Abstract. Tumor cell migration through the three-dimensional extracellular matrix (ECM) environment is an important part of the metastatic process. We have analyzed a role played by the integrin–tetraspanin protein complexes in invasive migration by culturing MDA-MB-231 cells within Matrigel. Using time-lapse video recording, we demonstrated that the Matrigel-embedded cells remain round and exhibit only limited ability for migration by extending short, highly dynamic pseudopodia. The α3β1-tetraspanin protein complexes were clustered on the thin microvilli-like protrusions extending from both the main cell body and pseudopodia. Ligation of the α3β1-tetraspanin protein complexes with monoclonal antibodies specifically stimulates production of matrix metalloproteinase 2 (MMP-2) and induces formation of long invasive protrusions within Matrigel. Accordingly, treatment with the monoclonal antibodies to various tetraspanin proteins and to the α3 integrin subunit increases invasive potential of the MDA-MB-231 cells in the Matrigel-penetration assay. A specific inhibitor of phosphoinositide 3-kinase (PI3K), LY294002, negated the effect of the monoclonal antibodies on the morphology of the Matrigel-embedded cells and on production of MMP-2. Interestingly, broad-spectrum inhibitors of protein tyrosine kinases (genistein) and protein tyrosine phosphatases (orthovanadate), and actin filament stabilizing compound (jasplakinolide), also block protrusive activity of the Matrigel-embedded cells but have no effect on the production of MMP-2. These results indicate that α3β1-tetraspanin protein complexes may control invasive migration of tumor cells by using at least two PI3K-dependent signaling mechanisms: through rearrangement of the actin cytoskeleton and by modulating the MMP-2 production. 

Key words: integrin • tetraspanin • invasion • matrix metalloproteinase • signaling
mediated by adhesion receptors of the integrin family may have a significant impact on production of MMPs by tumor cells (H€ino, 1996). In osteosarcoma cells, the $\alpha_{2}\beta_{1}$ integrin is a positive regulator of the expression of MMP-1 (Riikonen et al., 1995). Integrin-mediated adhesion to laminin and antibody-induced clustering of $\alpha_{3}\beta_{1}$ integrin enhanced the secretion of MMP-2 in rhabdomyosarcoma and glioblastoma cells (Chintala et al., 1996; Kubota et al., 1997). Likewise, production of MMP-2 during melanoma cell invasion was modulated by $\alpha_{v}\beta_{3}$ and $\alpha_{3}\beta_{1}$ integrins (Seftor et al., 1993). Signaling pathways that link activation of integrin receptors and production of MMPs in tumor cells are poorly understood. In osteosarcoma cells, wide range inhibitors of protein tyrosine kinases could prevent upregulation of MMP-1 by collagen (Riikonen et al., 1995). In ovarian cancer cells, both focal adhesion kinase and Ras are required for production of MMP-9 induced by fibronectin (Shibata et al., 1998).

Integrins also play a pivotal role in the regulation of a rapid turnover of cell-ECM adhesion contacts and actin cytoskeleton dynamics during invasive migration. A complex network of integrin-mediated signaling pathways, involving small Rho-family GTPases, phosphoinositide 3-kinase (PI3K), and nonreceptor tyrosine kinases of the Src family, sets the basis for migratory behavior of tumor cells (Keeley et al., 1997; Shaw et al., 1997; Thomas and Brugge, 1997). Interestingly, remodelling of actin cytoskeleton induced by ECM may be directly linked to activation and regulation of MMP production (Tomasek et al., 1997; Chintala et al., 1998). A though significant progress has been made recently towards identifying key elements within the invasion-related signaling network, relatively little is known about the initial steps of the signaling processes triggered by integrin receptors. A mong the numerous integrin-associated protein partners identified (Hemer, 1998), only a few seem to have a direct relevance to the invasive process. The receptor for urokinase type plasminogen activator interacts with various integrin receptors and may have an important role in tethering ECM-degrading activity to the adhesion sites (Chapman, 1997). Focal adhesion kinase, which is associated with the cytoplasmic tail of various integrin $\beta$ subunits and activated upon integrin ligation, is thought to regulate structural organization and turnover of adhesion complexes (Ilic et al., 1995; Guan, 1997).

Tetraspanins, or TM4SF proteins, are a large group of widely expressed cell surface transmembrane proteins, some of which can form complexes with various integrins (Hemler et al., 1996; Macker et al., 1997). A number of tetraspanins were described as tumor-specific antigens (Szala et al., 1990; Marken et al., 1992; Jankowski et al., 1994; Takagi et al., 1995), and recent evidence suggests that the function of some TM4SF proteins may be particularly relevant to tumor cell metastasis. For example, ectopic expression of CD9, CD63, or CD82 in tumor cells suppressed their metastatic potential in animal model systems (Keyama et al., 1993; Dong et al., 1995; Radford et al., 1995). Furthermore, elevated levels of expression of CD9 and CD82 were linked to a higher survival rate for various human cancers (Miyake et al., 1995; A dachi et al., 1996). It was hypothesized that tetraspanins may be implicated in the assembly of integrin-containing signaling complexes, thus modulating the function of integrin receptors in cell migration (Hemler et al., 1996; Berditchevski et al., 1997b; D O manco et al., 1997; M ecker et al., 1997; Y af€€ez-M€ö et al., 1998; Y auch et al., 1998). In this study we analyzed invasive migration of mammary carcinoma cells within the three-dimensional ECM environment and examined the involvement of tetraspanins in this process. Our results indicate that $\alpha_{3}\beta_{1}$-tetraspanin protein complexes play an important role in regulating proteolytic activity of the tumor cells and contribute to ECM-induced production of MMP-2.

