Pioneering studies on the structure and function of L1 have established this cell adhesion molecule (CAM) as a member of the immunoglobulin superfamily (IgSF) that plays a quintessential role in neural development (Lindner et al., 1983; Moos et al., 1988). Functions attributed to this neural CAM include such dynamic processes as cerebellar cell migration (Lindner et al., 1983) and neurite fasciculation and outgrowth (Lagenaur and Lemmon, 1987).

Human and mouse L1 and L1-related glycoproteins in the rat (nerve growth factor–inducible, large external glycoprotein [NILE]), chick (neuron–glial [Ng]CAM, 8D9, G4), and Drosophila (neuroglia) have been described (Gruen et al., 1984; Bock et al., 1985; Lemmon and McLoon, 1986; Mujoo et al., 1986). These homologues share an extracellular structure consisting of six Ig-like domains and five fibronectin type III–like repeats (Moos et al., 1988; Sonderegger and Rathjen, 1992). These extracellular domains are linked via a single transmembrane sequence to a short, highly conserved cytoplasmic domain (Reid and Hemperly, 1992). Limited structural variation within the human L1 molecule has been reported and can be attributed to variable glycosylation and two alternatively spliced mini exons (Reid and Hemperly, 1992; Jouet et al., 1995).
Reflecting its designation as a neural CAM (NCAM), L1 is highly expressed on postmitotic neurons of the central and peripheral nervous systems and on pre- or nonmyelinating Schwann cells of the peripheral nervous system (Lindner et al., 1983; Rathjen and Schachner, 1984; Martini and Schachner, 1986). Although classified a neural recognition molecule, L1 has also been identified on non-neuronal cell types of surprisingly diverse origin. Thus, we and others, have recently described L1 on human immune cells of both myelomonocytic and lymphoid origin (Ebeling et al., 1996; Pancook et al., 1997). In the first of these studies, we report an interaction between human L1 and the vitronectin receptor (Sibille et al., 1997). In a subsequent study, we further demonstrated that this CAM can support the attachment of both endothelial cells (α1β1) and activated platelets (α1β3). Given the interaction between L1 and the vascular integrins α1β3 and α1β1, it is significant that we also describe de novo L1 expression on blood vessels associated with certain neoplastic or inflammatory diseases. Based on these findings we suggest expanded and novel roles for L1-integrin interactions in vascular and thrombogenic processes.

**Materials and Methods**

**Antibodies**

Anti-integrin antibodies used include the following: anti-hamster α1β1 mAb PB1, anti-αv, and β3 integrin polyclonal (anti-vitronectic receptor [VNR]), anti-α5β1 mAb LM609, anti-αvβ3 mAb L3-CP8, anti-β3 integrin mAb 7E3, anti-β1, integrin mAb P4C10, and anti-αv, integrin mAb 17E6. PB1 was generated and provided by Dr. R. Juliano (University of North Carolina, Chapel Hill, NC), (Brown and Juliano, 1985). Anti-β3 integrin mAb P4C10 was provided by Dr. E.A. Wayner (University of Minnesota, Twin Cities, MN). The 7E3 antibody was originally generated and characterized by Coller et al. (1986) and the 17E6 antibody (Mitjans et al., 1995) was provided by Dr. S.L. Goodman (Merck KGaA, Darmstadt, Germany). LM609 (Cheresh and Spiro, 1987), anti-VNR, and L1-CP8 (Nijja et al., 1987) were generated within the Scripps Research Institute (La Jolla, CA). The anti-human L1 mAb 5G3 used in this study, was also generated and characterized within the Scripps Research Institute (Mujoo et al., 1986).

**Peptides**

L1 peptides were synthesized on a peptide synthesizer (ABI 430A; Applied Biosystems, Inc., Foster City, CA) within the Scripps Research Institute Core Facility. A 15-mer peptide was selected to include the single RGD site in human L1 (i.e., PSITWRGDGRDLQEL). Control peptides were substituted with alanine to give PSITWRQADGRDLQEL. For the purpose of immobilization an additional batch of these peptides was made with NH2-terminal cysteine residues. Peptides were prepared using Rink Amide MBHA or Wang resin (Calbiochem-Novabiochem, La Jolla, CA). After resin deprotection and assembly the peptides were cleaved from the resin with a cleavage cocktail (2.5% ethanedithiol, 5% thiouanisole, 5% water, 87.5% trifluoroacetic acid) and subsequently purified by preparative reverse phase HPLC. Peptides were characterized further by analytical HPLC and mass spectroscopy.
Cell Lines and Culture

The generation and characterization of CHO cells stably transfected to express normal human platelet αIIbβ3 (A5 cells) has been described in detail elsewhere (O’Toole et al., 1989, 1990; Frojmovic et al., 1991) and will be described only briefly here. CHO cells were cotransfected with equal amounts of human αIIb and β3 expression constructs and a CD8-my vector containing the neomycin resistance gene CDNeo at a ratio of 30:1 (O’Toole et al., 1989). Transfection was performed by the calcium phosphate method followed by glyceral shock. G418-resistant colonies were isolated and positive clones identified by flow cytometry using subunit-specific antibodies. The generation of CHO cells transfected to express the active extracellular domain of human αIIbβ3 (A5 cells) has been described (O’Toole et al., 1994). Briefly, the cytoplasmic sequence from the αIIb integrin subunit was engineered to contain a microcin D (MC-D) internal deletion mutant (αIIbΔMC) by ligating chimeric cDNA clones by PCR with the MC-D oligonucleotides designed to omit the αIIb cytoplasmic sequence V(ess) (i.e., αIIbΔMC). As previously described, the αIIbΔMC construct was ligated with a fragment encoding the extracellular and transmembrane domains of the αIIb integrin subunit. Coexpression of this chimeric internal deletion mutant (αIIbΔMC) with the wild-type β3 integrin subunit resulted in the stable expression of the αIIbΔMC,β3 heterodimer bearing an active, high affinity extracellular domain of human αIIbβ3 (O’Toole et al., 1994). Wild-type CHO-K1 cells and transfected cell lines were maintained in DME supplemented with 10% FCS, 1% glutamine, and 1% non-essential amino acids.

A spontaneously transformed, human umbilical vein endothelial cell line designated ECV304 (Hughes, 1986) was obtained from the American Type Culture Collection (Rockville, MD). A stable αβ3β3-negative variant of this line was obtained by repeated negative sorting using anti-αβ3 mAb LM609. Sorting of αβ3β3-negative unstained cells was performed using a FACStar® flow cytometer (Becton Dickinson, Co., Mountain View, CA). To obtain a stable, αβ3β3-negative population, the cells were sorted on five consecutive occasions. The ECV304 cells were maintained in M199 medium supplemented with 10% FCS and 1% glutamine.

Isolation of Human Platelets

Blood was collected from the antecubital vein of healthy adult donors through a 19-gauge needle into syringes containing, as anticoagulant, the thrombin inhibitor n-phenylalanyl-n-arginine chloromethyl ketone dihydrochloride (PPACK; Bachem Bioscience Inc., Philadelphia, PA) (50 nM final concentration) and supplemented, when indicated, with ADP scavenger apyrase (Sigma Chemical Co.) and centrifuged at 2,500 g for 10 min to isolate platelet-rich plasma. Platelet-rich plasma was removed and replaced with an equivalent volume of Heps-Tyrode’s buffer, pH 7.0 (5.0 mM Hepes, 140 mM NaCl, 0.3 mM KCl, 0.4 mM NaH2PO4, 5 mM NaHCO3, and 5 mM dextrose), containing 1 U/ml of apyrase. The resuspended blood cells were centrifuged again at 2,500 g for 10 min. The blood cells were washed twice using Heps-Tyrode’s buffer containing 0.2 U/ml apyrase in the next step and no apyrase in the last step. The final blood cell pellet was resuspended in 1 mM CaCl2, 1 mM MgCl2, and 100 μM MnCl2. The platelet count was adjusted to 100,000 platelets/μl. To analyze the effect of activation on platelet adhesion, the platelets were stimulated with ADP and epinephrine (20 μM final concentration) immediately before adding the platelet suspension to the assay plates. Adhesion of non-activated platelets was studied using unstimulated platelets prepared from PGE1-treated blood.

Construction and Expression of L1 Fusion Proteins

Two wild-type and two mutant L1–glutathione-S-transferase (GST) fusion proteins were used in this study. The wild-type fusion proteins consisted of Ig-like domains 4, 5, and 6 (L1-Ig4-6) or the sixth Ig-like domain alone (L1-Ig6). The mutant fusion proteins consist of the sixth Ig-like domain of L1 with the amino acid mutations Arg-554 and Asp-556 to Lys-554, and Glu-556, respectively (i.e., RGD−KGE) or the single amino acid mutation Asp-556 to Ala-556 (i.e., RGD−RGA). Amino acids are numbered as described by Bateman et al. (1996).

