Tetraspanins in intercellular adhesion of polarized epithelial cells: spatial and functional relationship to integrins and cadherins

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INTRODUCTION

Epithelial cells delimit two different milieu or tissues in the body. This cell type is polarized, exhibiting two different plasma membrane domains, apical and basolateral, that contain distinct arrays of proteins (Caplan, 1997; Rodriguez-Boulan and Powell, 1992). The polarized morphology allows epithelial cells to perform some of their functions as interfaces between two compartments in the body, such as directional transport of solutes and nutrients. The asymmetric distribution of several macromolecules in the membrane of these cells is achieved by different mechanisms (Caplan, 1997; Drubin and Nelson, 1996; Rodriguez-Boulan and Nelson, 1989; Yeaman et al., 1999), which include vectorial sorting from the trans-Golgi network (Brown and Stow, 1996; Keller and Simons, 1997; Rodriguez-Boulan and Powell, 1992; Simons and Wandinger-Ness, 1990), directed exocytic vesicle docking (Grindstaff et al., 1998; Low et al., 1998) and selective retention of the different subsets of proteins at specific membrane subdomains. Retention of the cell-surface proteins at their correct target membrane site involves both the tight junctions, which limit the diffusion between the apical and basolateral compartments (Dragsten et al., 1981; Stevenson and Keon, 1998), and the direct interaction with the membrane cytoskeleton (Bennett, 1990; Bennett, 1992; Nelson and Veshnock, 1987).

In the first steps of polarization, integrins are key receptors, as cell adhesion to ECM (extracellular matrix) proteins mediated by these molecules, in the absence of tight junctions, is sufficient to induce some of the characteristics of a polarized cell phenotype (Drubin and Nelson, 1996; Vega-Salas et al., 1987). These adhesion receptors orient the apicobasal axis through their interaction with ECM proteins (Eaton and Simons, 1995; Yeaman et al., 1999). On the other hand, some integrin heterodimers are also localized at cell-to-cell junctions (Lampugnani et al., 1991; Larjava et al., 1990; Schoenenberger et al., 1994; Yáñez-Mó et al., 1998), implicating them not only as extracellular matrix receptors but also as intercellular adhesion molecules. In a second step, intercellular adhesion mediated by cadherin superfamily members will nucleate the formation of the whole intercellular junction structure (Gumbiner et al., 1988).

Tetraspanins comprise a growing family of proteins that span the plasma membrane four times and have been widely reported to associate to other integral membrane proteins, including β1 integrins, on the surface of diverse cell types (Hemler et al., 1996; Maecker et al., 1997; Wright and Tomlinson, 1994). The localization of tetraspanin-integrin complexes has been described in leukocyte microvilli (Abitorabi et al., 1997) and at intercellular junctions in some adherent cell types (Nakamura et al., 1995; Yáñez-Mó et al., 1998).
MATERIALS AND METHODS

Cells
MDCK (Madin-Darby canine kidney cell), type II J-clone and Caco-2 human colon carcinoma cells were obtained from Dr W. J. Nelson (Department of Molecular and Cellular Physiology, Stanford, CA). Chinese hamster ovary (CHO) cell clones mock- and VE-cadherin-transfected (1α and 5α clones, respectively), were obtained from Dr E. Dejana (Istituto di Ricerche Farmacologiche Mario Negri, Milano, Italy) and have been previously described (Breviario et al., 1995).

CHO cells were grown in Dulbecco’s MEM (Gibco), supplemented with 10% fetal calf serum, 50 IU/ml penicillin and 50 μg/ml streptomycin. Caco-2 and MDCK cells were grown in low glucose DMEM (Gibco). For immunofluorescence studies, cells were cultured on polycarbonate porous filters 0.4 μm pore diameter (Costar, Cambridge, MA) coated with rat tail collagen, unless stated otherwise, and allowed to grow for more than 7 days for proper polarization.

