Tetraspanins associate on the cell membrane with several transmembrane proteins, including members of the integrin superfamily. The tetraspanin CD9 has been implicated in cell motility, metastasis, and sperm-egg fusion. In this study we characterize the first CD9 conformation-dependent epitope (detected by monoclonal antibody (mAb) PAINS-13) whose expression depends on changes in the activation state of associated β1 integrins. mAb PAINS-13 precipitates CD9 under conditions that preserve the association of this tetraspanin with integrins, but not under conditions that disrupt these interactions. Induction of activation of β1 integrins by temperature, divalent cation Mn2+, or mAb TS2/16 correlated with enhanced expression of the PAINS-13 epitope on a variety of cells. Through the use of different K562 myeloid leukemia transfectant cells expressing specific members of the β1 integrin subfamily we show that the expression of the PAINS-13 epitope depends on CD9 association with α6β1 integrin. The mAb PAINS-13 reactivity has been mapped to the CD9 region comprising residues 112–154 in the NH2 half of the large extracellular loop. Also, we show that the CD9 conformation recognized by mAb PAINS-13 is functionally relevant in β1 integrin-mediated cellular processes including wound healing migration, tubular morphogenesis, cell adhesion and spreading and in signal transduction involving phosphatidylinositol 3-kinase activation.

The tetraspanins (also called TM4SF) are integral membrane proteins with four transmembrane regions delimiting two extracellular regions of unequal sizes. All tetraspanins share the presence of a CCG motif and several conserved cysteine residues in the large extracellular domain. The tetraspanin family currently comprises more than 25 members, including the differentiation antigens CD9, CD37, CD53, CD63, CD81, CD82, and CD151. These proteins have been implicated in different cellular functions, including adhesion, migration, differentiation, and signal transduction (reviewed in Refs. 1–4).

The tetraspanins act as molecular facilitators or adaptors that organize a network of interactions among a subset of cell surface molecules, known as the “tetraspanin web.” Among the proteins identified as components of the tetraspanin web are many immunoglobulin superfamily protein members (including the CD4 and CD8 antigens on T cells), the CD19 molecule on lymphoid cells, the major histocompatibility complex class II molecules, and several members of the β1 subfamily of integrins on many cell types (Refs. 1–4, and references therein). The organization of the tetraspanin web is based on several levels of interactions (2, 5–7). The first level relates to primary interactions between a particular tetraspanin and specific proteins known as tetraspanin partners. These first level interactions are direct, resistant to detergents such as digitonin and Triton X-100, and of high stoichiometry (i.e. >50% in the complex). A second level of interactions are indirect, more numerous and resistant to Brij 96 detergent extraction, and arise as tetraspanins associate to each other linking multiple primary complexes. A third level of tetraspanin organization relates to the large and light complexes that are resistant to detergents such as Brij 99 or CHAPS3 and display partial lipid raft properties (8).

The tetraspanin CD9 is a 21–24-kDa surface molecule, which was initially identified as a lymphohemopoietic marker (9), and was later implicated in cell motility (10), metastasis (11, 12), neurite outgrowth (13), myotube formation and maintenance (14), and in sperm-egg fusion (15). CD9 seems to act as a suppressor of metastasis because its transfection into melanoma cells reduces their metastatic potential (16) and an inverse correlation between expression of CD9 and appearance of metastasis in melanoma, colon, lung, and breast cancers has been described (12, 17–19). The tissue and cellular distribution of CD9 is very wide, and most immortalized cell lines tested have been found to express detectable levels of this tetraspanin (20–22). Notable exceptions are several leukocytic cell lines of

**The abbreviations used are:** CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PI3K, phosphatidylinositol 3-kinase; PI4K, phosphatidylinositol 4-kinase; mAb, monoclonal antibody; EGFP, epidermal growth factor protein; HUVEC, human umbilical vein endothelial cells; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter, TBS, Tris-buffered saline; BSA, bovine serum albumin; LEL, large extracellular loop.

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both the lymphocytic (Daudi, Raji, and HS6-2) or the myeloid (U-937, KG1) lineages (21).

Some members of the $\beta_1$ integrin subfamily, particularly $\alpha_2\beta_1$ (VLA-3), $\alpha_4\beta_1$ (VLA-4), and $\alpha_6\beta_1$ (VLA-6), are among the tetraspanin-associated cell surface proteins found in the tetraspanin web in most cellular systems. The contribution of tetraspanins to adhesion-dependent signaling seems to be linked to their ability to recruit signaling components such as protein kinase C, PI3K, or PI4K into the integrin complexes (23, 24). The $\beta_1$ integrin subfamily comprises heterodimeric transmembrane proteins that mediate cell adhesion to extracellular matrix proteins, including laminin ($\alpha_1\beta_1$ and $\alpha_5\beta_1$), collagen ($\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_5\beta_1$), fibronectin ($\alpha_1\beta_1$ and $\alpha_5\beta_1$), and also cell-cell interactions ($\alpha_5\beta_1$/VCAM-1) (25). Integrins not only mediate cell adhesion but work as effective bidirectional signal transducers (25, 26). The adhesive and signaling capabilities of integrins depend on transitions between conformational changes in these receptors that reflect distinct “activation states” defined by their increased or decreased ability to mediate ligand interactions or signal transduction (27–29). Ligand binding, physiologic temperature (37 °C), the presence of divalent cations Mg$^{2+}$ and Mn$^{2+}$ in the extracellular medium, and several specific “stimulatory” monoclonal antibodies (mAbs) that provoke a conformational change upon binding to a particular integrin, are among the factors known to induce the activation of integrins. The activation state of many integrins can be monitored with the use of a different type of specific mAbs (“activation reporters”) that recognize conformation-dependent integrin epitopes whose expression reflect changes in the integrin avidity or/and affinity (29–32).