The generation and characterization of the L1-Ig4-6 GST fusion protein used in this study has been described in detail elsewhere (Zhao and Siu, 1995). The production of L1-Ig6 GST fusion protein (amino acids 518–641) was as follows. The region of interest was amplified from full-length L1 cDNA, which was provided by Dr. J. Hemperly (Becton Dickinson Research Center, Research Triangle Park, NC). Amplification was performed according to the manufacturer’s instructions for the Expand High Fidelity PCR System (Boehringer Mannheim Corp., Indianapolis, IN) using an upstream sense primer specific for nucleotides 1,542–1,562 of the human L1 open reading frame (ORF) and containing an engineered internal EcoRI restriction endonuclease site (5′-CTT CAC ATC ACT-3′) in conjunction with a downstream antisense primer specific for nucleotides 1,829–1,849 of the human L1 ORF, which was also engineered to contain an internal EcoRI site (5′-CGT GAA TTC GGC CCA GGC CTC-3′). The resulting product was digested with EcoRI and subcloned into a pGEX-1X vector (Pharmacia Biotech Sevage, Upplands Väsby, Sweden). Competent E. coli BL21 cells (Stratagene, La Jolla, CA) were transformed with this construct and resulting colonies were screened by PCR and examined for expression of appropriately sized GST fusion protein by SDS-PAGE, followed by immunoblotting with an anti-GST polyclonal antibody (Upstate Biotechnology, Inc., Lake Placid, NY). The chemiluminescent substrate PS-3 (Lumigen, Inc., Southfield, MI) was used for detection. Dideoxy sequencing of positive clones was performed to verify the integrity of the introduced coding sequence.

The mutant L1-Ig6 RGD−RGA was generated according to the manufacturer’s instructions for the Quickchange Site-Directed Mutagenesis Kit (Stratagene) using the plasmid DNA encoding the L1-Ig6 fusion protein (pGEX-1X-L1-Ig6) as template. Briefly, oligonucleotides corresponding to the sense and anti-sense sequences of bases 1,654–1,682 of the L1 ORF, which included a change from A to C at base 1,666 (sense: 5′-GCT GGC GTG GGG GGC GTC GAG ACC TTC AG-3′; antisense: 5′-CTG GAG GTG TCG ACC GGC CCC ACC CCA GG-3′) were annealed to a heat-denatured template, and the construct was replicated using Pfu DNA polymerase for 18 cycles. The resulting mixture was digested with the methylation-dependent endonuclease DpnI to degrade the wild-type template. Supercompetent E. coli strain XL-1 blue cells were transformed with this construct by heat shock and resulting colonies were screened and sequenced as described above.

To generate the mutant L1-Ig6 RGD−KGE the primers for an equivalent L1-Ig6 construct (forward primer: 5′-CTG GAG CAT CAC TGA GGC GC-3′; and the reverse primer: 5′-GGC AAC AAT TCT GGG ATC CCG GCC CAC GGC TCC CCA C-3′) encoding for amino acids 518–614, were used in conjunction with the mutagenic primers 5′-CAT CAC CTG GAA GGG GGA GGA TCG ACC AGA CC-3′ and 5′-GTA GTG GAC CCT CCC CCT CCC AAC TCT GTG-3′ in the fourth primer method (Higuchi, 1990). The amplified product was digested with BamHI and subcloned into this site of pGEX-3X for expression in the E. coli strain JM101. The nucleotide sequence of the insert was confirmed by double-strand DNA sequencing using the T7 Sequencing™ kit (Pharmacia Biotech Sevage).

Purification of the recombinant fusion proteins was performed using isopropylthio-β-D-galactoside–induced log-phase cultures essentially as described by the manufacturer for the GST Gene Fusion System (Pharmacia Biotech Sevage). Briefly, recovered bacteria were lysed by sonication and incubated with detergent before clarification on a Sepharose 4B column. Purification of the recombinant protein on a Sepharose 4B–coupled, glutathione affinity matrix (Pharmacia Biotech Sevage). After extensive washing, the GST fusion proteins were eluted from the matrix with 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0, and dialyzed extensively against PBS before use. The fusion proteins were subject to SDS-PAGE to confirm the correct mobility and to confirm purity.

Flow Cytometry

Integrin expression was assessed by FACs™ analysis. Subconfluent cultures were harvested and stained with anti-integrin mAbs at 20 μg/ml or polyclonals diluted 1:40. The cells were then treated with an anti-mouse or anti-rabbit IgG, FITC-conjugated antibody, and were analyzed with a FACScan® flow cytometer (Becton Dickinson, Co., Mountain View, CA). Control cells were treated with secondary FITC-conjugated antibody only.

Adhesion Assays

Adhesion experiments were performed as detailed by Lagaur and Lemmon (1987) with some modifications. Purified L1-Ig6 GST fusion proteins (L1-Ig6 or L1-Ig4-6) dialyzed into PBS were spotted (1-μl spots) and
coated onto the bottom of 96-well Titertek plates (ICN Pharmaceuticals, Inc., Costa Mesa, CA) as described (Montgomery et al., 1996). Unless otherwise noted, the fusion proteins were offered at a concentration of 40 μg/ml. Treated and control wells were blocked with 5% BSA for 1–3 h at 37°C. For adhesion studies involving immobilized peptides, wells were precoated overnight with murine IgG2a antibody at 20 μg/ml. Antibody-treated and washed wells were then incubated with the heterofunctional cross-linker, N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), at 30 μg/ml in PBS for 45 min. These wells were then washed and peptides added at 100–200 μg/ml for 2–3 h. Control wells received antibody and SPDP alone. Treated and control wells were blocked with 5% BSA for 1–3 h at 37°C.

CHO cells were harvested using EDTA (0.526 mM) in PBS (versene; Irvine Scientific, Santa Ana, CA) and ECV304 cells with a trypsin-versene mixture (Biowhittaker, Walkerville, MD). All the cells were then given a further wash with the EDTA solution to remove residual cations. The cells were then resuspended in adhesion buffer consisting of HBSS (without calcium and magnesium) supplemented with 10 mM Hepes, and BSA (0.2–1%), with the pH adjusted to 7.4. Divalent cations were added as indicated in the text and included MnCl₂ (0.4 mM), MgCl₂ (1–2 mM), and CaCl₂ (1–2 mM). Platelets were harvested and resuspended in Hepes-Tyrode’s buffer as described above. For inhibition studies, the cells and platelets were pretreated with polyvalent antibodies (1:30 dilution), mAbs (80 μg/ml) or peptides (25 μM) for 30 min before the addition of both cells and inhibitors to pretreated wells. Cells were added at 10⁵/well and platelets at 5 × 10³/well, and then these plates were spun at 700 rpm to give a continuous monolayer of cells or platelets on the floor of each well. Endothelial cells and CHO cells were allowed to adhere for 20–40 min at 37°C, while the platelets were allowed to adhere for 10 min. At the end of the assay the wells were carefully washed with PBS, and non-adherent cells removed under a constant vacuum. Remaining adherent cells were fixed with 1% paraformaldehyde, and enumerated with the aid of an inverted light microscope. Cells were counted per unit area using a ×15 high powered objective and an ocular grid with a minimum of four areas counted per well. Alternatively, adherent cells or platelets were stained for 20 min with 1% crystal violet in 0.1 M borate, pH 9.0. Dye was eluted with 10% acetic acid and its absorbance determined at 600 nm.

**Immunohistochemistry**

Frozen sections of normal human skin, squamous cell carcinoma, psoriatic skin, and synovial tissue from the knee joint of patients diagnosed with rheumatoid arthritis were stained for the L1 antigen using mAb 5G3 or rheumatoid arthritis were stained for the L1 antigen using mAb 5G3 or mAb LM609. Frozen sections were fixed in cold acetone before removal of endogenous peroxidase with 0.03% H₂O₂. Sections were blocked with 10% goat serum and 1% BSA in PBS. mAbs (80 μg/ml) or peptides (25 μM) were added at 100–200 μg/ml for 30 min before the addition of both cells and inhibitors to pretreated wells. Cells were added at 10⁵/well and platelets at 5 × 10³/well, and then these plates were spun at 700 rpm to give a continuous monolayer of cells or platelets on the floor of each well. Endothelial cells and CHO cells were allowed to adhere for 20–40 min at 37°C, while the platelets were allowed to adhere for 10 min. At the end of the assay the wells were carefully washed with PBS, and non-adherent cells removed under a constant vacuum. Remaining adherent cells were fixed with 1% paraformaldehyde, and enumerated with the aid of an inverted light microscope. Cells were counted per unit area using a ×15 high powered objective and an ocular grid with a minimum of four areas counted per well. Alternatively, adherent cells or platelets were stained for 20 min with 1% crystal violet in 0.1 M borate, pH 9.0. Dye was eluted with 10% acetic acid and its absorbance determined at 600 nm. All experimental treatments were performed in triplicate.