Antibodies
Monoclonal antibodies (mAbs) anti-CD151 (LIA1/1), anti-VE-cadherin (TEA1/31), anti-CD9 (VJ1/10 and VJ1/20), anti-α3 integrin (VJ1/18), anti-α2 integrin (TEA1/41), anti-human β1 integrin (TS2/16, VJ1/14 and HUTS-21) and anti-hamster β1 integrin (7E2) have previously been described (Arroyo et al., 1992; Brown and Juliano, 1988; Leach et al., 1993; Luque et al., 1996; Peñas et al., 2000; Yáñez-Mó et al., 1998). The anti-CD9 mAbs GR2110 and 200H19 were provided by Dr F. Garrido (Hospital Virgen de las Nieves, Granada, Spain) and by Dr A. Shaw (Cross Cancer Institute, Alberta, Canada), the 5αA6, anti-CD81 by Dr S. Levy (Stanford University School of Medicine, Stanford, CA), the I.33.22, anti-CD81 by Dr Vilella (Hospital Clinic, Barcelona, Spain), 8C3 anti-CD151 mAb by Dr Sekiguchi (Osaka University, Japan) and the anti-α5 integrin SAM-1 mAb by Dr C. Figdor (University Hospital Nijmegen, The Netherlands). Anti-pan-cadherin E2 rabbit polyclonal antibody was provided by Dr W. J. Nelson (Department of Molecular and Cellular Physiology, Stanford, CA). Anti-α3, -α2 and -β1 integrin polyclonal Abs were provided by Dr G. Tarone (Università di Torino, Italy). Anti-endoglin polyclonal Ab was provided by Dr C. Bernabeu (C.I.B., C.S.I.C., Spain). Anti-zonula occludens antigen-1 (ZO-1) rabbit polyclonal antibody was purchased from Zymed Laboratories (San Francisco, CA). Fab fragment of the anti-CD9 VJ1/10 mAb was obtained by papain digestion.

Flow cytometry, immunofluorescence and confocal microscopy
Flow cytometry was performed as previously described (Yáñez-Mó et al., 1998). For immunofluorescence experiments, cells were grown on glass coverslips or porous filters and fixed with 4% formaldehyde in PBS containing 1 mM CaCl₂ and 1 mM MgCl₂. Immunofluorescence on glass coverslips was performed as previously described (Yáñez-Mó et al., 1998). Lipid dye labeling was performed on fixed samples after 2 minutes incubation in serum-free medium with PKH2 Green Fluorescent Cell Linker Kit (Sigma, St Louis, MO) followed by extensive washes. Porous filters were blocked with 0.2% BSA and 1% normal goat serum. Primary and secondary antibodies (Amersham Life Science, Pittsburgh, PA) were added to both the apical and basolateral compartments of the transwell. After washing, porous membranes were cut from the insets and mounted with Vectashield (Vector Laboratories, Burlingame, CA), and analyzed in a MultiProbe 2010 (Molecular Dynamics), in a MCR 1024 (BioRad Laboratories, Hercules, CA) or in a Leica TCS-SP (Leica Microsystems, Heidelberg, Germany) confocal microscopes.