In this study we characterize the first CD9 conformation-dependent epitope whose expression depends on changes in the activation state of associated $\beta_1$ integrins. Through the use of different K562 transfectant cells expressing specific members of the $\beta_1$ integrin subfamily we have determined that the expression of the PAINS-13 epitope depends preferentially on the presence of $\alpha_5\beta_1$ integrin. These interactions are functionally relevant in $\beta_1$ integrin-dependent cellular processes including wound healing, migration, tubular morphogenesis, cell adhesion and spreading, and signal transduction involving PI3K activation and may therefore play a regulatory role in cell migration during angiogenesis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human microvascular endothelial cell line HMEC-1 (33) was grown in MCD8-131 medium (Sigma) supplemented with 20% fetal bovine serum, 2 mM glutamine, 50 IU/ml penicillin, 50 $\mu$g/ml streptomycin, 10 ng/ml recombinant human epidermal growth factor (Promega), and 1 mM hydrocortisone (Sigma) on gelatin-coated (0.5%) flasks. Human umbilical vein endothelial cells (HUVEC) were obtained and cultured as previously described (34) and used within the first 6 passages. Human T lymphocytes (primary T lymphoblasts obtained from healthy blood donors as previously described (35), HS2-2, and Jurkat cell lines), B lymphocytes (Raji, Ramos, and JY cell lines), K562 erythroblasts, and HMEC-1 (obtained from Dr. M. Hemler (Dana-Farber Cancer Institute, Boston, MA) (37) and $\alpha_5$-K562 transfectants were a gift from A. Sonnenberg (Netherlands Cancer Institute, Amsterdam, The Netherlands) (38). Stable K562 transfectants were maintained in 0.2 mg/ml streptomycin and 50 $\mu$g/ml G418 as selection agent.

**Antibodies**—Several anti-integrin monoclonal antibodies were used in this study, including the anti-$\alpha_1$ mAb 5E6D9 (39), the stimulatory anti-$\beta_1$ integrin mAb TS2/16 (40), the “blocking” anti-$\beta_1$ integrin mAb L1ia/2 (41), and the “activation reporter” mAb HUTS-21 that recognizes the activated conformation of $\beta_1$ integrin (40). The anti-CD9 antibodies used in this study were VJ1/20 and VJ1/10 (42), and mAb 10B1, which detects an epitope located in the second (COO-2) half of the large extracellular loop of CD9. The anti-CD9 mAb T882 has been previously described (41). Other antibodies were mAb Tea 1/51 (anti-VE-cadherin) and mAb TP1/15 (anti-CD31) (42). The monoclonal IgP3X63 myeloma protein (IgG1,κ) was used as a negative control (41). The anti-p85 subunit of PI3K antibody was purchased from Transduction Laboratories (Lexington, KY). When necessary, purified antibodies were biotinylated with 3-ethyl-N-hydroxysuccinimide ester (Sigma) as previously described (30).

**Adhesive Proteins**—Matrigel basement membrane matrix was obtained from the Engelbreth-Holm-Swarm sarcoma cells as previously described (43). Fibronectin and poly-l-lysine were purchased from Sigma. Laminin 1 was either purchased from Sigma or purified from Engelbreth-Holm-Swarm sarcoma cells (44).

**Generation of PAINS-13 MAb**—Confluent HMEC-1 endothelial cells were collected using 5 mM EDTA, pH 8, and intraperitoneally injected into Balb/c mice on days −22 and −12 and intravenously on day −3. Spleen cells were fused on day 0 with SP2 mouse myeloma cells at a 4:1 ratio and hybridomas were distributed in 96-well culture plates. After 7 days the hybridoma supernatants were harvested and their reactivity against the HMEC-1 used in the immunization was assayed by flow cytometry. Positive clones obtained by the limiting dilution method were selected and inoculated into Balb/c mice to generate ascitic fluid. Monoclonal antibodies were purified from ascitic fluid and concentrated by affinity chromatography using a column of protein G-Sepharose and eluted using sodium citrate 0.1 M, pH 5.0. MAbs PAINS-13 is an IgG1 antibody that detects an epitope located in the second (COO-2) half of the large extracellular loop of CD9. PAINS-13 and MAbs against CD9 associated with Pains-13 were used in all experiments as control for CD9-EGFP expression (209).