**Materials and Methods**

**Reagents**

Herbimycin B, sodium orthovanadate, genistein, cytochalasin B, and aprotinin were purchased from Sigma Chemical Co. L Y 294002, ML7, KT5720, bisindolylmaleimide I, and human recombinant tissue inhibitor of metalloproteinases (TIMPs) were from Calbiochem. Jasplakinolide was obtained from Cambridge Bioscience. A broad-range inhibitor of MMPs, BB3103, was provided by British Biotech Pharmaceuticals Ltd. Rat tail collagen, type I was purchased from Collaborative Biomedical Products.

**Antibodies and Cells**

A nintegrin mAb was used: anti-$\alpha_{2}$, A2-IIE10 (Lee et al., 1995) and A2-VIIIE6 (Lee et al., 1995); anti-$\alpha_{3}$, P1B5 (Carter et al., 1990) and A3-IIIF5 (Weltzman et al., 1993); anti-$\alpha_{6}$, A6-BB (Tachibana et al., 1997); anti-$\beta_{1}$, clone P5D2 (provided by Dr. N. Hotchin, University of Birmingham, Birmingham, UK). A nti-TM4SF5 mAb was used: anti-C9-D9, C9-BB (Tachibana et al., 1997); anti-C9D3, H1 (Berditchevski et al., 1995); anti-C9D3, M38 (Imai and Yoshie, 1993); anti-C9D2, M104 (Imai and Yoshie, 1993); and anti-C9D1, SC11 (Berditchevski et al., 1997a). Other mAb was used: anti-iemannin, 8G6 and 7E7 (Berditchevski et al., 1997a); anti-MMP-9, 4H3; and anti-MMP-1, SE600 (British Biotech Pharmaceuticals, Ltd.). A nti-MMP-2 polyclonal antibody, ABB08, was from Chemicon International. A ntiphosphorytrosine (pTyr) mAb, clone 4G10, was purchased from Cambridge Bioscience. A ntiphosphatidyl serine mAb, clone P3K, and anti-Z1, clone GoH3, were purchased from Serotech. Various anti-$\alpha$-cat mAb was purchased from New England Biolab. The MDA-MB-231 human breast cancer cell line was purchased from American Type Culture Collection and maintained in L-15 Leibovitz medium (Sigma Chemical Co.) supplemented with 15% FCS. HT3080zeo and HT1080/C9 cells (Berditchevski et al., 1996) were cultured in DMEM supplemented with 10% FCS.

**Three-Dimensional Cell Culture and Time-Lapse Video Recording**

To study the cell behavior in the three-dimensional ECM environment, the cells were embedded into Matrigel (Becton Dickinson) according to the manufacturer’s recommendations. In brief, MDA-MB-231 cells were detached from culture dishes and suspended in Matrigel solution at the final concentration of 5 $\times$ 10$^6$ cells/ml at 4°C. The cell suspension was aliquoted (250–300 $\mu$l) into a 24-well plate and left to solidify at 37°C for 30 min. The cells embedded into Matrigel were cultured in L-15 growth medium with or without FCS. For time-lapse video recording, Matrigel-embedded cells were cultured in the temperature controlled humidified box mounted on the stage of the inverted microscope (Axiovert 25; Carl Zeiss). Migration of MDA-MB-231 cells within Matrigel was recorded using the Hitachi CCD camera connected to the computer (model P5-233; Gateway 2000) and the captured images were processed using the Image PC software package (Scion Co.).

**Immunofluorescence Staining of MDA-MB-231 Cells in Three-Dimensional Matrices**

Cellular localization of integrins and tetraspanins on the surface of embedded MDA-MB-231 cells was analyzed by indirect immunofluorescence and confocal immunofluorescence microscopy. The three-dimensional cultures were fixed with 1%
paraformaldehyde/PBS for 20 min and washed three times with PBS for 1 h. The primary antibodies were applied to the cultures and incubated for 1 h at room temperature. A filter three 10-min washes in PBS, samples were incubated for 1 h at room temperature with FITC-conjugated goat anti-mouse antibody (Sigma Chemical Co.). A filter subsequent washes, the samples were analyzed using a Zeiss A xioscop. The immunofluorescence images were captured using the Coolview CCD camera (Photonic Sciences) and subsequently processed for analysis by using the Openlab software package (Improvision).