Labeling of cell-surface proteins at specific plasma membrane subdomains
Cell monolayers grown on porous filters for 7 days were washed with Ringer’s buffer (10 mM Hepes, pH 7.4, 154 mM NaCl, 7.2 mM KCl, 1.8 mM CaCl₂), Sulfo-NHS-biotin (200 μg/ml) (Pierce Chemical, Rockford, IL) in DMSO was added either to the apical or to the basal compartment of the Transwell. The integrity of the monolayers was assessed with 3H-inulin and filters with permeability greater than 1% were discharged. After 30 minutes at 4°C, biotin was washed with TBS (Tris-buffered saline) and cell lysis was performed under stringent (0.5% Triton X-100 in 50 mM NaCl, 300 mM sucrose, 10 mM Pipes pH 6.8, 3 mM MgCl₂) and non-stringent (1% Brij 96, 1% BSA, TBS) conditions for 10 minutes at 4°C. After scraping, cell lysates were centrifuged at 13,000 rpm for 15 minutes. In stringent conditions, the soluble fraction was separated (supernatant), whereas the pellet was resuspended in 1% SDS in 10 mM Tris-HCl pH 7.5, 2 mM EDTA, boiled for 5 minutes and diluted 1:10 in 0.5% Triton X-100 containing lysis buffer. Then, both supernatants and pellets were pre-cleared with Protein-A (Pharmacia Biotech, Sverige) and immunoprecipitated with Protein-A coupled to rabbit anti-mouse Ig (Dako-PATTS, Copenhagen, Denmark) (when appropriate) and the different Abs. Washes for stringent conditions were sequentially made with HS-B buffer (0.1% SDS, 1% Deoxycholate, 0.5% Triton X-100, 120 mM NaCl, 25 mM KCl, 5 mM EDTA, 5 mM EGTA, 20 mM Tris Buffer pH 7.5), 1 M NaCl HS-B and LSWB (2 mM EDTA 10 mM Tris Buffer pH 7.5), and several times with lysis buffer for non-stringent conditions. Immunoprecipitates were resolved in reducing 12% SDS-PAGE gels, transferred to nitrocellulose membrane (Transblot Transfer Medium, BioRad Laboratories) and developed with Vectastain ABC Peroxidase Standard Kit (Vector).

Reprecipitation experiments
MDCK cells were labeled with biotin as described above. Cell lysis and first immunoprecipitation were performed in non-stringent conditions. After extensive washes immunoprecipitates were eluted in TBS 1% NP40 and reimmunoprecipitated with TS2/16 anti-β1 integrin or E2 anti-pan cadherin Ab as negative control. Immunoprecipitates were then boiled in Laemmli buffer, separated under non-reducing conditions in a 10% SDS-PAGE gel, and revealed with Vectastain ABC Peroxidase Standard Kit.

Affinity chromatography
Purified laminin 5 (Roussel et al., 1995) and collagen I (Sigma) were coupled to CN-Br-sepharose (Pharmacia Biotech) at 2 mg/ml following manufacturer’s instructions, columns saturated with 1% BSA and washed with lysis buffer. Caco-2 cell lysates obtained under non-stringent detergent conditions were treated, when indicated, with 5 mM Mn²⁺ prior to addition to the column. Columns were extensively washed with wash buffer (lysis buffer without BSA) and eluted by passing six times the column volume of 50 mM EDTA in TBS or by boiling the sepharose in Laemmli buffer. Samples were resolved in a 12% SDS-PAGE gel under non-reducing conditions and immunoblotted with specific antibodies against CD9 and CD151 tetraspans or α2 and α3 integrins.

Cell aggregation and morphogenesis
Cell aggregation assays were performed as previously described.
(Caveda et al., 1996) with slight modifications. Briefly, cells were detached with trypsin-EDTA, washed twice and resuspended in 150 mM NaCl, 20 mM Hepes, 1 mM Ca²⁺, 1mM Mg²⁺, 1% BSA containing (or not) 10 µg/ml of the different mAbs or 1 mM Mn²⁺. Cell suspensions at 8x10⁵ cell/ml were allowed to aggregate on BSA-coated plates for 1 hour at 37°C and rotation (100 rpm) and fixed by addition of 1/10 of the final volume of formaldehyde 37%. The number of particles before and after the aggregation were quantified in a FACScan® flow cytometer (Becton Dickinson Labware, Lincoln Park, NY). For immunofluorescence staining, aggregates were washed with TBS after fixation, permeabilized with 0.5% Triton X-100, when appropriate, incubated at room temperature with primary and secondary Abs in V-bottom 96-well plates and resuspended in 2% DABCO (Sigma) in TBS. Images were acquired with a low-magnification objective in a non-confocal microscope to capture the whole fluorescence of the sample. Fluorescence quantification was performed using Optimas 5.2 (Bioscan, Edmonds, WA) and no digital treatment of the images was performed in order to sharpen the fluorescence signal. In Caco-2 aggregates, images were acquired at a higher magnification and a three-dimensional reconstruction of the cell aggregate is shown.