**Results**

**Characterization of CHO-K1 Cells and Transfectants**

Currently, α₃β₃ is the only integrin that has been shown to interact with human L1 (Ebeling et al., 1996; Montgomery et al., 1996; Duzcma et al., 1997; Pancook et al., 1997). To determine whether L1 is also a ligand for the platelet integrin α₁₇β₃, we used a CHO cell line (A5) genetically altered to express human platelet α₃β₃ (O’Toole et al., 1989, 1990; Fromovitch et al., 1991). To further determine whether α₁₇β₃ needs to be in an active state to recognize L1 we used a CHO cell line (α₁₇β₃Δβ₃) transfected to express α₁₇β₃Δβ₃ in a constitutively active state (O’Toole et al., 1994). Activation was achieved by chimerization of extracellular and transmembrane α₁₇ with a cytoplasmic deletion mutant of the α₇ integrin subunit (O’Toole et al., 1994). The integrin profile of these transfected cell lines and the CHO wild type was determined by flow cytometry using anti–hammer or anti–human integrin–specific antibodies (Fig. 1A). A number of salient conclusions can be drawn from this analysis. First, both transfected cell lines have been successfully manipulated to express high levels of human α₁₇β₃ (L1-CP8 reactivity). Second, transfection of the human β₃ integrin subunit has also resulted in a pairing with endogenous hamster α₇, and consequently expression of chimeric α₇β₃ (LM609 reactivity). Finally, and of immediate relevance for this study, wild-type and transfected CHO express high levels of endogenous α₃β₃ (PB-1 reactivity) and endogenous α₁₇ integrin(s) (VN1 reactivity).

**Wild-Type and Transfected CHO Cells Display Concentration-dependent Adhesion to L1-Ig6**

We have previously demonstrated that purified full-length L1 and a recombinant L1 fusion protein (L1-Ig4-6) can support the adhesion of melanoma cells via the integrin α₇β₃ (Montgomery et al., 1996). We further proposed that it is the sixth Ig-like domain of human L1 that is likely to be relevant for such adhesion by virtue of the presence of a single RGD motif in this domain. Based on this proposition, and for the purposes of this study, we generated a fusion protein consisting of this L1 domain alone (i.e., L1-Ig6). As a first step, this L1-Ig6 recombinant protein was tested for its ability to support adhesion by wild-type and transfected CHO cells.

Importantly, the L1-Ig6 fusion protein supported significant concentration-dependent adhesion by all three CHO cell lines (Fig. 1B). Furthermore, transfected CHO cells exhibited greater adhesion than the wild-type CHO-K1 cells (Fig. 1B). These data clearly indicate the importance of the sixth Ig-like domain of L1 for mediating cellular adhesion, and also suggest that transfection and expression of β₃ integrins (α₃β₃ and/or α₁₇β₃) can lead to enhanced binding to L1-Ig6. Significantly, CHO cells bearing the active α₁₇β₃Δβ₃ (α₁₇β₃Δβ₃ cells) showed the greatest level of adhesion, particularly at lower L1-Ig6 concentrations (Fig. 1B). When offered at saturating concentrations (i.e., >50 μg/ml), L1-Ig6 supported >90% attachment of the α₁₇β₃Δβ₃ cells in contact with the substrate, resulting in a continuous monolayer of spreading cells and these cells were resistant to detachment by the large shear forces generated during the washing of the 96-well plates. When offered at a saturating concentration, vitronectin gave an equivalent response (data not shown).

**Wild-type CHO-K1 Cells Interact with L1-Ig6 Using α₃β₃ and an α₁₇ Integrin(s) and These Heterophilic Interactions Are Differentially Regulated by Divalent Cations**

From the data presented in Fig. 1B it is evident that the wild-type CHO-K1 cells can interact with the L1-Ig6 fusion protein. To characterize this wild type adhesion we looked for evidence of either β₃ or α₁₇ integrin involvement.
In the presence of Ca\(^{2+}\), Mg\(^{2+}\), and Mn\(^{2+}\), CHO-K1 cell adhesion was completely abrogated by a VNR polyclonal antibody, indicating the involvement of one or more \(\alpha_5\) integrins (Fig. 2A, left). Despite high levels of \(\alpha_5\beta_1\) expression, a function blocking mAb specific for hamster \(\alpha_5\beta_1\) (PB1) had no impact on adhesion in this cation environment (Fig. 2A, left). However, in the presence of Mn\(^{2+}\) alone, CHO-K1 adhesion to L1-Ig6 could only be abrogated using a combination of VNR polyclonal antibody and the anti-\(\alpha_5\beta_1\) mAb PB1 (Fig. 2A, right). This finding indicates that in the presence of Mn\(^{2+}\) alone, \(\alpha_5\beta_1\) can also recognize human L1-Ig6. However, it is also clear that binding by \(\alpha_5\beta_1\) must be acutely susceptible to inhibition...
by either Ca\(^{2+}\) or Mg\(^{2+}\) since, as stated, we did not observe \(\alpha_5\beta_1\) involvement when all three cations were present in the adhesion buffer (Fig. 2 A, left).

Since Ca\(^{2+}\) has been shown to inhibit \(\beta_1\) integrin ligation (Kirchhofer, 1991; Mould et al., 1995) we determined whether this cation was responsible for a lack of \(\alpha_\text{Rb}1\) involvement in a mixed cation environment (Fig. 2 A, left). To this end, CHO-K1 cells were allowed to adhere to L1-Ig6 in the presence of Mn\(^{2+}\) and increasing concentrations of exogenous Ca\(^{2+}\). To discriminate between \(\alpha_\text{Rb}1\) and \(\alpha_{\text{III}}\beta_3\) integrin–dependent binding, the cells were treated with either PB1 or anti-VNR antibody. Using this approach, a significant inverse correlation was observed between the concentration of exogenous Ca\(^{2+}\) and \(\alpha_\text{Rb}1\)-dependent binding (Fig. 2 B) such that the addition of 2 mM calcium reduced the level of \(\alpha_\text{Rb}1\)-dependent binding by >80\% (Fig. 2 B). In contrast, the same concentration of calcium reduced the \(\alpha_{\text{III}}\) integrin(s)–dependent binding by only 20\%.

It is important to note that when the divalent cations were added to the adhesion buffer individually only Mn\(^{2+}\) could support significant wild-type CHO-K1 adhesion (Fig. 2 C). Together these findings indicate, not only an absolute requirement for Mn\(^{2+}\), but also a pivotal role for Ca\(^{2+}\) in the differential regulation of integrin binding to L1.

**CHO Transfectants Use Both \(\alpha_\text{Rb}1\) and Activated \(\alpha_{\text{III}}\beta_3\) to Interact with L1-Ig6**

Thus far, it has been shown that the CHO transfectants used in this study have been successfully manipulated to express significant levels of both \(\alpha_\text{Rb}1\) and \(\alpha_{\text{III}}\beta_3\) (Fig. 1 A) and that these same cells have an increased ability to bind to the sixth Ig-like domain of L1 (Fig. 1 B). A further series of experiments was performed to determine whether these \(\beta_1\) integrins can indeed recognize L1 and, if so, how the binding of these integrins is regulated.