Cell Adhesion Assay
A standard static adhesion assay (30–35 min) was carried out as described previously (Weitzman et al., 1993). When the effect of mAb on adhesion was studied, cells were preincubated with mAbs at 4°C for 30 min and then aliquoted into 96-well plates precoated with various concentrations of Matrigel.

In Vitro Invasion Assay
For the invasion assay, an upper surface of 8-μm framed polycarbonate filters (NeuroProbe, Inc.), was coated at 17.5 μg/cm² of Matrigel for 30 min at 37°C. The lower compartments of the 96-well chemotactic Boyden chamber were filled with L-15 medium supplemented with 5% FCS. MDA-MB-231 cells (4 × 10⁴) were suspended in serum-free L-15 media containing 30 μg/mL of BSA (no treatment) or mAb at a similar concentration. In the experiments using a mixture of anti-TM 4SF antibodies, the mAb E9-B8, 6H1, and 4C11 were combined at a final concentration of 10 μg/mL each. Untreated and mAb-treated cells were incubated for 1 h at 37°C before they were applied to the upper compartment of the chamber (chamber well). A filter incubation for 20 h at 37°C in 5% CO₂ and 95% air, the filters were fixed with methanol and stained with 1% crystal violet (Sigma Chemical Co.). The cells from the upper surface of the filter were wiped off, and the migrated cells were counted in five to eight randomly selected microscopic fields (×1,200) for each chamber well. All experiments were performed in triplicate.

Gelatin Zymography
Production of MMPS by MDA-MB-231, HT1080/neo, and HT1080/CD9 cells was analyzed by gelatin zymography as described previously (Huesmann and Dowdle, 1980). Cells suspended in the complete media were plated for 4 h on the 100-mm bacteriological dish precoated either with ECM ligands or immobilized antibodies. A filter three washes with serum-free L-15 media, cells were incubated in serum-free media for an additional 24 or 48 h before the conditioned media were collected for the analysis. The conditioned media were supplemented with Laemmli sample buffer, and the loading volumes of each sample were subsequently adjusted according to the cell number. The samples were resolved in 9% acrylamide SDS-PAGE containing 1 mg/ml gelatin. The gels were washed three times with 2.5% Triton X-100 for 1 h, and incubated for 24 h at 37°C in 50 mM Tris-HClpH 7.4, 10 mM CaCl₂, 150 mM NaCl with 0.02% NaN₃. The lytic bands were visualized by Coomassie brilliant blue R250 staining. A filter staining, the gels were scanned using the GI6000 Gel densitometer (Bio-Rad) and analyzed using the Molecular Analyst software package (Bio-Rad).

Immunoprecipitation and PI3K Assay
Serum-starved MDA-MB-231 cells were plated on the immobilized mAb bs or ECM ligands in serum-free DMEM for 1 h. Cells were lysed for 3 h at 4°C in 1% Brij 98 buffer containing 20 mM Tris HCl, pH 7.4, 140 mM NaCl, 10% glycerol, 1 mM NaVO₄, 1 mM NaF, 10 mM Na-pyrophosphate, 2 mM PM SF, 5 μg/ml aprotinin, and 10 μg/ml leupeptin. Insoluble material was pelleted at 13,000 rpm and the supernatants were precleared with 30 μl of phosphatidylinositol (Sigma Chemical Co.) and 5 μCi (γ³²P)ATP. The reactions were terminated after 15 min with 60 μl 2 N HCl, and lipids were extracted with 160 μl of 1:1 (vol/vol) chloroform/methanol. The lipids were subsequently analyzed by TLC (Y ach et al., 1998).

Immunoblotting
Serum-free growth medium, conditioned by MDA-MB-231 cells for 24 h, was incubated with gelatin-conjugated Sepharose beads for 2 h at 4°C. The beads were washed three times with 50 mM Tris HCl, pH 7.6, 150 mM NaCl, 5 mM CaCl₂, and the captured material was eluted from the beads into Laemmli loading buffer at 95°C. Proteins were resolved in 10% SDS-PAGE, transferred to the nitrocellulose membrane, and developed with the specific anti-MMP antibody. Protein bands were visualized using HRP-conjugated secondary antibodies and Enhanced Chemiluminescence Reagent (Amersham Pharmacia Biotech).

Results
In this study we investigated whether integrin–tetraspanin protein complexes play a role in controlling invasive behavior of tumor cells. As a model system we used highly invasive mammary epithelial cells, MDA-MB-231. In preliminary experiments, we have established that MDA-MB-231 cells express five TM4SF proteins (e.g., CD 9, CD 63, CD 81, CD 82, and CD 151) and each of them form complexes with α3β1 integrin (data not shown).