**Flow Cytometry Analysis**—Cells were washed twice, incubated with primary antibodies at 4 °C for 20 min, washed, and incubated for 30 min on ice with fluorescein isothiocyanate-conjugated anti-mouse IgG (Sigma). After washing three times, cells were fixed in 2% formaldehyde in PBS, and fluorescence was measured using a FACScan™ flow cytometer (BD Biosciences). To study the effect exerted by temperature on the expression of different cellular surface molecules, an aliquot of cells was incubated at 4 °C and another aliquot at 37 °C with the primary antibody for 20 min and subsequently FACS analysis was performed as described above. The effect of divalent cations was studied in cells washed with Hepes buffer (20 mM Hepes, 150 mM NaCl, 1 mg/ml glucose) containing 2 mM EGTA, and then resuspended in Hepes buffer. The primary antibody was supplemented or not with MnCl$_2$ (1–6 mM), depending on the cellular type) during 20 min at 37 °C. For direct activation of $\beta_1$ integrins, cells were incubated for 20 min at 37 °C with the stimulatory mAb TS2/16 (10 $\mu$g/ml) in Hepes buffer containing 1 mM CaCl$_2$, 1 mM MgCl$_2$, and biotinylated PAINS-13 or HUTS-21 (20 $\mu$g/ml) mAbs were then added and incubated at 37 °C for an additional period of 20 min and finally incubated with avidin-fluorescein isothiocyanate (Sigma) and processed for FACS analysis. To increase the HUTS-21 epitope expression on the K562 transfectants, N-acetylcyanate (50 mM) was used, and the FACs assay was performed after eliminating this agent before the primary antibody was added to the cells.

**Immunofluorescent Staining**—HUCV endothelial cells were grown on 12-mm glass coverslips precoated with 1% gelatin. Cells were incubated with the appropriate primary antibody for 1 h at room temperature, washed twice, and incubated for 30 min in the presence of secondary antibody Alexa Fluor® 488 goat anti-mouse IgG (Molecular Probes, Leiden, Netherlands). After washing with Tris-buffered saline (TBS) containing 1 mM CaCl$_2$, 1 mM MgCl$_2$, cells were fixed with 3% formaldehyde in PBS for 10 min at room temperature and mounted with Mowiol (Calbiochem). In some cases, cellular fixation was performed before the incubation with the primary antibody and nonspecific binding sites were blocked with TNN (0.1 mM Tris-HCl, 0.15 mM NaCl, 0.5% bovine serum albumin; Roche Molecular Biochemicals) for 20 min. Samples were examined with a Nikon Labophot-2 photomicroscope and images were acquired with a COHU high performance CCD camera (Cohu, Tokyo, Japan) connected to a LEICA Q550 CW work station (Leica Imaging Systems, Ltd., Cambridge, UK) using the LEICA QFISH V1.01 Software.

**Transient Transfections**—Mouse fibroblast L929 cells were trypsinized and resuspended in RPMI 1640, 10% fetal calf serum medium supplemented with 5 $\mu$l of 15 mM NaCl, 20 $\mu$g of pBlueScript (Stratagene), and 5 $\mu$g of the CD9-EGFP or CD151-EGFP cDNAs. Cells were electroporated at 975 microfarads/200 V in a Gene Pulser II (Bio-Rad) and used for immunofluorescence experiments 24 h after transfection. The CD9-EGFP fusion protein construct was obtained by PCR amplification of CD9-cDNA followed by cloning in the pEGFP-N1 vector (Clontech Laboratories) (45). For epitope mapping, the CD9 and

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$^a$ E. Rubinstein, unpublished results.
Cell Labeling and Immunoprecipitations—HMEC-1 confluent monolayers were labeled using 0.3–0.5 mg/ml biotin 3-sulf-N-hydroxysuccinimide ester (Sigma) in PBS with 1 mM CaCl2, 1 mM MgCl2, for 30 min at 4°C. Cells were lysed in 1% digitonin, 1% Brij 96, or in different concentrations of Triton X-100 buffer (in TBS supplemented with 1% BS-A, 1 mM MgCl2, 1 mM CaCl2, 1 mM phenylmethylsulfonyl fluoride, 0.2 μg/ml leupeptin, and 5 μg/ml aprotinin) for 30 min at 4°C. Cellular material was recovered using a cellular scraper and the insoluble portion was removed by centrifugation at 12,000 rpm for 15 min. Protein G-Sepharose was precleared with cellular lysates from unlabeled cells, and coupled with the specific antibodies for 2–4 h at 4°C. After removing the excess antibodies, they were incubated overnight at 4°C with the biotinylated lysates. Beads were then rinsed three times with diluted (1:10) lysis buffer in TBS and immune complexes were eluted by boiling in Laemmli buffer and resolved in a 12% acrylamide/bisacrylamide SDS-PAGE gel, followed by transfer to nitrocellulose membranes. Blots were blocked with 5% BSA in TBS and the chemiluminescence was measured using the Immunopore ABC peroxidase—staining kit (Pierce) in TBSB (TBS, 0.1% Tween 20) and chemiluminescence (ECL detection kit, Amersham Biosciences).