Morphogenesis experiments were carried out by plating 3x10⁵ CHO cells per well in 24-well plates in complete medium. After 5 hours of adhesion, medium was removed and replaced by new medium containing (or not) 10 µg/ml of the different mAbs. After 48 hours, DIC images of cells were taken with a 20x objective on a Leica DMRB microscope.

RESULTS

CD81 localizes at the basolateral membrane whereas CD9 is also present on apical microvilli in MDCK cells

Some epithelial cell lines possess the capacity to form polarized cell monolayers when cultured on porous filters. These monolayers display distinct apical and basolateral plasma membrane domains delimited by functional tight junction structures that prevent the diffusion of lipids and proteins between both subdomains. The MDCK cell line is a classical model of polarized kidney epithelial cell (Eaton and Simons, 1995; Hammerton et al., 1991), displaying tight junction structures in the most apical portion of the lateral intercellular junctions, as revealed by staining of ZO-1 (Fig. 1b, part A). We were therefore interested in studying the distribution of endogenous tetraspanin proteins in both apical and basolateral compartments of these epithelial cells. Interestingly, CD9 staining was observed both at apical microvilli, in both horizontal (Fig. 1a, part A, inset) and vertical confocal sections of MDCK cell monolayers cultured on filters, and in the lateral membrane (Fig. 1a, part A and Fig. 1b, part B). By contrast, CD81 was detected only on the lateral membrane (Fig. 1a, part B and Fig. 1b, part C). The anti-CD151 mAb was not included in these studies since this antibody does not crossreact with its homologous canine antigen (data not shown).

The above observations were confirmed by biochemical analysis. The apical or the basolateral subdomains of polarized monolayers of MDCK cells were labeled with biotin and endogenous antigens immunoprecipitated with either an anti-pan-cadherin polyclonal antibody, or monoclonal antibodies to CD9 or CD81 (Fig. 2a). Both CD9 and CD81 tetraspanins were readily extracted with 0.5% Triton X-100, and appeared only

in the supernatant fraction, suggesting that these proteins are not directly associated to actin cytoskeleton. In accordance with the immunofluorescence staining, CD9 was observed both in the apical and basolateral fractions, whereas CD81 was only immunoprecipitated from the basolateral domain. An anti-pap-cadherin Ab was included as a control of basolateral localization (Fig. 2a).

We next determined whether tetraspanin proteins were associated to integrins and whether these complexes were apically or basolaterally localized in these cells. When immunoprecipitation was performed under stringent detergent conditions, β1 integrins were found mainly in the basolateral compartment, and surprisingly in the supernatant fraction, in which tetraspanins were also found (Fig. 2a). Immunofluorescence studies confirmed that β1 integrin localized mainly basolaterally (Fig. 1b, part D) and was present also at the level of cell-substratum contact. When immunoprecipitation was carried out under non-stringent detergent conditions, tetraspanin complexes were predominantly observed at the basolateral surface (Fig. 2b, lanes F,H). Complexes immunoprecipitated with both anti-tetraspanin mAbs contained several associated bands. The identity of the high molecular weight bands as β1 integrins
was assessed by reprecipitation of proteins in tetraspanin immunoprecipitates with anti-β1 mAb TS2/16 (Fig. 2c). It should also be noticed that a band of around 35 kDa, whose identity remains to be determined, was only detected in basolateral complexes co-immunoprecipitated with both anti-CD9 and anti-CD81 mAbs (Fig. 2b, lane F and faintly seen in lane H), indicating that CD9-containing complexes in both apical and basolateral subdomains are different at the molecular level.