Migration of MDA-MB-231 Cells within Matrigel
Migration on planar surfaces coated with ECM proteins is a widely used paradigm for analyzing tumor cell invasion. Indeed, the data accumulated in these in vitro studies provided important insights into the molecular mechanisms that may control invasive behavior of tumor cells. However, it became increasingly obvious that cellular responses may change when cells are placed within a three-dimensional ECM environment (Boudreau and Bissell, 1998). Thus, we rationalized that migratory behavior of tumor cells within a three-dimensional ECM could reflect an invasion process more accurately and would allow better understanding of the molecular mechanisms that control invasive migration. In the course of this study we examined the behavior of MDA-MB-231 cells within artificial EHS matrix (Matrigel). Time-lapse video microscopy has shown that within the first 2–4 h after embedding into Matrigel, characteristic short pseudopodial protrusions began to appear (Fig. 1). These protruding structures were dynamic and typically retracted within 15–40 min. Although they remained round, most cells had undergone various types of motion, including wobbling, turning around, and slow directional movement (Fig. 1, A–E). The average rate of cellular migration within Matrigel was 3.44 ± 0.8 μm/h (calculated observing 19 cells in 12 separate experiments). The random protrusive activity persisted over 48–72 h, with the protrusions becoming longer and more stable at later times, and then decreased in number when cells started to form round colonies. The cells exhibited similar behavior when they were plated on top of polymerized Matrigel (data not shown). These experiments have demonstrated that migration through three-dimensional ECM environment does not require cell spreading, but may rely on short invasive protrusions developed by tumor cells (see also below).
The α3β1–Tetraspanin Protein Complexes Are Abundant at the Periphery of Thin Invasive Protrusions and Control Protrusive Activity of MDA-MB-231 Cells in Matrigel

We began investigating the role of α3β1-tetraspanin protein complexes in invasive migration by examining their distribution on the Matrigel-embedded MDA-MB-231 cells. In the initial experiments, we carried out whole-mount immunofluorescence staining using nonpermeabilized cells. When analyzing various focus planes, we found that α3β1 integrin and tetraspanins were concentrated within distinctive large clusters covering the cell body and at the tips of short thread-like protrusions (Fig. 2A–D). These microvilli-like protrusions extended from the main cell body and from the pseudopodia (Fig. 2G and H), thus producing characteristic hairy images. In contrast, clusters of α2β1 integrin were visibly smaller and not detected on the thread-like protrusions (Fig. 2E and F).

Given their abundant presence at the tips of the microvilli-like cellular extensions, we addressed the question as to whether the α3β1-tetraspanin complexes have the potential to affect protrusive activity of MDA-MB-231.
cells. Thus, we examined the effect of antitetraspanin mAbs on the morphology of the cells embedded in Matrigel. These experiments were performed under serum-free conditions to avoid growth factor interference. As in the presence of serum, during the first 24 h after embedding, the cells incubated without mAbs remained rounded, and only 25–30% of them extended one or two short (0.25–0.5 size of the cell body diameter) pseudopodial protrusions (Fig. 3 A). In contrast, 45–70% of the cells incubated with mAbs to CD9, CD63, CD81, and CD151 developed long thin extensions that often exceeded the cell body diameter by 2–2.5-fold (Fig. 3, B–E). When analyzed using time-lapse video microscopy, we found that in the presence of antitetraspanin mAbs, protrusive activity of the cells was not appreciably affected, whereas retraction of the invasive protrusions was attenuated, thus leading to their extended elongation (Fig. 4). Notably, we observed that the mAb-treated cells could use these elongated protrusions to generate traction forces and facilitate directional movements within Matrigel (Fig. 4). Importantly, the morphogenetic effect of the mAb to α3 integrin subunit was comparable to that of antitetraspanin mAbs (Fig. 3 F). On the other hand, mAbs to α2 integrin subunit and emmprin, an abundant cell surface protein that also form complexes with α3β1 integrin (Berditchevski et al., 1997a), had no appreciable effect on cell morphology (Fig. 3, G and H, respectively). Taken together, these results indicate that α3β1-tetraspanin complexes play an important role in controlling protrusive activity of the MDA-MB-231 cells.

**Effect of Pharmacological Inhibitors on the mAb-induced Protrusive Activity of MDA-MB-231 Cells**

One explanation for the observed changes of cell morphology is that the mAbs could directly affect adhesive interactions between MDA-MB-231 cells and Matrigel. Thus, we tested the effect of the antitetraspanin and anti-integrin mAbs on attachment of the cells to various concentrations of Matrigel. These experiments demonstrated that mAbs to the α3 integrin subunit or to tetraspanins (tested separately or in various combinations) neither diminished nor increased adhesion of MDA-MB-231 cells to Matrigel (Fig. 5). Interestingly, we found that the function inhibitory mAb to the α2 or α6 integrin subunits also failed to interfere with the adhesion of the cells to Matrigel. On the other hand, an inhibitory mAb to the β1 integrin subunit almost completely blocked adhesion of MDA-MB-231 cells to the substrate. While these results clearly demonstrate that adhesive interactions of MDA-MB-231 cells with Matrigel matrix involve various β1 integrins, they also argue against a direct modulation of cell adhesiveness by the α3β1-tetraspanin protein complexes. We surmised that clustering with the antitetraspanin or anti-α3 mAbs may trigger signaling events that induced changes of morphology of the Matrigel-embedded cells.