Adhesion and Cellular Spreading Assays—For cellular adhesion and spreading assays, 96-microwell plates (Costar) were coated with different extracellular matrix components (30 μg/ml laminin I, 5 μg/ml fibronectin, or 50 μg/ml poly-l-lysine as a control) overnight at 4°C and blocked with 1% milk-denatured BSA for 1 h at room temperature. The trypanized HMEC-1 cells were washed and resuspended in MDBC-131 medium (without serum and growth factors, and supplemented with 20 mM Hepes and 0.1% BSA) containing 10 μg/ml of each purified mAb. 1.25 × 105 cells (for adhesion assays) or 1.5 × 106 (for spreading assays) were added to the coated wells and incubated for 10 min at 4°C to allow cells to sediment onto the well bottom, followed by 15 (for laminin) or 5 min (for fibronectin) incubations at 37°C. In adhesion assays, unbound cells were removed by filling the wells with PBS, 1 mM CaCl2, 1 mM MgCl2, 20 mM Hepes, 0.1% BSA, sealing with plastic, and inverting the plates for 30 min at 37°C. After fixation of bound cells in 3.7% formaldehyde and treatment with 2% methanol followed by 70% ethanol dehydration, immunoprecipitations were analyzed by measuring the absorbance of eluted crystal violet at 540 nm. For cellular spreading assays, the fixed adherent cells were photographed in a phase-contrast Nikon Diaphot 300 inverted microscope equipped with a Sony SSC-M350 CE CCD video camera and VCR and the cellular perimeter was calculated using the image analysis software Optimas 5.2 (Bioscan, Edmonds, WA).

In Vitro Angiogenesis Assay—80 μl of Matrigel were added into flat-bottomed 96-well tissue culture plates at 4°C and allowed to gel during 1 h at 37°C. Then, 25 × 103 HMEC-1 cells were seeded at the top of the gel in a volume of 80 μl with 20 μg/ml purified mAbs. After 6 h incubation, tubular structures were formed and images were recorded on TMAX 400 film (Kodak) with a phase-contrast microscope (Nikon E600 D, 0.5×-1.1×). Tubes were defined as cellular extensions linking cell masses (46).

Wound Healing Assays—Wound healing assays were performed as a modification of the previously described method (42). HMEC-1 cells were seeded on 0.5% gelatin-coated dishes to confluence and medium without growth factors was added 16 h before the assay. Antibodies (20 μg/ml) were added for 20 min at 37°C. After two washes using cold PBS, 1 mM CaCl2, 1 mM MgCl2, an additional cross-linking was performed with a rabbit anti-mouse IgG (1:200, Sigma) for 10 min at 4°C, followed by 30 min at 37°C. Then, chilled lysis buffer (20 mM Tris buffer, pH 7.4, 140 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, pepstatin, and leupeptin) was added and cells were removed using a cellular scraper. Lysis was performed during 30 min at 4°C and the nonsoluble material was removed by centrifugation at 12,000 rpm for 10 min. Cellular lysates were incubated overnight at 4°C with an anti-p58 subunit of PI3K antibody bound to Protein G-Sepharose. The immunoprecipitates were washed three times with diluted lysis buffer, and twice in kinase buffer (20 mM Tris, pH 7.5, 75 mM NaCl, 20 mM Hepes, 10 mM MgCl2, and 200 μM adenosine). Beads were then resuspended in 40 μl of kinase buffer containing 10 μg of t-α-phosphatidylinositol (Sigma), the reaction was started by addition of 5 μCi of [γ-32P]ATP, carried out for 15 min at room temperature, and stopped on ice. Phospholipids were extracted in chloroform/methanol/HCl, desiccated under N2 flow, and dissolved in chloroform/methanol/H2O (75:25:2 in volume) prior to being resolved by thin layer chromatography on Silica Gel 60 plates (Merck) and analyzed by autoradiography. Total precipitated enzyme was quantified by Western blot using the same anti-p58 subunit antibody.

Results

Mab PAINS-13 Is Specific for Human CD9 Tetraspanin—The monoclonal antibody PAINS-13 was selected as a CD9-specific antibody based on the following criteria: (i) its high reactivity and specific pattern of immunofluorescence staining of monolayers of primary HUVEC endothelial cells (Fig. 1A); (ii) PAINS-13 immunoprecipitates from Brij 96 lysates of human microvascular HMEC-1 cells were identical to immunoprecipitates obtained with other characterized anti-CD9 mAbs (Fig. 1B); (iii) the anti-CD9 specific mAb VJ1/20 recognizes a band of 24 kDa corresponding to CD9 in Western blots of PAINS-13 and VJ1/20 immunoprecipitates from HMEC-1 lysates (Fig. 1C); (iv) mab PAINS-13 specifically recognizes mouse fibroblast L929 cells transsected with a human CD9-EFGP cDNA construct (Fig. 1D) but was ineffective with mock-transfected cells or cells transfected with other tetraspanin-coding DNA constructs (CD151-EFGP, data not shown), and (v) mab cross-competition binding assays on HMEC-1 cells in which cell preincubation with anti-CD9 mab VJ1/20 completely abolished subsequent reactivity of mab PAINS-13, but preincubation with different antibodies to CD31, VE-cadherin, α1, α2, α4, α6, α9, α6, or β1 integrin had no blocking effect (data not shown).