**CD9, CD81 and CD151 localize to the basolateral membrane in Caco-2 cells**

Caco-2, a human colon carcinoma cell line is also able to develop a polarized phenotype in culture. Immunofluorescence studies on these cells grown on filters demonstrated the junctional staining of the three different tetraspanins studied, CD9, CD81 and CD151 (Fig. 3a, parts B-D, respectively). When a vertical section of these cultures was observed by confocal microscopy, all three tetraspanin antigens localized at the lateral portion of the basolateral subdomain (Fig. 3b, parts B-D), in a pattern similar to that observed with an anti-pan-cadherin Ab (data not shown, and Grindstaff et al., 1998), but they were not detected at the cell-substratum contact area. Further analyses on polarized cultures of Caco-2 cells by immunofluorescence revealed that β1 integrin localization was basolateral (Fig. 3c, part A), although apical microvilli were also stained. As expected from its function as extracellular matrix (ECM) receptors, β1 integrins stained the basal portion of the membrane (Fig. 3c, part A). To study the conformational state of the subset of integrins associated to tetraspanins, immunofluorescence staining on polarized cultures of Caco-2 was performed with HUTS21, a monoclonal antibody that specifically recognizes an activation epitope (LIBS: Ligand-induced binding site) on the β1 integrin chain (Luque et al., 1996). The HUTS21 staining was mostly restricted to the cell-substratum contact sites, and this epitope was not expressed by those integrins localized to cell-cell junctions (Fig. 3c, part E). These results concur with previous reports describing that lateral integrins in keratinocytes are not ligand occupied (Kim and Yamada, 1997; Peñas et al., 1998). The subcellular localization of different integrin α chains was also studied in polarized monolayers. As shown in Fig. 3c, α5 (part D), and in a lower extent α2 (part B) stained the cell-matrix contact, even though cells were initially seeded on collagen matrix. This is consistent with the fact that Caco-2 cells assemble a fibronectin matrix. Intercellular junctions were stained by both anti-α2 and anti-α3 mAbs (parts B,C, respectively). α3 staining correlates with that observed with anti-tetraspanin mAbs, in that it is mainly excluded from the cell-matrix contact.

When Caco-2 cells were allowed to grow for 9 days on ECL substratum, containing the ligand for α3β1 integrin entactin (Dedhar et al., 1992), the culture exhibited a tubular-like structure (Fig. 4). On this substratum, both α3 integrin and...
Fig. 3. Confocal microscopy analysis of the localization of CD9, CD81 and CD151 tetraspanins in polarized Caco-2 cells. (a) Horizontal (xy) sections of polarized Caco-2 cell monolayers stained with an anti-ZO-1 polyclonal Ab (A), or with anti-CD9 GR2110 (B), anti-CD81 I.33.22 (C) and anti-CD151 LIA1/1 (D) mAbs. Sections shown correspond to those in which a maximal intensity of fluorescence was observed. (b) Vertical (zy) sections of polarized Caco-2 cell monolayers stained with the polyclonal anti-ZO-1 Ab (A), or with the mAbs GR2110 anti-CD9 (B), I.33.22 anti-CD81 (C), LIA1/1 anti-CD151 (D). (c) Vertical (zy) sections of polarized Caco-2 monolayers stained with mAbs to the different integrin chains: anti-β1 TS2/16 (A), anti-α2 TEA1/41 (B), anti-α3 VJ1/18 (C), anti-α5 SAM-1 (D) or the LIBS β1 mAb HUTS21 (E).

Fig. 4. Confocal analysis of the tubular-like structures developed by Caco-2 cells on ECL substratum. Cells were grown for 9 days on filters coated with ECL substratum, fixed and stained with anti-CD151 LIA1/1 (upper panels) or anti-α3 integrin VJ1/18 (lower panels) mAbs. Confocal sections were taken every 8 μm in CD151 staining and every 12 μm for α3 integrin starting at the top of the structure. Scale bar: 20 μm.
CD151 staining were observed at intercellular contacts and surrounding the tubular structure (Fig. 4).

**Tetraspanin-α3β1 integrin complexes are ligand binding competent on high affinity ligands**

All the data above suggested a role of tetraspanin-integrin complexes in intercellular adhesion in a non-ligand-occupied state. In order to determine whether these complexes were able to bind to ECM proteins, we performed affinity chromatography experiments with laminin 5 columns (Dogic et al., 1998). These columns were able to specifically retain α3 integrin as well as CD151 (Fig. 5, lane C). As a control of specificity, collagen I columns were able to retain α2 but not α3 integrin or CD9 and CD151 tetraspanins (Fig. 5, and data not shown), although the association between β1 and tetraspanins was conserved as determined by co-precipitation experiments from the non-retained fraction (data not shown). These data demonstrate that tetraspanin-integrin complexes were ligand-binding competent and that this binding did not disrupt their association.