To begin investigating which signaling pathway(s) is involved in the MAb-mediated morphogenetic responses, MDA-MB-231 cells (untreated or treated with the anti-CD63 mAb) were cultured in Matrigel in the presence of various pharmacological inhibitors. The results of these experiments are shown in Fig. 6. Orthovanadate and

| Antibodies | Mean(%) | SD  |
|------------|---------|-----|
| No-treatment | 24.93 | 5.87 |
| C9BB | 59.70 | 7.80 |
| 6H1 | 64.80 | 6.07 |
| M38 | 62.80 | 4.02 |
| 5C11 | 66.34 | 7.58 |
| A3-IIF5 | 56.29 | 7.63 |
| A2-IIIE10 | 33.16 | 7.13 |
| 7E7 | 29.27 | 9.54 |

Figure 3. Antitetraspanin mAbs induce cellular protrusions on the Matrigel-embedded MDA-MB-231 cells. MDA-MB-231 cells were treated for 30 min at room temperature with 25 µg/ml of either BSA (A) or purified mAbs: C9BB, anti-CD9 (B); 6H1, anti-CD63 (C); M38, anti-CD81 (D); 5C11, anti-CD151 (E); A3-IIF5, anti-α3 integrin subunit (F); A2-IIIE10, anti-α2 integrin subunit (G); 7E7, antiemmprin (H). The cells were embedded into Matrigel and cultured under serum-free conditions for 24 h. Quantitative data on the effect of the mAbs are shown in the table. Percentage of cells that developed protrusions was calculated in five randomly selected microscopic fields. A cell was scored positive (e.g., cell with the protrusions) if it had at least one protrusion with length equal or exceeding the diameter of the cell body. All experiments were performed in triplicate. Bar, 10 μm.
genistein inhibited protrusive activity of both untreated and mA b-treated cells, suggesting that the basal and antibody-induced protrusive activities involve functions of tyrosine phosphatase(s) and protein tyrosine kinase(s) (Fig. 6, G and H). In agreement with this notion, herbimycin, another tyrosine kinase inhibitor, could mimic the morphogenetic effect of the mAb (Fig. 6, B and E). Notably, a specific inhibitor of PI3K, LY294002, inhibited only the mAb-mediated morphogenetic response and did not affect the basal protrusive activity of MDA-MB-231 cells (Fig. 6 I). In contrast, inhibitors of myosin light chain kinase (ML7), protein kinase C (bisindolylmaleimide I), and cAMP-dependent protein kinase (KT5720) had no appreciable effects on the morphology of the Matrigel-embedded cells (Fig. 6, K and L, and data not shown, respectively). We also analyzed the effect of the drugs that affect the actin cytoskeleton. Interestingly, we found that jasplakinolide, a macrocyclic peptide that induces actin polymerization (Sheikh et al., 1997), negated the effect of the antitetrascranin complexes with mA bs can affect production of MMsPs, MDA-MB-231 cells spread on tissue culture plastic were incubated with anti-CD81 and antitetraspanin mAbs (Fig. 8 C). Interestingly, we have noticed that the efficacy of the stimulatory signals is dependent on presentation of the particular antitetraspanin mAb. Indeed, clustering with soluble anti-CD81 and anti-CD151 mA bs caused only a modest increase in production of MMP-2 when compared with anti-CD9 and

**Figure 4.** Effect of the anti-CD 151 mA b on morphology and migration of MDA-MB-231 cells in M atrigel. MDA-MB-231 cells were incubated with the anti-CD 151 mA b (as described in the legend to Fig. 3), embedded into M atrigel, and cultured in a temperature-controlled humidified chamber for 24 h. (A–E) A sequence of phase-contrast video images taken at 5 min (A), 190 min (B), 305 min (C), 495 min (D), and 895 min (E) after the embedding. (F) A digital overlay of A and E showing contours of the cell in the beginning (white dashed line) and at the end (black solid line) of the recording. A row indicates direction of the cell body translocation. Bar, 10 μm.