The Epitope Recognized by Mab PAINS-13 on the CD9 Tetraspanin Is Dependent on the Activation State of Associated β Integrins—When the cellular reactivity of anti-CD9 mAb PAINS-13 was compared with that of the previously described anti-CD9 antibody VJ1/20 in a variety of cells from different lineages (Fig. 2a), striking differences were observed: whereas the reactivity of mab VJ1/20 was strong for all the CD9-positive cell types tested, PAINS-13 only yielded a high reactivity in HUVEC and HMEC-1 endothelial cells but a weak to moderate staining in NB100, DX3, A375, and Jurkat cell lines. These striking differences in cellular reactivity between mab PAINS-13 and other “classical” anti-CD9 antibodies led us to consider the possibility that PAINS-13 might be recognizing a subpopulation of CD9 molecules displaying a specific conformation. To test this hypothesis, we compared the PAINS-13 immunoprecipitates from HMEC-1 cells solubilized in increasing stringency conditions (i.e. 0.1% Triton X-100, 0.5% Triton X-100, 1% Triton X-100) under which the interactions formed among TM4SF and associated membrane proteins are disrupted. As Fig. 2b shows, a 24-kDa band corresponding to CD9 is clearly visible in the mab PAINS-13 precipitates from ly-
sates obtained with 0.1% Triton X-100 (panel A). In contrast, mAb PAINS-13 was no longer able to precipitate the CD9 molecule under more stringent conditions such as 0.5% Triton X-100 (panel B) or 1% Triton X-100 (panel C) or under 1% digitonin lysis conditions (data not shown). In addition, the fact that PAINS-13 is unreactive in Western blotting (not shown)
also suggests that PAINS-13 could be specific for a CD9 conformational epitope.

As the motion in the plane of membrane, the level of association and changes in conformation of many cell surface molecules are all processes characterized by their temperature dependence, we decided to analyze whether the expression of the PAINS-13 epitope on the CD9 molecules was also affected by this parameter. For this purpose, we compared the expression of the PAINS-13 epitope on different cell types at 4 and 37 °C. Fig. 3A shows that in DX3 or NB100 cells, which display a high expression of the CD9 molecule and moderate basal expression of PAINS-13, this epitope was up-regulated at 37 °C compared with PAINS-13. In contrast, the expression of the CD9 epitopes detected by two classical anti-CD9 mAbs, VJ1/20 and VJ1/10 (not shown), was not affected. As expected, 37 °C was also ineffective at inducing any expression of PAINS-13 epitope in all the CD9-negative cell lines tested, including HSB2, Ramos, and Raji (not shown). Most importantly, the PAINS-13 epitope was not inducible on the CD9-positive B lymphocytic cell line JY, which lacks β1 integrin expression (Fig. 3A).

The presence of β1 integrins in PAINS-13 immunoprecipitates together with the modulation by temperature of the expression of this epitope on some cell lines but not on the β1-negative JY cells, prompted us to investigate whether the expression of the CD9 conformation detected by mAb PAINS-13 was dependent on changes in conformation of β1 integrins. In this regard, we first studied the effects of the divalent cation Mn2+ (a well characterized stimulus for the activation of the adhesive and signaling properties of β1 integrins) on the PAINS-13 expression. As shown in Fig. 3B, an important increase in the expression of the PAINS-13 epitope on DX3 and NB100 cells occurred upon replacement of extracellular divalent cations Ca2+ and Mg2+ by a concentration of 1 mM Mn2+, which correlated with a parallel increase in the expression of the activation-reporter epitope HUTS-21 on the β1 integrin molecules. As with temperature, treatment with Mn2+ did not induce any detectable increase in the expression of PAINS-13 either on the CD9-positive/β1-negative cell line JY nor on the CD9-negative cell lines HSB2 (Fig. 3B), Ramos, or Raji (not shown), further pointing out the β1 integrin dependence for expression of the PAINS-13 epitope. The Mn2+-induced up-regulation of PAINS-13 expression was specific, as the total expression of CD9 (detected by mAb VJ1/20) or β1 integrin (detected by mAb TS2/16) remained unaltered after Mn2+ treatment (Fig. 3B).