In contrast to the situation with the wild-type CHO-K1 cells, cells transfected to express \(\alpha_\text{Rb}1\) and \(\alpha_{\text{III}}\beta_3\) (A5 and \(\alpha_{\text{III}}\alpha_\text{Rb}1\Delta\beta_3\), cells) were able to recognize L1 in the presence of either Ca\(^{2+}\) alone or Mg\(^{2+}\) alone (Fig. 3 vs. Fig. 2 C). Most importantly, this de novo adhesion could be attributed to both the chimeric \(\alpha_\text{Rb}1\) and active \(\alpha_{\text{III}}\beta_3\). Thus, CHO cells engineered to express active \(\alpha_{\text{III}}\beta_3\) (\(\alpha_{\text{III}}\alpha_\text{Rb}1\Delta\beta_3\), cells) showed significant adhesion to L1-Ig6 in the presence of Ca\(^{2+}\) or Mg\(^{2+}\), and this adhesion could only be significantly reduced using a combination of antibodies to both \(\alpha_\text{Rb}1\) and \(\alpha_{\text{III}}\beta_3\) (Fig. 3, bottom). It is important to note, that in the presence of Ca\(^{2+}\) alone, \(\alpha_{\text{III}}\beta_3\)-dependent binding to L1-Ig6 was only evident in the CHO cells engineered to express active \(\alpha_{\text{III}}\alpha_\text{Rb}1\beta_3\) (\(\alpha_{\text{III}}\alpha_\text{Rb}1\Delta\beta_3\), cells). Thus, in A5 cells bearing \(\alpha_{\text{III}}\beta_3\) in its resting state, adhesion to L1-Ig6 in the presence of Ca\(^{2+}\) was fully inhibited by mAb LM609 (i.e., \(\alpha_\text{Rb}1\) dependent) with no evident contribution by \(\alpha_{\text{III}}\beta_3\) (Fig. 3, top left). This finding implies that \(\alpha_{\text{III}}\beta_3\)-dependent recognition of L1 requires this integrin to be in its activated state. However, it is interesting to note that Mg\(^{2+}\) alone is sufficient to activate \(\alpha_{\text{III}}\beta_3\) binding by the A5 cells. Thus, the adhesion of these cells, in the presence of Mg\(^{2+}\) alone, could only be abrogated using antibodies to both \(\alpha_{\text{III}}\beta_3\) and \(\alpha_\text{Rb}1\) (Fig. 3, top right). Because of endogenous \(\alpha_{\text{III}}\) integrin and \(\alpha_\text{Rb}1\) binding, adhesion in the presence of Mn\(^{2+}\) could only be abrogated using a combination of polyclonal antibodies to both \(\alpha_\text{Rb}1\) and \(\beta_1\) integrins (VNR and PB1, data not shown). The ability of the different cations to support adhesion of the transfected cell lines was in the order Mn\(^{2+}\) > Mg\(^{2+}\) > Ca\(^{2+}\) (Fig. 3; Mn\(^{2+}\) not shown).

**Platelets Interact with L1-Ig6 Via Activated \(\alpha_{\text{III}}\beta_3\)**

Having identified activated \(\alpha_{\text{III}}\beta_3\), as a heterophilic ligand for L1-Ig6 in our CHO model we wished to determine whether this integrin will also mediate platelet attachment. An interaction between L1- and platelet \(\alpha_{\text{III}}\beta_3\) would then suggest a novel physiological function in thrombogenic processes. In this regard, L1 could contribute either as a cellular ligand expressed on myelomonocytic cells (Pancook et al., 1997), metastatic neuroectodermal tumors (Linnemann et al., 1989), or endothelial cells, or as a shed ligand in solution or associated with subcellular matrix (Martini and Schachner, 1986; Pottorak et al., 1990; Montgomery et al., 1996).

In adhesion assays comparable to those performed with the CHO cells we observed that L1-Ig4-6, L1-Ig6, and the immobilized L1-derived peptide (C)PSITWRGDGRDQEL could all support the rapid and significant attachment of activated platelets (Fig. 4, A–C). This adhesion was not affected by anti-\(\alpha_\text{Rb}1\) mAb LM609 alone, but was...
abrogated by the mAb 7E3, which is a potent function-blocking antibody, specific for both \( \alpha_{\text{IIb}} \beta_3 \) and \( \alpha_{\text{IIb}} \beta_3 \). The affinity of activated platelets for immobilized L1-Ig6, and the strong inhibitory effect of mAb 7E3, is also evident from the photomicrographs presented in Fig. 4, A–C. The contribution of \( \alpha_{\text{IIb}} \beta_3 \) was also confirmed using the specific anti--\( \alpha_{\text{IIb}} \beta_3 \) mAb LI-CP8, but this mAb was generally less effective than 7E3 (data not shown). It is important to note that these results were only obtained using platelets bearing active \( \alpha_{\text{IIb}} \beta_3 \) as a result of stimulation with ADP and epinephrine. Thus, unstimulated platelets, prepared from PGE_1-treated blood, showed only minimal adhesion to L1-Ig4-6 (Fig. 4 A). These data, like the data obtained using the CHO cells, clearly indicate that \( \alpha_{\text{IIb}} \beta_3 \) needs to be in an active conformation to bind L1. However, as described for other peptides, recognition of the L1-derived peptide was not fully dependent on platelet activation (Fig. 4 C). When offered at saturating concentrations, the L1 fusion proteins supported the rapid attachment of a uniform monolayer of platelets such that the majority of platelets adhered and in contact with the substrate appeared to attach (Fig. 4 D). When used as a positive control, vitronectin supported comparable levels of platelet attachment (data not shown). However, as with L1-Ig4-6 significant attachment to vitronectin required platelet activation.

**Figure 4.** Activated \( \alpha_{\text{IIb}} \beta_3 \) can mediate the attachment of platelets to immobilized L1 fusion proteins and the L1-derived peptide (C)PSITWGRDGRDLQEL. (A–C) Stimulated or unstimulated human platelets were allowed to adhere to immobilized L1 fusion proteins (L1-Ig4-6 or L1-Ig6; A and B) or to immobilized, L1-derived RGD peptide (C). Some of the platelets were pretreated with antibody to \( \alpha_{\text{IIb}} \beta_3 \) (mAb LM609; 80 \( \mu \)g/ml), or to \( \alpha_{\text{IIb}} \beta_3 \) and \( \alpha_{\text{IIb}} \beta_3 \) (mAb 7E3; 80 \( \mu \)g/ml). The platelets were allowed to adhere for 10 min in the presence or absence of the antibodies and the adherent platelets subsequently stained with crystal violet for quantification. Experimental treatments were performed in triplicate. Error bars represent \( \pm 1 \) SD. (D and E) Photomicrographs of stimulated platelets adhering to L1-Ig6 in the absence (D) or presence of mAb 7E3 (E). Adherent platelets were stained with crystal violet and are confined to the circular area coated with the fusion protein. Individual platelets cannot be discriminated because of their small size and some aggregation. The platelets were allowed to adhere for 10 min in the presence or absence of 7E3. Any minimal residual adhesion to BSA-blocked plastic alone has been subtracted from the OD values shown. Bar, 300 \( \mu \)m.

**Endothelial Cells Interact with L1-Ig6 Using \( \alpha_{\text{IIb}} \beta_3 \) and \( \alpha_{\text{IIb}} \beta_3 \) and These Heterophilic Interactions Are Differentially Regulated by Divalent Cations**

L1 is expressed on a variety of cell types known to interact with endothelium (Ebeling et al., 1996; Pancook et al., 1997); and shed L1 may also associate with components of the subendothelial matrix (Hall et al., 1997). This distribution raises the question of if and how endothelial cells interact with L1. To address this issue we used a transformed cell line (ECV304) derived from human umbilical vein endothelial cells (Hughes et al., 1996). These wild-type cells were found to express both \( \alpha_{\text{IIb}} \beta_3 \) and a high level of \( \beta_3 \) integrins (Fig. 5 A, *left column*). We also detected expression of \( \alpha_{\text{IIb}} \beta_3 \) (mAb P1F6) at a median fluorescence comparable to that found for \( \alpha_{\text{IIb}} \beta_3 \) (not shown). Consistent with our findings using the transfected CHO cells, we observed that endothelial \( \alpha_{\text{IIb}} \beta_3 \) can also promote adhesion to L1-Ig6 and this adhesion is evident in the presence of calcium (Fig. 5 B). Adhesion by these wild-type endothelial cells was only marginally inhibited by the anti–\( \beta_3 \) integrin mAb P4C10 (Fig. 5 B). A similar pattern of adhesion was obtained using primary human dermal microvascular endothelial cells (Clonetics, San Diego, CA) (data not shown).