**Effect of Antiintegrin and Anti-TM4SF Protein Antibodies on Production of MMPs**

We hypothesized that MMsPs, a group of ECM-degrading enzymes, may play an important role in controlling cellular behavior within M atrigel. Indeed, both basal and mA b-induced protrusive activities of MDA-MB-231 cells were completely abolished when the cells were cultured in the presence of BB 3103, a wide-range MMP inhibitor (Fig. 7). On the other hand, aprotinin, a common inhibitor of serine proteases, caused only ~25% inhibition of the mA b-induced protrusive activities (Fig. 7). Next, we addressed the question whether or not α3β1-tetraspanin complexes may be involved in the regulation of MMP production. When grown on tissue culture plastic MDA-MB-231 cells secrete two major gelatinolytic activities of 92 and 56 kD (Fig. 8 A, lane a). In addition, we consistently detected a weaker gelatinolytic band of 72 kD. Western blotting analysis carried out with MMP-specific antibody has shown that 92- to 72- and 56-kD proteins corresponded to MMP-9, MMP-2, and MMP-1, respectively (Fig. 8 A, lanes b–d). To examine whether clustering of the α3β1-tetraspanin complexes with mA bs can affect production of MMPs, MDA-MB-231 cells spread on tissue culture plastic were incubated with anti-α3 or various antitetraspanin mA bs for 48 h and the samples of conditioned media were analyzed by gelatin zymography. As illustrated in Fig. 8 B, treatment with anti-α3 and all four tested antitetraspanin mA bs enhanced the production of the latent form of MMP-2 by 2–15-fold. Notably, this treatment did not affect production of MMP-9 or MMP-1 (Fig. 8 B, and data not shown, respectively). In the control experiments, anti-α2 or antiemmprin mA bs did not alter the amounts of MMP-2 and MMP-9 secreted by the MDA-MB-231 cells. Likewise, a specific increase in production of MMP-2 was observed when the cells were plated on the immobilized anti-α3 and antitetraspanin mA bs (Fig. 8 C). Interestingly, we have noticed that the efficacy of the stimulatory signals is dependent on presentation of the particular antitetraspanin mAb b. Indeed, clustering with soluble anti-CD 81 and anti-CD 151 mA bs caused only a modest increase in production of MMP-2 when compared with anti-CD 9 and
Figure 5. Anti-tetraspanin and anti-integrin mAbs do not influence adhesion of the MDA-MB-231 cell to Matrigel. BCECF-AM–labeled MDA-MB-231 cells were preincubated with anti-tetraspanin mAbs (25 μg/ml) and tested for adhesion to a 96-well microtiter plate coated with Matrigel as described in Materials and Methods. The mAbs used were: P5D2, anti-β1; GOH3, anti-α6; A2-IIE10, anti-α2; A3-IIF5, anti-α3; C9BB, anti-CD9; 6H1, anti-CD63; 5C11, anti-CD151; and 7E7, anti-emmprin. The results are presented as percentage of attached cells.

anti-CD63 mAbs (Fig. 8 B, compare lanes e and g with lanes d and f). On the other hand, the stimulation induced by the immobilized mAbs was comparable for all four tetraspanins (Fig. 8 C, lanes b–f). Not only do these data sug-
experiments were performed in triplicate. To obtain direct evidence that tetraspanins are involved in the adhesion-dependent production of MMP-2, we examined gelatinolytic activities present in culture media conditioned by HT1080/zeo and HT1080/CD9 cells. The HT1080/CD9 line has been established by expressing human CD9 cDNA in HT1080, the human fibrosarcoma cell line, whereas HT1080/zeo was developed as a control drug-resistant cell line (see Materials and Methods for details). Both cell lines expressed similar levels of β1 integrins and were comparable in their ability to attach to various ECM ligands (data not shown). When plated on collagen or laminin 5-containing ECM, both cell lines secrete comparable amounts of MMP-9 (Fig. 9, lanes a–d). Strikingly, the amount of MMP-2 secreted by the HT1080/CD9 cells plated on laminin 5-containing ECM was significantly higher (approximately fourfold) than that secreted by the HT1080/zeo cells (Fig. 9, lanes b and d). On the other hand, growth media conditioned by the cells plated on collagen contained similar amounts of MMP-2 (Fig. 9, lanes a and c). Given the fact that attachment of the HT1080 cells to laminin 5-containing ECM is mediated by α3β1 integrin (Wayner et al., 1993), these results provide strong support for the idea that α3β1-tetraspanin protein complexes (and the α3β1–CD9 complex in particular) may be involved in the ECM-induced production of MMP-2.