Although changes in the temperature and divalent cations regulate the activation state of β1 integrins, these conditions could also be affecting a variety of other cellular targets. To address more specifically the issue of the dependence of the PAINS-13 epitope expression on β1 integrin activation, we directly induced the activation of β1 integrins by using the stimulatory mAb TS2/16. As shown in Fig. 3C, pretreatment of DX3 cells with mAb TS2/16 induced an important increase in expression of the PAINS-13 epitope, which correlated with the enhanced expression of the β1 integrin activation epitope HUTS-21. Taken together, our results clearly demonstrate that mAb PAINS-13 recognizes an epitope on the CD9 molecule in cell lysates is lost under increasing detergent stringency conditions. Surface-biotinylated HMEC-1 cells were lysed using 0.1% Triton X-100 (A), 0.5% Triton X-100 (B), and 1% Triton X-100 (C). After overnight immunoprecipitations using mAbs TS2/16 (β1 integrin), VJ1/20 (CD9), PAINS-13, and the x63 control mAb (∗), the immune complexes were resolved by nonreducing SDS-PAGE, and the total surface-biotinylated proteins were revealed by blotting using the ABC avidin peroxidase system.
whose expression is dependent on conformational changes conveyed through members of the \( \beta_1 \) integrin subfamily.

At this point we decided to answer whether the CD9 conformation detected by mAb PAINS-13 was preferentially dependent on a particular integrin subunit. For this purpose, we employed the erythroblastoid K562 cells (wt-K562), which display moderate levels of the CD9 molecule and only express the \( \alpha_5 \) integrin subunit associated to \( \beta_1 \) (\( \alpha_5 \)-K562), and K562 transfected cells expressing \( \alpha_6 \) (\( \alpha_6 \)-K562), \( \alpha_3 \) (\( \alpha_3 \)-K562), or \( \alpha_5 \) (\( \alpha_5 \)-K562) integrin subunits. All these cells were treated with 5 mM Mn\(^{2+}\) to induce activation of the \( \beta_1 \) integrins. We also used \( N \)-acetylcysteine, an antioxidant agent that induces important changes in the conformation of \( \beta_1 \) integrins (presumably by reduction of disulfide bonds in the cysteine-rich region of the \( \beta_1 \) integrin subunit),\(^3\) as revealed by the strong expression of the HUTS-21 epitope. As shown in Fig. 4, Mn\(^{2+}\) or \( N \)-acetylcysteine only induced a detectable increase of PAINS-13 expression in the \( \alpha_5 \)-K562 cells, clearly implying that the \( \alpha_5 \beta_1 \) integrin molecules in the tetraspanin web are preferentially responsible for transmitting the conformational changes to CD9 molecules resulting in expression of PAINS-13 epitope.

**Location of the PAINS-13 Epitope on the CD9 Molecule**—To identify the CD9 region that is detected by mAb PAINS-13 we used the following CD9/CD82 chimeric molecules (depicted in Fig. 5): CD9/82 (comprising the NH\(_2\) portion of the CD9 molecule up to the beginning of the large extracellular loop (LEL)); CD82/9 (comprising the NH\(_2\) portion of CD82 molecule up to the beginning of the LEL); CD82LEL9 (comprising the large extracellular loop of CD9) and CD82CCG9 (comprising the NH\(_2\) portion of CD82 molecule up to the CCG motif of LEL). Transient expression of full-length CD9, full-length CD82, and of each of these cDNA chimeric constructions in the human CD9/CD82 Colo320 cell line and subsequent FACS analysis clearly showed that mAb PAINS-13 reacts with the region comprising the NH\(_2\) half of CD9 LEL, which encompasses CD9 residues 112–154.

**Relationship between CD9 Conformation and \( \beta_1 \) Integrin Function**—The tetraspanin CD9 has been proposed to mediate some of the functional activities of associated cell surface molecules, including regulation of cell migration and adhesion-dependent signaling through \( \beta_1 \) integrins (23, 24, 48, 49).

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\(^3\) A. Luque and C. Cabañas, unpublished observations.
To probe the functional consequences of CD9 engagement with mAb PAINS-13, we analyzed the effect of this antibody in in vitro wound healing assays of HMEC-1 endothelial cell monolayers. As shown in Fig. 6A, both the inhibitory anti-β1 integrin mAb Lia1/2 and the stimulatory β1 integrin-specific mAb TS2/16 caused an important (43.6 and 50.7%, respectively) inhibition in the repaired area of the wounds over control conditions, clearly indicating the dependence on a balanced level of β1 integrin adhesion for endothelial motility in this assay. The mAb PAINS-13 caused a 64.2% inhibition of endothelial cell motility in this type of assay. Interestingly, two conformation-independent mAbs specific for CD9, VJ1/10 and VJ1/20, exerted very different effects on endothelial cell motility, with mAb VJ1/20 causing a 81.2% inhibition, whereas mAb VJ1/10 caused only a marginal 19.6% inhibition. These results show that the functional consequences after ligation of the tetraspanin CD9 clearly depend on the specific antibody engagement of distinct epitopes on CD9 molecules.