Whereas the contribution of \( \alpha_{\text{IIb}} \beta_3 \) to a variety of vascular processes is well documented it is also clear that this integrin is either absent or only marginally expressed by quiescent endothelial cells (Brooks et al., 1994). Accordingly, we wished to determine whether L1 could also be recognized by \( \alpha_{\text{IIb}} \beta_3 \)-negative endothelial cells. To address this we exploited ECV304 endothelial cells that had been repeatedly FACs® sorted for a lack of \( \alpha_{\text{IIb}} \beta_3 \) expression. This approach proved successful, resulting in the generation of a stable population of \( \alpha_{\text{IIb}} \beta_3 \)-negative cells (Fig. 5 A, *right column*). The levels of \( \alpha_{\text{IIb}} \beta_3 \) and \( \beta_3 \) integrin expression in these cells remained unchanged (not shown; and Fig. 5 A, *right column*). Remarkably, these \( \alpha_{\text{IIb}} \beta_3 \)-negative cells also showed significant adhesion to L1-Ig6 (Fig. 5 C). However, in contrast to the wild-type cells, adhesion was unaffected by an antibody to \( \alpha_{\text{IIb}} \beta_3 \) (LM609) but was fully inhibited by a mAb to \( \beta_3 \) integrins (i.e., P4C10) (Fig. 5 C). Furthermore, such adhesion was also completely abrogated with an antibody specific for \( \alpha_3 \) integrins (Fig. 5 C). Together these results are consistent with an interaction between L1 and endothelial \( \alpha_{\text{IIb}} \beta_3 \).

Given that both \( \alpha_{\text{IIb}} \beta_3 \) and \( \alpha_{\text{IIb}} \beta_3 \) can interact with L1-Ig6, we wished to determine why \( \alpha_{\text{IIb}} \beta_3 \) is the dominant integrin in wild-type ECV304 cell adhesion (Fig. 5 B). In this re-
Cells were treated with antibodies to rabbit antibodies and were analyzed using a FACScan® flow cytometer. Control cells were treated with secondary fluorescein-conjugated antibody only. (A) Integrin expression is represented by FACS® histograms. Cells were treated with antibodies to \( \alpha_v \beta_3 \) integrins (mAb P4C10; 80

![Figure 5](Image 64x398 to 304x750)

Figure 5. (A) Integrin profiles of wild-type ECV304 human umbilical vein endothelial cells or ECV304 cells repeatedly sorted for a lack of \( \alpha_v \beta_3 \) expression. (B and C) \( \alpha_v \beta_1 \) and \( \alpha_v \beta_3 \)-dependent adhesion of wild-type or sorted ECV304 endothelial cells to L1-Ig6. (A) Integrin expression is represented by FACS® histograms. Cells were treated with antibodies to \( \alpha_v \) integrins (mAb 17E6), to \( \alpha_v \beta_3 \) (mAb LM609), to \( \alpha_v \) or \( \beta_3 \) integrin subunits (polyclonal VNR), or to \( \beta_3 \) integrins (mAb P4C10). These cells were subsequently stained with fluorescein-conjugated goat anti–mouse or goat antirabbit antibodies and were analyzed using a FACScan® flow cytometer. Control cells were treated with secondary fluorescein-conjugated antibody only. (B and C) Wild-type or sorted cells were allowed to adhere to immobilized L1-Ig6 fusion protein offered at 40 \( \mu \)g/ml. Adhesion was performed in the presence of Ca\(^{2+} \) (2 mM), Mg\(^{2+} \) (2 mM), and Mn\(^{2+} \) (0.4 mM). Some cells were pretreated with antibody to \( \alpha_v \beta_3 \) (mAb LM609; 80 \( \mu \)g/ml), to \( \beta_3 \) integrins (mAb P4C10; 80 \( \mu \)g/ml), to \( \alpha_v \) integrins (mAb 17E6; 80 \( \mu \)g/ml), or with a combination of antibodies. After 40 min non-adherent cells were removed by washing and the remaining adherent cells were counted per unit area with a \( \times 15 \) high powered objective. Both, a combination of the two preceding antibodies. Experimental treatments were performed in triplicate with a minimum of four areas counted per well. Error bars represent \( \pm 1 \) SD.

![Figure 6](Image 322x448 to 562x750)

Figure 6. Usage of \( \alpha_v \beta_1 \) by wild-type ECV304 human umbilical vein endothelial cells is dictated by a requirement for Mn\(^{2+} \) and by inhibition by Ca\(^{2+} \). (A) Wild-type ECV304 endothelial cells were allowed to adhere to immobilized L1-Ig6 in the presence of Ca\(^{2+} \) alone (1 mM), Mg\(^{2+} \) alone (1 mM), or Mn\(^{2+} \) alone (0.4 mM). Some cells were pretreated with antibody to \( \alpha_v \beta_3 \) (mAb LM609; 80 \( \mu \)g/ml), to \( \beta_3 \) integrins (mAb P4C10; 80 \( \mu \)g/ml), to \( \alpha_v \) integrins (mAb 17E6; 80 \( \mu \)g/ml), or with a combination of antibodies. (B) Wild-type or sorted endothelial cells were allowed to adhere to immobilized L1-Ig6 fusion protein in the presence of Mn\(^{2+} \) (0.4 mM) alone or in combination with increasing concentrations of Ca\(^{2+} \) (0.5–2 mM). After 40 min non-adherent cells were removed by washing and the remaining adherent cells were counted per unit area with a \( \times 15 \) high powered objective. Both, a combination of the two preceding antibodies. Experimental treatments were performed in triplicate with a minimum of four areas counted per well. Error bars represent \( \pm 1 \) SD.

of Ca\(^{2+} \) could be abrogated with anti-\( \alpha_v \beta_3 \) mAb LM609 (Fig. 5 B), the adhesion of these cells in the presence of Mn\(^{2+} \) alone could only be blocked using a combination of antibodies reactive with both \( \alpha_v \beta_3 \) and \( \alpha_v \beta_1 \) (i.e., LM609 and P4C10), or with a mAb reactive with both \( \alpha_v \) integrins (i.e., 17E6) (Fig. 6 A, right). Second, minimal wild-type adhesion was observed in the presence of Ca\(^{2+} \) or Mg\(^{2+} \) alone and this appeared to be fully dependent upon \( \alpha_v \beta_3 \) (Fig. 6 A). Finally, in an experiment analogous to that performed with the wild-type CHO cells (Fig. 2 B), we observed that the \( \alpha_v \beta_3 \)-mediated (Mn\(^{2+}\)-dependent) adhesion of the sorted, \( \alpha_v \beta_3 \)-negative endothelial cells was significantly more susceptible to inhibition by exogenous Ca\(^{2+} \) than the wild-type adhesion (Fig. 6 B). Together these data indicate that in the absence of \( \alpha_v \beta_3 \) expression, quiescent endothelial cells may use \( \alpha_v \beta_1 \) to bind to L1,
However with the induction of $\alpha_5\beta_3$ expression, physiological levels of calcium are likely to favor the use of this integrin. It is also clear that whereas $\alpha_5\beta_1$-mediated adhesion is susceptible to inhibition by exogenous $Ca^{2+}$, this inhibition is less pronounced than that seen when adhesion is mediated via $\alpha_5\beta_1$ (Fig. 2B).

**The Interaction between L1-Ig6 and the $\beta_3$ or $\beta_1$ Integrins Is RGD-dependent**

Thus far we have demonstrated that a single Ig-like domain of L1 can support multiple integrin interactions. This same domain contains an RGD integrin recognition motif that may provide the binding site for all the aforementioned integrins. However, it is also clear that a given RGD site may or may not support adhesion depending on flanking sequences, conformational restraints and accessibility (D’Souza et al., 1991; Haas and Plow, 1994). Furthermore, although $\alpha_5\beta_3$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_5\beta_3$ have all been reported to interact with RGD motifs, $\alpha_5\beta_1$ and the $\beta_3$ integrins have also been shown to interact with non-RGD sequences (Koivunen et al., 1994). To help address this issue we generated a 15-mer peptide based on a sequence in the sixth Ig-like domain of L1 and inclusive of the RGD recognition motif (i.e., PSITWRGDGRDLQEL). This peptide was tested for its efficacy both as an immobilized ligand and as soluble inhibitor of L1 integrin binding.

Supporting the concept of a RGD-dependent interaction we observed that our L1-RGD peptide, once immobilized, could support significant endothelial cell attachment via $\alpha_5\beta_3$, and $\alpha_5\beta_1$ (Fig. 7, Endothelial) and CHO cell attachment via endogenous $\alpha_5\beta_1$ and transfected $\alpha_5\beta_3$ (Fig. 7, CHO). Specific integrin–peptide interactions were confirmed using the function blocking antibodies indicated (Fig. 7). When offered at the same concentration, a control peptide (C)PSITWRADGRDLQEL was ineffective as an adhesive ligand. However, it should be noted that because of the conservative glycine to alanine mutation (i.e., RGD→RAD) this control peptide could also support some attachment but only at significantly higher concentrations (not shown).