**Role of tetraspanins-integrin complexes in intercellular adhesion and morphogenesis independent of cadherin expression and function**

To determine whether the localization of tetraspanins and integrins to the lateral junctions was dependent on cadherin-mediated intercellular adhesion, we explored the expression of these antigens in CHO cells, that do not express members of the cadherin superfamily and are unable to assemble tight junction structures (Breviario et al., 1995). These cells expressed both CD9 and CD81, as determined by flow cytometry (Fig. 6a). When indirect immunofluorescence was performed on confluent
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cultures of CHO cells, both CD9 and CD81 were localized at cell-cell junctions (Fig. 6b, parts C,D, respectively). Vertical reconstruction of the confocal sections (lower parts in Fig. 6b) further confirmed that both tetraspanins were at intercellular junctions compared to a lipid dye (Fig. 6b, part A) and also stained apical microvilli. β1 integrin staining was also present at intercellular contacts and apical microvilli as well as in cell-substratum contact areas (Fig. 6b, part B). The same pattern of intercellular integrin-tetraspanin staining was observed in CHO cells transfected with VE-cadherin although, because of the presence of cadherin, junctions were more organized and cells presented a greater contact-growth inhibition than non-transfected CHO cells (not shown). These data indicate that the localization of tetraspanin-integrin complexes at lateral junctions takes place independently of the cadherin-catenin molecular system, which has been reported to be necessary for the assembly of other intercellular adhesion structures, such as tight junctions (Gumbiner et al., 1988).

To assess the functional role of tetraspanin-integrin complexes in intercellular adhesion we performed aggregation assays in CHO cells. As shown in Fig. 7a, anti-CD9 mAb GR2110 was able to induce high levels of cell aggregation. HUTS21, an anti-β1 mAb that locks the integrin in the high-affinity state (Gómez et al., 1997) or Mn2+ treatment, which changes the conformation of the integrin to the high-affinity state, did not exert any effect on cell aggregation (Fig. 7a). When immunofluorescence staining was carried out on anti-CD9-induced cell aggregates, CD9 was concentrated at cell-cell boundaries (Fig. 7b, part B). Interestingly, β1 integrin also concentrated at cell-cell contact sites in the aggregates induced by the anti-CD9 mAb (Fig. 7b, part C). To rule out the possibility of an artefactual enhancement of fluorescence at cell-to-cell contacts, owing to apposition of cell membranes, aggregates were labeled with a lipid dye (Part A) and with an Ab directed to an unrelated plasma membrane antigen such as endoglin (Part D). Although fluorescence slightly increased at points of cell contact, fluorescence intensity measurements on a line traced on the fluorescent image showed that the ratio of intercellular staining versus membrane signal was much lower in lipid dye- or endoglin than in CD9- or β1- labeled aggregates (Fig. 7b), HUTS21, recognizing a LIBS epitope, was negative in these aggregates (data not shown). These data are in accordance with the non-ligand-occupied conformational state of intercellularly located integrins in polarized cells. Furthermore, cell-cell aggregation induced through tetraspanins seems to be regulated in a different manner than adhesion to ECM, in that it neither induces a conformational change in the integrin moiety nor is the conformational change necessary for it.