Although the wound healing assay is dependent on β1 integrin adhesion, it does not permit discrimination between agents that act through augmentation or inhibition of adhesive activity of these integrins. We therefore analyzed the formation of anastomosing angiotubular cord-like cellular structures on Matrigel basement membranes, which has been used as an in vitro model for cell morphogenesis and angiogenesis. As shown in Fig. 6B, while the inhibitory anti-β1 mAb Lia1/2 completely blocked the formation of angiotubes, the stimulatory anti-β1 mAb TS2/16 and the anti-CD9 mAbs VJ1/20 and PAINS-13 all induced an important enhancement in the number of these cellular cord-like structures. As the functional effects of PAINS-13 epitope engagement were very similar to those exerted upon activation of β1 integrin with the stimulatory mAb TS2/16 in both functional cellular assays tested (inhibition of in vitro wound healing and enhancement of formation of angiotubular cellular structures), we decided to probe the direct effects of PAINS-13 engagement.
on $\beta_1$ integrin activation. For this aim, we first quantitated the effects of mAb PAINS-13 on the adhesion of HMEC-1 endothelial cells to laminin-1 (a ligand for $\alpha_6\beta_1$ integrin) and fibronectin (a ligand for $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins). Interestingly, PAINS-13 engagement only induced an increase in cell adhesion to laminin-1 but had no effect on cell adhesion to fibronectin (Fig. 7A), again supporting the specific dependence on $\alpha_6\beta_1$ integrin for PAINS-13 functional effects. As expected, the stimulatory mAb TS2/16 induced a 149% increase in cellular spreading on laminin-1 over control conditions (100%), and mAb PAINS-13 similarly induced a 169% increase, reflecting the activation of the $\alpha_6\beta_1$ integrin. As expected, mAb TS2/16 also increased the spreading of HMEC-1 cells on fibronectin but, in contrast, mAb PAINS-13 had no effect on cells spreading on this substrate, clearly indicating that the observed effects are specifically mediated by $\alpha_6\beta_1$ integrin.

Engagement of the PAINS-13 Epitope Activates PI3K—Activation of PI3K is a hallmark of $\beta_1$-mediated integrin signaling. Because mAb PAINS-13 recognizes a CD9 epitope whose expression depends on $\alpha_6\beta_1$ integrin activation, we used this antibody to probe the functional implication of the specific subpopulation of CD9 molecules expressing this epitope. As shown in Fig. 8, cross-linking of CD9 molecules with mAb PAINS-13 induced a strong activation of PI3K in endothelial HMEC-1 cells. This activation was similar to that induced after direct activation of $\beta_1$ integrin with mAb TS2/16. Cross-linking of other conformation independent anti-CD9 mAbs (VJ1/10 and VJ1/20) or cross-linking of other abundantly expressed molecules (anti-CD31 mAb TP1/15) on HMEC-1 cells only exerted a minimal effect on activation of PI3K. Taken together, these results show that the functional effects after engagement of the...
PAINS-13 epitope are very similar to those obtained after ligation of β1 integrin with the stimulatory antibody TS2/16.

DISCUSSION

The tetraspanin CD9 is a widely distributed cell surface molecule found in numerous tumor cells, as well as in neural, vascular, and leukocytic cells. CD9 expression in several types of cancer is inversely correlated with their metastatic potential and patient survival (12). Through the use of transfection and monoclonal antibodies specific for the CD9 molecule, this tetraspanin has been implicated in cell motility and migration (10), in vitro wound healing repair and angiogenesis (50), metastasis (17), neurite outgrowth (13), myotube formation and maintenance (14), and in sperm-egg fusion (15). Recently, some of us have described the implication of CD9 in transendothelial migration of melanoma cells through a mechanism involving strengthening of melanoma-endothelial cell heterotypic interactions (45). The involvement of CD9 in all these functions seems to depend on its association with other proteins, and particularly those belonging to the integrin family, in molecular complexes known as the tetraspanin web.

In the present study we have characterized the first CD9 epitope, recognized by mAb PAINS-13, whose expression depends on conformational changes transmitted upon activation of the associated β1 integrins, and particularly of the α6β1 integrin heterodimer. This has been demonstrated by immunoprecipitation analysis under different detergent stringency conditions, direct induction of β1 integrin activation, and the use of K562 transfectants expressing specific β1 integrin members.

In a recent paper by Geary et al. (51) several monoclonal antibodies to the tetraspanin CD151 have been characterized and found to react differentially with this molecule based on tissue staining and binding to hemopoietic cells. These authors demonstrate that these differences in mAb reactivity are because of masking/unfolding of the conformational epitopes recognized in the integrin-CD151 protein complexes. Interestingly, in this study it is shown that transfection of K562 cells with either α6 or α6 integrins results in increased expression of a subset of conformational epitopes and reduced expression of others. One of the anti-CD151 mAbs included in this report is TS151r, originally described by Rubinstein and co-workers (5), which detects a CD151 conformational epitope that is lost upon interaction with the α6β1 integrin. The expression of the CD9 epitope recognized by mAb PAINS-13 as described in this paper shows the opposite behavior, as it depends on the presence of β1 integrins and particularly α6β1 on the cell surface, and is increased upon integrin activation.