Next, we carried out two sets of experiments to address the question of whether the effect of the mAbs on the morphology of the Matrigel-embedded cells is related to the increased production of MMP-2. First, MDA-MB-231 cells were cultured in Matrigel in the presence of both anti-CD63 mAb and TIMP-2, a potent inhibitor of MMP-2. The gelatinolytic activities present in culture media conditioned by MDA-MB-231 cells were resolved in 10% PAGE and analyzed by a gelatin zymography (lane a) or by Western blotting with mouse mAb 4H3, anti–MMP-9 (lane b), rabbit polyclonal antibody 808, anti–MMP-2 (lane c), and rabbit polyclonal antibody SE600, anti–MMP-2 (lane d). (B) MDA-MB-231 cells were plated on a 24-well plate in serum-containing growth media for 8 h. After washes with PBS, the cells were incubated for 48 h in serum-free media supplemented with 25 μg/ml BSA (control sample, lane a); mAb B2-VIIIC6, anti-α2 integrin (lane b); mAb 4A3-IIF5, anti-α3 integrin (lane c); mAb 6H1, anti-CD63 (lane d); mAb M38, anti–CD81 (lane e); mAb C9-BB, anti–CD9 (lane f); mAb 5C11, anti–CD151 (lane g); and mAb 7E7, antiemmprin (lane h). Conditioned media were analyzed by gelatin zymography as described in Materials and Methods. Gelatinolytic activities of the samples were quantified by scanning densitometry using the Molecular Analyst software package, and the data are presented as percentage relative to a control sample. (C) MDA-MB-231 cells were plated on immobilized mAbs in serum-containing growth media for 4 h. After washes with PBS, the cells were incubated for 24 h in serum-free media before the samples of the conditioned media were analyzed by gelatin zymography. Gelatinolytic activities of the samples were quantified as above, and the data are presented as percentage relative to a sample collected from the cells plated on antiemmprin mAb. The immobilized mAbs were: P1B5, anti–α3 (lane a); C9-BB, anti–CD9 (lane b); 4H3, anti–CD63 (lane c); M38, anti–CD81 (lane d); M104, anti–CD82 (lane e); 5C11, anti–CD151 (lane f); and 7E7, antiemmprin (lane g). (B and C) White columns represent MMP-9, and black columns represent MMP-2.
As shown in Fig. 7, TIMP-2 only partially negated the mAb-induced protrusive activity of the MDA-MB-231 cells. Second, we analyzed the effect of pharmacological inhibitors on the mAb-induced production of MMP-2. These experiments were carried out with MDA-MB-231 cells plated on the immobilized antitetrarpsalin mAb. Preliminary experiments have demonstrated that the presence of pharmacological inhibitors during the time of the experiment had no significant effect on the morphology or viability of the cells attached to the immobilized antitetrarpsalin mAbs. However, we decided against applying the inhibitors to the mAb-treated cells grown on the uncoated glass coverslips (as in the experiment shown in Fig. 8 B), since some of them induced dramatic changes of cell morphology and might, therefore, affect the MMP production in a nonspecific fashion. The MDA-MB-231 cells were seeded on immobilized anti-CD151 mAb, the strongest stimulus of MMP-2 production (Fig. 8 C, lane f), and the effect of the pharmacological inhibitors was analyzed by gelatin zymography. Gelatinolytic activities of the samples were quantified as described in the legend to Fig. 8, and the data are presented in arbitrary densitometric units.

A Role of the α3β1-Tetraspanin Protein Complexes in Tumor Cell Invasion

To analyze whether antibody-induced production of MMP-2 and changes of cell morphology could affect invasiveness of MDA-MB-231 cells, we performed the Matrigel penetration assay. As shown in Fig. 11 A, continuous presence of various antitetrarpsalin mAbs during the time of the assay (tested separately or in combinations) increased invasiveness of MDA-MB-231 cells 1.5–3.5-fold. Similarly, the mAb to α3 integrin subunit also facilitated cell invasion through Matrigel. Interestingly, we found that the inhibitory mAb to α2 integrin subunit also increased invasiveness of the MDA-MB-231 cells (up to 1.6-fold). In the control experiments, mAbs to α6 integrin subunit or to emmprin had no effect on cell invasion (Fig. 11 A). Next we investigated the effect of pharmacological inhibitors on invasiveness of MDA-MB-231 cells. As illustrated in Fig. 11 B, inhibitors to PI3K, myosin light chain kinase, and one of the protein tyrosine kinase inhibitors, genistein, efficiently blocked cellular invasion induced by the antitetrarpsalin mAb, with only a minimal effect on the basal level of invasion. Similarly, treatment with jasplakinolide specifically negated the effect of the mAb on invasion of MDA-MB-231 cells. On the other hand, orthovanadate, cytochalasin B, and to a certain extent protein kinase C inhibitor were effective in blocking both basal and mAb-induced cellular invasion (Fig. 11 B). Interestingly, herbimycin, a more selective inhibitor of tyrosine kinases, had no effect on the mAb-induced invasion but facilitated basal invasiveness of the MDA-MB-231 cells. Taken together, these results demonstrate that the invasive process involves multiple signaling proteins, and there is only a partial correlation between the ability of MDA-MB-231 cells to develop invasive protrusions and their overall invasive potential. Finally, we investigated the contribution of MMPs in cellular invasion through Matrigel. We found that both broad-range (BB 3103) and more selective (TIP-2) inhibitors of MMPs decreased invasiveness of the MDA-MB-231 cells (Fig. 11 C).
The Role of the α3β1–Tetraspanin Protein Complexes in Activation of PI3K

Given a notable consistency of LY294002 inhibiting both morphological and biochemical consequences of the mAb clustering, we wanted to investigate whether there is a direct link between activation of the α3β1–tetraspanin complexes and signaling pathways involving PI3K. To this end, two sets of experiments were carried out. First, we examined whether or not plating of MDA-MB-231 cells on the mAbs facilitated an interaction of PI3K with tyrosine phosphorylated cellular proteins. Lipid kinase assays performed on the anti-pTyr immunoprecipitates have shown that the activities of the associated enzyme in cells plated on different substrates (including various mAb bs and ECM proteins) were unchanged relative to a control sample (e.g., cells kept in suspension) (Fig. 12, A–C). Second, we assessed phosphorylation levels of c-Akt, a serine/threonine kinase whose phosphorylation (and activation) is regulated by PI3K (Downward, 1998). We found that plating of MDA-MB-231 cells on various antitetraspanin and particular anti-α3 mAb bs induced a small (≤1.8–4-fold, in 3 separate experiments) but reproducible increase in the phosphorylation levels of c-Akt (Fig. 12 D, upper panel, lanes c–e and lane b, respectively, and Fig. 12 E). On the other hand, c-Akt phosphorylation level in the cells attached to the antiemmprin mAb b was comparable to that found in the control sample (e.g., cells kept in suspension) (Fig. 12 D, upper panel, lanes f and a, respectively). Although activation of c-Akt may not be directly related to cell invasion (Shaw et al., 1997) or induction of cellular protrusions within Matrigel, these results clearly indicate...
the α3β1-tetraspanin protein complexes can modulate signaling pathway involving PI3K.