Further support for RGD-dependent interaction with L1 was obtained using the same L1-RGD peptide as a soluble inhibitor. At a concentration of 25 $\mu$M, the peptide effectively abrogated endothelial cell adhesion to L1-Ig6 in the presence of Mn$^{2+}$ alone (Fig. 8, left). We have previously demonstrated that adhesion by these cells in the presence of this cation involves both $\alpha_5\beta_3$ and $\alpha_5\beta_1$ (Fig. 6 A, right). Likewise, we also observed a complete inhibition of wild-type CHO-K1 adhesion in the presence of Mn$^{2+}$ (Fig. 8, middle). According to our previous data, this is consistent with an inhibition of endogenous $\alpha_5\beta_3$ and the $\alpha_5$ integrin(s) (Fig. 2 A, right). Adhesion by the CHO cells transfected to express active $\alpha_5\beta_3$ was less sensitive to inhibition via the RGD peptide when used at 25 $\mu$M (Fig. 8, right), but could be fully inhibited at higher peptide concentrations (not shown). Used at an equivalent concentration our control peptide was ineffective at preventing adhesion. But again because of the conservative mutation of this peptide (i.e., RGD→RAD) this peptide could also inhibit attachment at high concentrations (e.g., 250 $\mu$M).

Aforementioned work with the L1-RGD peptide supports the concept of a RGD-dependent interaction between integrins and L1. However, to address this issue definitively, we sought to demonstrate that mutation of the RGD site in L1-Ig6 is sufficient to abrogate integrin binding. To this end, we generated additional L1-Ig6 fusion proteins containing the mutations RGD→KGE or RGD→RGA. Importantly, the conservative RGD→KGE mutation completely abrogated $\alpha_5\beta_3$- and $\alpha_5\beta_1$-dependent binding by the endothelial cells (Fig. 9, Endothelial). This same mutation and the RGD→RGA mutation also significantly abrogated $\alpha_5\beta_3$-dependent binding by wild-type CHO cells (Fig. 9, CHO, wild-type). Some residual low level binding to both mutations was still observed, perhaps indicating that the mutated sequences can still be recognized to some extent by some integrins.

*Figure 7.* All of the integrins characterized can recognize the L1-derived peptide (C)PSITWRGDGRDLQEL. Wild-type or sorted ECV304 endothelial cells and wild-type or transfected CHO cells ($\alpha_5\beta_1$, $\Delta\beta_3$ cells) were allowed to adhere to immobilized 16-mer peptide (C)PSITWRADGRDLQEL or to control peptide (C)PSITWRADGRDLQEL. Adhesion was performed in the presence of $Ca^{2+}$ (2 $mM$), $Mg^{2+}$ (2 $mM$), and Mn$^{2+}$ (0.4 $mM$) or in the presence of Mg$^{2+}$ (1 $mM$) alone, or Mn$^{2+}$ alone (0.4 $mM$). Some cells were pretreated with antibody to human $\alpha_4\beta_3$ (mAb LJ-C8; 80 $\mu$g/ml), to $\alpha_5\beta_3$ (mAb LM609; 80 $\mu$g/ml), to $\beta_1$, integrins (mAb P4C10; 80 $\mu$g/ml), to $\alpha_5$ integrins (mAb 17E6; 80 $\mu$g/ml), to hamster $\alpha_5\beta_3$ (PB1 ascites 1:30) or to VNR (polyclonal anti-VNR; 1:30), or with a combination of antibodies. After 40 min, non-adherent cells were removed by washing and the remaining adherent cells stained with crystal violet. Both, a combination of the two preceding antibodies. Experimental treatments were performed in triplicate with a minimum of four areas counted per well. Error bars represent ±1 SD.
degree by α3β1. Alternatively, a second α3β1-binding motif may exist within the L1-Ig6 domain. Interestingly the RGD→KGE mutation could still be recognized to a significant level by αIIbβ3 (Fig. 9, CHO, Active αIIbβ3). However, the alanine substitution mutant (RGA) was sufficient to fully abrogate αIIbβ3-mediated binding.

**L1 Expression Can Be Induced on Endothelial Cells In Vivo**

We have identified a variety of endothelial and platelet integrins that can interact with the sixth Ig-like domain of L1. Such heterophilic interactions prompted us to determine whether L1 can be expressed on endothelial cells. This expression would suggest the potential for L1 integrin interactions in vascular processes such as angiogenesis and thrombosis; these are processes that require homotypic or heterotypic (platelet) interactions involving endothelial cells.

To address this issue, we looked for L1 expression on normal or quiescent blood vessels and on activated or angiogenic vessels associated with neoplastic or inflammatory diseases. In normal human skin, L1 was absent or minimally expressed by the dermal vessels (Fig. 10 G). However, significant expression of L1 was observed on vessels proximal to a squamous cell carcinoma (Fig. 10, A and B). These proximal vessels also expressed high levels of α6β1 (Fig. 10 C); a documented marker of angiogenic blood vessels (Brooks et al., 1994). Importantly, α6β1 and its heterophilic ligand L1, could be colocalized on these angiogenic blood vessels (Fig. 10, D and E). It is important to note, that L1 was not detected on all the angiogenic vessels identified by α6β1 (Fig. 10, D and E); and that whereas α6β1 expression was evident on intra-tumor vessels, L1 was only detected on angiogenic vessels in normal skin tissue peripheral to the tumor (Fig. 10 F). This pattern of expression may indicate the induction of L1 expression during a limited phase of blood vessel maturation. In a preliminary analysis, we did not detect L1 on vessels associated with ei-

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**Figure 8.** Integrin-dependent adhesion to L1-Ig6 can be abrogated or reduced using soluble L1-derived peptide PSITWRG-DGRDLQEL. Wild-type ECV304 endothelial cells and wild-type or transfected CHO cells (αIIbβ3Δβ2 cells) were allowed to adhere to immobilized L1-Ig6. Some cells were pretreated and subsequently adhered in the presence of the soluble peptide PSITWRG-DGRDLQEL (25 μM) or the control peptide PSITWRADGRDLQEL (25 μM). Some CHO cells were pretreated with antibody to human αIIbβ3 (mAb LJ-CP8; 80 μg/ml). Adhesion was performed in the presence of Mg2+ (1 mM) alone, or Mn2+ alone (0.4 mM). After 40 min, non-adherent cells were removed by washing and the remaining adherent cells counted per unit area with a ×15 high powered objective. The level of control adhesion achieved in the absence of peptide inhibitors was taken as 100%. In the case of wild-type endothelial and CHO cells this was equivalent to 188 and 160 adherent cells per field, respectively; in the case of the CHO cells bearing active αIIbβ3, this was equivalent to 232 adherent cells per field. Experimental treatments were performed in triplicate with a minimum of four areas counted per well. Results are expressed as a percent of the adhesion observed on wild-type L1-Ig6. Error bars represent ±1 SD.

**Figure 9.** Mutation of the RGD sequence in the sixth Ig-like domain of L1 abrogates binding by α3β1, α6β1, and αIIbβ3 and reduces binding mediated by α5β1. Wild-type or sorted ECV304 endothelial cells and wild-type or transfected CHO cells (αIIbβ3Δβ2 cells), were allowed to adhere to immobilized L1-Ig6 or to L1-Ig6 containing the mutated sequences RGD→KGE or RGD→RGA. Adhesion was performed in the presence of Ca2+ (2 mM), Mg2+ (2 mM), and Mn2+ (0.4 mM), or in the presence of Mg2+ (1 mM) alone, or Mn2+ alone (0.4 mM). Some cells were pretreated with antibody to human αIIbβ3 (mAb LJ-CP8; 80 μg/ml). After 40 min, non-adherent cells were removed by washing and the remaining adherent cells counted per unit area with a ×15 high powered objective. The level of control adhesion achieved on the wild-type fusion protein was taken as 100%. In the case of wild-type and sorted endothelial cells this was equivalent to 168 and 127 adherent cells per field, respectively, in the case of the wild-type CHO cells this was equivalent to 82 (mixed cations) and 203 (Mn2+ alone) adherent cells per field and in the case of CHO cells bearing active αIIbβ3 this was equivalent to 224 cells per field. Experimental treatments were performed in triplicate with a minimum of four areas counted per well. Results are expressed as a percent of the adhesion observed on wild-type L1-Ig6. Error bars represent ±1 SD.
ther breast or lung tumors suggesting that induction of vascular L1 may be tissue or organ specific.