Through the use of different CD9 × CD82 intertetraspanin chimeric constructions we have demonstrated that the reactivity of mAb PAINS-13 requires the integrity of the CD9 region comprising the first NH2-half LEL, which encompasses residues 112–154. The regions of the CD9 molecule involved in the association of this tetraspanin with integrins have not been characterized yet, but interestingly, Berditchevski and co-workers (49, 50) have recently described that the region of tetraspanin CD151 necessary and sufficient for stable association with integrin α6β1 is the second COO2-half of CD151 LEL. As it is likely that equivalent regions in different tetraspanins are responsible for their molecular association with β1 integrins, we could infer that the tetraspanin CD9 associates with integrin α6β1 through its LEL COO2-half and upon integrin activation a change in conformation is conveyed to CD9 that is
motility is inhibited by mAbs that affect the activity of extracellular matrix components including laminin, type IV collagen, and those produced as they move a deposition of different extracellular matrix components. To determine the expression of the HUTS-21 epitope we have tested the possibility that PAINS-13 engagement might change the affinity of associated \( \beta_1 \) integrins. In this regard, we have not been able to detect such \( \beta_1 \) conformational changes (not shown) suggesting that the observed increase in cell adhesion and spreading onto laminin-1 are mainly dependent on changes in integrin avidity. Because cell motility requires subtle and dynamic changes in the activity of \( \beta_1 \) integrins, another attractive possibility to explain the effect of PAINS-13 on cell motility is that upon engagement of this CD9 epitope the conformation of associated integrins might be kept in a more rigid or fixed conformation that would be incompatible with motility. The fact that two anti-CD9 mAbs (VJ1/10 and VJ1/20) exerted different effects on endothelial cell motility clearly reflects that depending on the CD9 epitope engaged the functional effects on associated integrins can be very diverse.

The formation of cord-like structures by endothelial cells plated on basement membrane matrix (Matrigel) depends on \( \beta_1 \) integrin-dependent cell adhesion and migration. Matrigel contains abundant laminin-1 and type IV collagen, which are ligands for distinct members of the \( \beta_1 \) integrin subfamily, including \( \alpha_5 \beta_1 \) and \( \alpha_6 \beta_1 \), and indeed, cellular morphogenesis characterized by formation of these structures depends on a correct balance of the adhesive capacities of this subfamily of integrins on the endothelial cells. In this regard, our results have confirmed the previous results of Sincock et al. (46), by showing that a blocking anti-\( \beta_1 \) integrin antibody (Lia1/2) completely inhibits the formation of angiotubular structures and by demonstrating that a stimulatory anti-\( \beta_1 \) integrin mAb (TS2/16) exerts the opposite effect, i.e., augments the formation of these structures. In a recent paper by Zhang et al. (53) it has been established that endothelial cell morphogenesis in Matrigel is inhibited by blocking antibodies directed to either \( \alpha_5 \beta_1 \) integrin or the tetraspanin CD151, demonstrating the relevance of the \( \alpha_6 \beta_1 \)-CD151 complexes in angiogenesis. Our results showing in this case the augmentation of endothelial morphogenesis by mAb PAINS-13, clearly establish the relevance of the \( \alpha_6 \beta_1 \)-CD9 complexes in these phenomena.

Signal transduction through integrins in many different cellular systems involves the activation of PI3K, an event which in turn regulates many other important cell functions such as cell survival and apoptosis and the activity of Rhein family small GTPases, which control cell morphology, polarization, and migration (26, 47, 55, 56). So far, however, very few reports have described the implication of tetraspanins in integrin-mediated signal transduction. Sugira and Berditchevski (24) have reported that different \( \alpha \beta \) tetraspanin protein complexes control invasive migration of tumor cells through two distinct PI3K-dependent mechanisms. The levels of activation of PI3K on serum-starved human breast cancer MDA-MB-231 cells after antibody engagement of \( \alpha_5 \) integrin or of tetraspanins CD9, CD63, and CD81, remained, however, nearly unaltered. In this paper, we have confirmed that, in agreement with previous reports, engagement of \( \beta_1 \) integrins with stimulatory mAb TS2/16 induces activation of PI3K in human endothelial HMEC-1 cells. We report here that engagement of the CD9 tetraspanin on HMEC-1 endothelial cells with mAb PAINS-13 directed to a conformation-dependent epitope induced an important increase in the activity of PI3K. Interestingly, engagement of the CD9 molecule with two other anti-CD9 mAbs did not induce activation of this lipid-phosphorylating enzyme.

Taken together, our results obtained in these four different functional assays clearly show that the functional biological effects after engagement of the CD9 conformation-dependent epitope PAINS-13 are similar to those exerted by the stimulatory anti-\( \beta_1 \) integrin antibody TS2/16 and underline the functional relevance of the \( \beta_1 \) integrin-CD9 protein complexes in

![Fig. 8. PAINS-13 induces the PI3-kinase activation in HMEC-1 cells.](image-url)
the regulation of cellular morphogenesis, migration, and signal transduction. A general conclusion from our studies is that different conformational states exist for the CD9 tetrapsan that are likely dependent on multiple molecular associations, which can be monitored adequately by specific mAbs and whose functional relevance is just beginning to be unraveled.

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A Functionally Relevant Conformational Epitope on the CD9 Tetraspanin Depends on the Association with Activated β1 Integrin

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