Fig. 7. Anti-tetraspanin mAbs induce cellular aggregation independently of integrin conformational change. (a) Cellular aggregation was performed with CHO cells as described under Material and Methods in the presence (or absence) of 10 μg/ml of the following mAbs: anti-β1 integrin HUTS21; anti-CD9 VJ1/20, GR2110 and 50H19; anti-CD81 I.33.22; or 1 mM Mn2+. Data are shown as the mean percentage of aggregation±s.d. in a representative experiment. (b) Immunofluorescence staining of CD9-induced aggregates of CHO cells. Lipid dye (A), CD9 staining (B) was obtained by directly incubating aggregates with anti-mouse secondary Ab. β1 integrin (C) and endoglin (D) stainings were obtained with polyclonal sera. Insets show the corresponding phase-contrast images. Histograms show the fluorescence intensity quantification on the white line traced on the immunofluorescence images. Start and end points are labeled in both the image and the histograms. Scale bar: 20 μm.
Aggregation experiments performed with Caco-2 cells treated with trypsin to avoid cadherin-mediated intercellular adhesion revealed that tetraspanin-induced intercellular adhesion can also be triggered by anti-CD9 and anti-CD151 mAbs (Fig. 8a). Furthermore, this aggregating effect was independent of antigenic crosslinking, since monovalent Fab fragments of the aggregating anti-CD9 VJ1/10 mAb also induced the effect (Fig. 8a). As observed in CHO cells, both CD9 and CD151 tetraspanins (Fig. 8c,b, respectively) as well as \( \alpha_3 \) integrin (Fig. 8f) but not endoglin (Fig. 8d) were relocalized to intercellular contact sites upon CD9-induced aggregation.

To further assess the possible role of tetraspanins in intercellular adhesion, CHO cell cultures on plastic dishes, lacking cadherin-mediated adhesion, were treated with anti-tetraspanin mAbs. After 48 hours, branching structures were observed in cultures treated with anti-CD9 50H19 but not GR2110 (Fig. 9C,D respectively). HUTS21 mAb also induced the formation of these structures (Fig. 9B). Thus, tetraspanin-integrin complexes are functionally relevant in regulating intercellular adhesion, even in the absence of cadherin-mediated adhesion.

**DISCUSSION**

Members of the tetraspanin family of proteins play diverse functions in the cell, regulating their proliferation, adhesion and migration (reviewed by Maecker et al., 1997). Different tetraspanin proteins associate among themselves and to different transmembrane receptors in a cell type-dependent manner, suggesting the existence of a tetraspanin web on the cell surface (Hemler et al., 1996; Maecker et al., 1997; Rubinstein et al., 1996; Wright and Tomlinson, 1994). Herein, we studied in detail the expression of three tetraspanins CD9, CD81 and CD151 in different epithelial cell lines, as well as their functional role in conjunction with \( \alpha_3 \beta_1 \) integrin. All three tetraspanins localized to cell-to-cell junctions in different epithelial cell types, whereas CD9 also stained apical microvillae in MDCK cells. CD9 has been previously reported to play distinct roles in comparison to other tetraspanins (Lagaudriere-Gesbert et al., 1997). In renal epithelial cells it is induced by osmotic stress (Sheikh-Hamad et al., 1996), a fact that might point to a specific role of CD9 in this particular cell type. Moreover, CD9 has been located to the apical surface of certain regions of the uterine epithelium (Chen et al., 1999). Nevertheless, our study is the first report showing the coexistence of at least two differentiated tetraspanin multimolecular complexes in the same cell type, with different subcellular localization and different molecular partners.

We have shown here that the junctional character of these antigens is independent of cadherin-mediated intercellular adhesion, as it was also observed in CHO cells that lack expression of members of the cadherin family of proteins. Cadherins act as nucleators of intercellular adhesion structures like desmosomes and tight junctions in polarized epithelial cells (Gumbiner et al., 1988). The fact that integrins and tetraspanins
Intercellular adhesion and tetraspanins are localized to the cell-cell contact areas in cells lacking cadherins may indicate that these complexes act in a step in cell-cell adhesion or recognition prior to cadherin adhesion. Recent reports showing that cadherin-mediated intercellular adhesion is altered in α3-deficient keratinocytes also supports this issue (Wang et al., 1999). Tetraspanin-induced cell aggregation has previously been reported in leukocyte models both dependent and independent of β1 integrin (Cao et al., 1997; Fitter et al., 1999; Lagaudriere-Gesbert et al., 1997; Masellis-Smith and Shaw, 1994; Rubinstein et al., 1994; Takahashi et al., 1990). All these data further suggest a direct role of tetraspanins in intercellular adhesion, independent of other intercellular adhesion mechanisms.