Interestingly, expression of vascular L1 was also observed in synovial tissues obtained from three out of five patients diagnosed with rheumatoid arthritis (Fig. 10). In a preliminary study, L1 was also detected on vessels in psoriatic skin (not shown). Furthermore, whereas we detected little or no L1 expression on cultured human dermal microvascular endothelial cells (Clonetics) we did detect significant L1 levels on the surface of the ECV304 endothelial cell line (data not shown). Together these findings may indicate that de novo L1 expression can be induced on endothelial cells as a result of stimulation by specific, tumor-associated or inflammatory cytokines. Such vascular L1 may then function as a receptor either for itself or for the vascular and platelet integrins identified in this study.

Discussion

In this work we have detailed the interaction between a single Ig-like domain within L1 and multiple integrins including \( \alpha_{v}\beta_3, \alpha_{v}\beta_1, \alpha_\text{IIb}\beta_3 \), and \( \alpha_\text{IIb}\beta_3 \). To our knowledge this is the first observation that both \( \alpha_{v}\beta_1 \) and \( \alpha_\text{IIb}\beta_3 \) can interact with a member of the IgSF. Indeed, L1 may be unique within this family in its capacity to interact with multiple RGD-dependent integrins. Key structural and regulatory issues have also been addressed including the central importance of a single RGD motif and the critical regulatory effect of physiological levels of calcium. Based on these novel integrin–CAM interactions, and the novel observation that L1 can be expressed on blood vessels under various pathogenic conditions, we propose that L1 may have an expanded and unexpected role in various vascular and thrombogenic processes.

The \( \beta_3 \) and \( \beta_1 \) integrins identified in this study as heterophilic receptors for the sixth Ig-like domain of L1 share a collective ability to recognize RGD motifs within their respective ligands (D’Souza et al., 1991; Haas and Plow, 1994; Marshall et al., 1995). In this regard, the single RGD sequence present in L1-Ig6 (Reid and Hemperly, 1992) would appear to be a legitimate putative recognition motif. However, it is also apparent that the conformational and sequential environment of a given RGD site and its accessibility will ultimately dictate whether it can truly function as a recognition motif for a given integrin. Furthermore, it is now widely documented that non-RGD motifs can also be recognized by \( \alpha_{v}\beta_1 \), \( \alpha_\text{IIb}\beta_3 \), and \( \alpha_\text{IIb}\beta_3 \) (Koivunen et al., 1994). To demonstrate definitively that the single RGD sequence present in human L1 is indeed critical for binding

Figure 10. L1 expression can be induced on blood vessels. L1 expression associated with vessels proximal to a squamous cell carcinoma (A and B). L1 staining was performed using the anti-L1 mAb 5G3 and the substrate AEC to give a red stain. L1 is evident on the small vessels in the dermis (arrows). Vessels proximal to the tumor also expressed \( \alpha_\text{IIb}\beta_3 \): a marker of angiogenic blood vessels (C). Staining for \( \alpha_\text{IIb}\beta_3 \) was performed using LM609 and the substrate AEC to give a red stain. Vascular L1 and \( \alpha_\text{IIb}\beta_3 \) expression could be colocalized on a subset of vessels (D and E). Vessels staining for \( \alpha_\text{IIb}\beta_3 \) are red (AEC), whereas vessels also costaining with L1 (arrow) are darker brown to blue-black (AEC and VIP). Angiogenic vessels expressing L1 were detected in normal dermis between the tumor mass (left arrow) and the epidermis (right arrow) (F). The tumor and epidermis are stained for cytokeratin (F). Vessels in the dermis of normal human skin were found to be negative or very weakly positive for L1 (G). Vessels in synovial tissue derived from the joint of a patient with rheumatoid arthritis also showed evidence of L1 expression (H). L1 staining was performed using the anti-L1 antibody 5G3 and the substrate AEC to give a red stain. Bars: (A) 100 μm; (B) 50 μm; (C and D) 20 μm; (E) 25 μm; (F) 200 μm; (G and H) 100 μm.
by $\alpha_5\beta_1$, $\alpha_\beta_3$, $\alpha_\beta_1$, and $\alpha_\mbox{Iib} \beta_3$ we demonstrate that two mutations of this site in L1-Ig6 reduce or abrogate binding by all four of these integrins. Furthermore we demonstrate that a L1-RGD peptide with the relevant flanking sequences (i.e., PSITWRGDRDLOELE) is effective both as an immobilized substrate and as a soluble inhibitor for all of the integrins identified.

As stated, the sequence environment of a given RGD site is important in determining the strength and specificity of integrin interactions (Kunicki et al., 1997). In the case of L1, the RGD site is flanked by both a tryptophan and a glycine (i.e., WRGDG). In this regard, phage display libraries have identified numerous $\alpha_\beta_1$ and $\alpha_\beta_3$ peptide ligands that include a glycine residue COOH-terminal to the RGD (Healy et al., 1995; and Koivunen et al., 1994). Interestingly, peptides with a strong affinity for $\alpha_\mbox{Iib} \beta_3$ generally have a large hydrophobic residue COOH-terminal to the RGD (O’Neil et al., 1992). This preference may explain why at low concentrations our L1 peptide was more efficient at supporting attachment via $\alpha_\beta_3$ than via $\alpha_\mbox{Iib} \beta_3$ (data not shown). Integrin-binding peptides containing a tryptophan residue NH$_2$-terminal to the RGD have rarely been identified by phage display libraries, but one has been reported with specificity for $\alpha_\beta_3$ (Healy et al., 1995). It is interesting to note, that although we detected significant $\alpha_\beta_3$ expression on the ECV304 endothelial cells used in this study (data not shown), we found no evidence for an interaction between L1-Ig6 (or the L1-RGD peptide) and this RGD-dependent integrin (data not shown). This would suggest that the sequence environment of the RGD site in L1 is not suitable for recognition by $\alpha_\beta_3$.

The conformational or stereochemical presentation of the RGD site is also a key element in dictating receptor recognition and affinity. Secondary structural analysis of RGD recognition motifs in fibronectin (FNIII$_{10}$) and Foot and Mouth Disease Virus support an emerging model of the RGD being presented at the apex of a flexible loop that extends outwards from the protein core. The side chains of Arg and Asp are purported to face away from each other and are flexible enough to adopt the proper conformation for high affinity integrin binding (Haas and Plow, 1994). By using a combination of electron microscopic analysis and computer-assisted modeling, Drescher et al. (1996) conclude that the RGD sites of murine L1, and the single conserved RGD motif of human L1, are exposed at the molecular surface in a loop or turn between two $\beta$-strands. Furthermore they suggest that in the context of the whole molecule, the sixth $\mbox{Ig-like}$ domain of L1 possesses greater surface hydrophobicity than the other $\mbox{Ig-like}$ domains suggesting that it, and contained RGD site(s), can participate in intermolecular interactions.

As a general rule, all integrins require divalent cations for ligand recognition (D’Souza et al., 1994) and two potential mechanisms have been proposed for this dependence. First, specific cations may induce a conformational change in the integrin that favors ligand binding (Mould et al., 1995). Second, the cation may be required to form a ternary complex with the ligand (e.g., RGD) and the integrin; this complex is envisaged to be an unstable but required intermediate with the cation eventually being displaced as the ligand–receptor complex stabilizes (D’Souza et al., 1994). Irrespective of the mechanism, it is now well documented that different divalent cations can dramatically and differentially influence integrin-binding affinity and selection. The findings of this study provide a case in point. Thus, whereas we observed that Ca$^{2+}$ is able to support a limited interaction between L1 and $\alpha_\beta_3$ or $\alpha_\mbox{Iib} \beta_3$, this same cation profoundly suppressed a Mn$^{2+}$-dependent interaction with $\alpha_\beta_1$ or $\alpha_\beta_1$. It is important to note, that such a dichotomy between these $\beta_1$ integrins and the $\beta_3$ integrins has been documented for other ligands. Thus, Ca$^{2+}$ has been shown to support both $\alpha_\beta_3$ or $\alpha_\mbox{Iib} \beta_3$ attachment to RGD peptides or vitronectin (Kirchhofer, 1991; Suehiro et al., 1996), whereas this same cation suppresses the attachment of $\alpha_\beta_1$ and $\alpha_\beta_1$ to fibronectin and an RGD peptide, respectively (Kirchhofer, 1991; Mould et al., 1995). Recently, Suehiro et al. (1996) described a novel classification for defining $\beta_1$ ligands depending upon their ability to support the attachment of $\alpha_\beta_3$ and/or $\alpha_\mbox{Iib} \beta_3$ in the presence of different cations. Thus, class I $\beta_3$ ligands should be able to support attachment of both integrins either in the presence of Ca$^{2+}$ or Mn$^{2+}$. A class II ligand will also support attachment of $\alpha_\mbox{Iib} \beta_3$ in the presence of either Ca$^{2+}$ or Mn$^{2+}$, but $\alpha_\beta_3$ attachment is suppressed by Ca$^{2+}$. Finally, class III ligands bind exclusively to $\alpha_\beta_3$ under all cation conditions, whereas class IV ligands bind exclusively to $\alpha_\beta_3$. According to this classification scheme we propose that L1 is a novel class I ligand for $\beta_3$ integrins, together with vitronectin, RGD peptides and disintegrin group A (Suehiro et al., 1996).