Integrin-mediated adhesion is regulated at two levels: clustering of receptors and affinity, only the second one implying a conformational change (Sánchez-Mateos et al., 1996). It has been shown that some of the signaling moieties recruited by integrins are found associated to these receptors, even in the absence of ligand binding, and in some cases the crucial step is the clustering of integrin receptors (Miyamoto et al., 1995). The tetraspanin web may thus represent a ligand-independent mode of clustering integrin heterodimers. Finally, tetraspanins themselves are constitutively associated to some signaling molecules (Berditchevski et al., 1996). Our data on cell aggregation suggest that tetraspanins are involved in the regulation of intercellular adhesion and that this functional activity does not require or induce a conformational change in the integrin partner nor does it need cadherin-mediated...

Fig. 9. Anti-CD9 and anti-β1 mAbs induce branching structures in CHO cells. CHO cells were grown for 48 hours in the absence (A) or the presence of 10 μg/ml of anti-β1 integrin HUTS21 (B) and anti-CD9 50H19 (C), or GR2110 (D) mAbs. Scale bar: 50 μm.
adhesion. However, the α3β1 integrin complexed to tetraspanins can be activated by Mn2+ in a classical mode and thus it binds its ECM ligand without altering its association to the tetraspanin moieties. Thus, the combination of different conformational states of the integrin and its association to tetraspanins might lead to different signaling complexes.

During epithelial cell polarization, extrinsic spatial cues from cell-cell and cell-matrix adhesion determine the final polarity of the cell (Yeaman et al., 1999). Different integrin-containing signaling complexes would be implicated at different steps along this process. A first step would be cell adhesion to ECM, probably mediated by LIBS-positive, tetraspanin-non-associated integrins, since integrin-tetraspanin complexes are not found on complex focal adhesion structures (Berdichevsky and Odintsova, 1999). This adhesion step has been shown to be sufficient to induce some of the characteristics of a polarized cell phenotype in the absence of tight junctions (Drubin and Nelson, 1996; Vega-Salas et al., 1987), and would orient the apicobasal axis of the cell (Eaton and Simons, 1995). In a second step, LIBS-negative integrin-tetraspanin complexes might signal intercellular adhesion. A third signaling complex would be formed by ligand-bound tetraspanin-associated integrins that could signal induction of morphogenesis. Our results on the induction of tubule-like and branching structures in a cadherin-independent manner in CHO cells by engagement of CD9 tetraspanin or β1 integrins support this issue. Accordingly, anti-α3 mAbs have been reported to induce branching morphogenesis in a growth factor-independent way (Berdichevsky et al., 1994). Alternatively, delocalization of integrin-tetraspanin complexes from intercellular contacts by antibody crosslinking or ligand binding might induce the morphogenesis signal.

α3β1 integrin has recently been shown to be involved in cytoskeletal organization (Wang et al., 1999; Kreidberg, 2000) and to act as a trans-dominant inhibitor of integrin receptor function (Dogic et al., 1998; Hodivala-Dilke et al., 1998), suggesting that interactions via α3β1 integrins induce inhibitory signals to other integrins. The particular characteristics of this integrin heterodimer might lie on its strong association with certain tetraspanin proteins (Yauh et al., 1998). Further studies on the function of this integrin should thus take into account the tetraspanin contribution as well as whether tetraspanin proteins can affect other integrin heterodimer functions in the absence of α3β1.

We thank all people in Dr W. J. Nelson’s laboratory, especially K. Siemers, for their support and help with polarity experiments; C. Domínguez-Jiménez for crucial experimental advise, P. Sánchez-Mateos for the papain digestion, Dr E. Dejana for providing us with the CHO cell clones; M. A. Olazcarrizqueta for technical assistance with confocal microscopy; M. A. García-López for her help with thyroid epithelium, and Drs R. González-Amaro and M. A. Alonso for critical reading of the manuscript. This work was supported by grants SAF99-0034-C02-01 and 2FD97-0630-C02-02 from the Ministerio de Educación y Cultura, by QLRT-1999-01036 from the European Community to F. S.-M., and by grant from the Asociación para el recherche sur le cancer (9334) to P. R.

REFERENCES


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