From the findings presented it is probable that Ca$^{2+}$ will act as a potent physiological regulator of L1–integrin interactions. Thus, given the presence or absence of a given integrin, physiological calcium concentrations are likely to favor a hierarchy of L1–integrin interaction such that $\alpha_\beta_3$ or activated $\alpha_\mbox{Iib} \beta_3$ > $\alpha_\beta_1$ > $\alpha_\beta_3$. This hierarchy is likely to be further compounded by transnegative dominance, a recently described phenomenon in which the ligation of a $\beta_3$ integrin has been shown to suppress the function of a $\beta_1$ integrin (Diaz-Gonzalez et al., 1996). It could legitimately be argued from our data that physiological levels of calcium would effectively preclude an interaction between $\alpha_\beta_3$ and L1-Ig6. However, it is important to note that an interaction between $\alpha_\beta_3$ and murine L1 has been observed in the presence of calcium but only after an undefined activation event after ligation of CD24 (heat stable antigen) (Kadmon et al., 1995). Thus, $\alpha_\beta_3$ may indeed have a physiological role in L1 binding, but it is likely to be regulated by a requirement for additional activation signals.

The observation that L1 can support an RGD-dependent interaction with $\alpha_\beta_3$ confirms our earlier study that demonstrated that melanoma cells can interact with either full-length L1 or an L1 fusion protein (L1-Ig4-6) via $\alpha_\beta_3$ (Montgomery et al., 1996). The interaction between human L1 and $\alpha_\beta_3$ has also been confirmed using lymphocytic cell lines (Ebeling et al., 1996). The finding that human L1-Ig6 can also interact with $\alpha_\mbox{Iib} \beta_3$, $\alpha_\beta_1$, and $\alpha_\beta_3$ has not (to our knowledge) been reported, and significantly expands the potential repertoire of heterophilic L1 interactions that this CAM can support and the range of cell types that may be involved. It is important to note that, contrary to our findings, a previous study did not detect any significant interaction between human L1 and $\alpha_\beta_1$ (Ebeling et al., 1996). The most obvious explanation for this is the strict cation
requirement of this interaction and perhaps transnegative

The observation that human L1 can interact with αβ3. It should also be noted that our CHO
cells express very high levels of αβ3. The observation that
human L1 can interact with αβ3 is, however, in agreement
with a report demonstrating an interaction between this
integrin and murine L1 (Ruppert et al., 1995). In this
regard, it is noteworthy that the sixth Ig-like domain of mu-
rine L1 contains an additional RGD sequence (LGD in
human L1) and that this sequence, like the human motif,
may well be available for interaction on an exposed loop
(Drescher et al., 1996). It is conceivable that the absence of
this second RGD sequence in humans may make it a
less favorable ligand for αβ3. However, it is also of inter-
est that human L1 retains some additional non-RGD tri-
peptide sequences such as NGR (fibronectin [FN]-like do-
main 3), STF (FN-like domain 2), and ETA (IG-like
domain 4), which if exposed in the right stereochemical
configuration, could augment the interaction between L1
and αβ3. Thus, all three of these tripeptide sequences
have been identified as important for the interaction of
non-RGD, 7-mer peptides with αβ3 (Koivunen et al., 1993).

L1 and the integrin counter receptors identified in this
study are expressed on multiple cell types of diverse histo-
logical origin. This suggests the potential for a plethora of
interactions and functions that have yet to be described.
This is especially true given the observation that L1 ex-
pression is not strictly confined to cells of the nervous sys-
tem. Thus, we and others have recently described L1 ex-
pression on human cells of both myelomonocytic and
lymphoid origin (Ebeling et al., 1996; Pancook et al., 1997).
L1 has also been described on epithelial cells of the intest-
tine and urogenital tract (Thor et al., 1987; Kowitz et al.,
1992; Kujat et al., 1995) and on transformed cells of di-
verse histological origin, including melanoma, neuroblas-
toma, embryonal carcinoma, osteogenic sarcoma, squamous
lung carcinoma, squamous skin carcinoma, pheochromo-
cytoma, rhabdomyosarcoma and retinoblastoma cell lines
(Mujoo et al., 1986; Linnemann et al., 1989; Reid and
Hemperly, 1992). Finally, in this study we have also shown
that L1 expression can be induced on certain endothelial
cells. This said, however, the extent to which L1-Ig6–inte-
grin pairing contributes to either homotypic or heterotypic
cell–cell interaction among these diverse cell types re-
mains to be determined. It is important to note that Rup-
pert et al. (1995) have demonstrated that the interaction
between murine L1 and αβ3 can promote significant ho-
motypic cell aggregation. It is also of interest that L1–inte-
grin pairing may be modulated by other molecules that as-

strue adhesion molecule. Thus, a number of studies have

have demonstrated a novel interaction between L1-Ig6 and
endothelial cells via either vascular αβ3 or αβ1. It is con-

ceivable that these interactions will contribute to the roll-
ing, arrest, and/or attachment of L1-expressing cells on, or
to, endothelium. This possibility is particularly intriguing
given the expression of L1 on trafficking immune cells and
metastatic tumor cell lines (Linnemann et al., 1989; Reid
and Hemperly, 1992; Ebeling et al., 1996; Pancook et al.,
1997). Our observation that L1 expression can be induced
on blood vessels associated with certain neoplastic or in-
flammatory diseases may indicate a role for L1–integrin or
L1–L1 interactions in the maturation of new blood vessels
and/or reflect an induction of de novo L1 expression by in-
flammatory or tumor-associated cytokines.

Whereas the focus of this study has been on non-neural
cell types, the data presented may have some interesting
implications for neuronal processes. For example, both
αβ3 and αβ1 have been implicated in avian neural crest
cell adhesion and migration (Delannet et al., 1994). In a
recent study, Milner et al. (1996) demonstrated the impor-
tance of αβ1 for migration by oligodendrocyte precu-
sors. Interestingly, the authors speculate that, among other
ligands, L1 present within axonal tracts might serve as a
potential αβ1 ligand, providing a mechanism for guiding
migrating oligodendrocytes. Whereas this is merely specu-
lation, our data clearly support the potential for L1–αβ1
interactions during neural development. At this stage, no
evidence yet exists to show that L1–integrin interactions
promote neurite extension or other neuronal processes in-
volving L1; these processes appear to be primarily depen-
dent upon a homophilic interaction. However, consider-
ation needs to be given to the integrin repertoire of the
cell being tested. For example, αβ1 is expressed on oligo-
dendrocyte precursors but is lost on differentiation. Simi-
larly, attention needs to be given to the appropriate cation
environment. Thus, it is evident from this study that ex-
pression of αβ1 adhesion is dependent upon the presence
of Mn2+ but is inhibited by Ca2+.
The findings of this study extend the range and significance of L1–integrin interactions and add to our understanding of how these heterophilic interactions are regulated. In addition to the documented interaction between human L1 and α5β1, we have demonstrated that this CAM is a relatively promiscuous ligand, supporting further novel heterophilic interactions with α5β1, α6β4, and αHβ3. It is further illustrated that these integrins share a collective ability to recognize a single RGD motif in the sixth Ig-like domain of human L1 and that the binding of these integrins to this motif is critically and differentially regulated by physiological levels of calcium. Based on the novel interactions involving α6β1 and αHβ3, we have shown that the sixth Ig-like domain of human L1 can support significant endothelial cell and platelet attachment. Based on these findings, and the observation that L1 expression can be induced on endothelial cells, we propose an expanded role for this CAM in vascular and thrombogenic processes.

The authors wish to thank Dr. R.A. Reifeld of the Scripps Research Institute for his support and encouragement. This is Scripps manuscript number 10821-1MM.

This study was supported by National Institutes of Health ROI Grant CA69112-01 (J.M.P. Montgomery), by National Institutes of Health R29 Grant CA67988 (B. Felding-Habermann), and by the Medical Research Council of Canada (C.H. Siu). P. Yip is supported by an Ontario Graduate Studentship (Canada), and S. Silletti by a National Cancer Institute research fellowship (1/F32/CA72192-01).

Received for publication 2 May 1997 and in revised form 22 August 1997.

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