Discussion

Penetration through the basement membrane is an important step during tumor dissemination. To gain an insight into cellular and molecular mechanisms that control this process we examined invasive behavior of tumor cells in response to Matrigel, a widely used mimic of the basement membrane. Numerous earlier studies have provided a detailed morphological analysis of tumor cells migrating across planar surfaces, and uncovered a number of signaling pathways controlling cell motility. A critical novel aspect of our study is the choice to use three-dimensional Matrigel to analyze specifically invasive migration. By culturing cells within a three-dimensional ECM environment, we were aiming to observe cellular responses that would more accurately reflect invasive processes occurring in vivo. Our data clearly indicate that significant differences exist between planar and three-dimensional migration. Migration of MDA-MB-231 cells on absorbed Matrigel could be described by a well-defined three-step extension-retraction model: extension of lamellipodial protrusions → generation of traction forces, jerky cell body translocation → retraction of the trailing edge (Sugiura, T., and F. Berditchevski, unpublished results). In contrast, cells embedded in or plated on top of Matrigel remain rounded and generate highly dynamic short pseudopodial extensions that may play a major part in governing limited ability for invasive movement (mainly, wobbling, turning around, and slow directional movement). The evidence presented in this report suggests that α3β1-tetraspanin protein complexes could make two important contributions into the invasive process. First, the α3β1-tetraspanin protein complexes can control elongation of invading pseudopodia. Second, α3β1-tetraspanin protein complexes can control elongation of invading pseudopodia. The rapid retraction of the invasive pseudopodia within three-dimensional ECM environment, saturated with potential integrin-binding sites, implies that the signaling pathways triggered within extending protrusions are di-
rected against generating strong cell–ECM interactions along the protrusion length. Given the effect of the anti-tetraspanin mAb on the morphology of the MDA-MB-231 cells, we hypothesize that the function of the α3β1–tetraspanin protein complexes may be linked to stabilization of the invasive protrusions. Furthermore, our data indicate that the α3β1 integrin in complexes with tetraspanins plays a modulatory/signaling role in this process. Although it is theoretically possible that the morphogenetic effect of the mAb is caused by direct modulation of adhesive capacity of the α3β1 integrin, two lines of evidence argue against this notion. First, treatment of MDA-MB-231 cells with anti-TM4SF mAb did not influence adhesion of the cells to Matrigel (Fig. 5) or to any other ECM ligands that were tested in a short-term adhesion assay (Berditchevski, F.B., unpublished results). Second, the α3β1–tetraspanin complexes were clustered at the tips of short, thread-like protrusions that resembled microvilli, distinct morphological structures that are thought to mediate transient rather than stable adhesive interactions (Vestweber and Blanks, 1999). Indeed, the α3β1–tetraspanin clusters were devoid of vinculin (Sugiura, T., unpublished results), a cytoskeletal protein associated with focal complexes and focal adhesions (both are stable adhesion complexes) that can be seen in MDA-MB-231 cells (unpublished). Nevertheless, these data indicate that the ectopic expression of the truncated form of ezrin/radixin/moesin (ERM) family may be among the potential candidates. In this regard, it has been reported that the ectopic expression of the truncated form of ezrin and moesin could destabilize cortical cytoskeleton and induce formation of long filopodia-like extensions (Martín et al., 1995; A mieva and Furthmayr, 1995). The fact that jasplakinolide blocks the mAb-induced extension of protrusions whereas cytochalasin B induces their formation even in the absence of the mAb suggests that the actin cytoskeleton is the ultimate target for the signals triggered by the α3β1–tetraspanin complexes. Although a particular intermediary component that may be involved in the α3β1–tetraspanin-induced reorganization remains unknown, actin-binding proteins of the ezrin/radixin/moesin (ERM) family may be among the potential candidates. In this regard, it has been reported that the ectopic expression of the truncated form of ezrin and moesin could destabilize cortical cytoskeleton and induce formation of long filopodia-like extensions (Martín et al., 1995; A mieva and Furthmayr, 1995). Furthermore, thrombin-induced phosphorylation of moesin on threonine 558 in platelets, a modification that potentiates its binding to F-actin, closely correlated with the formation of long filopodial protrusions (Nakamura et al., 1995).

Numerous earlier studies have shown that the dynamics of actin cytoskeleton can be controlled at various levels, with different tyrosine kinases, tyrosine phosphatases, and P13K being intimately involved in this process (Schmidt and Hall, 1998). Which signaling pathways are utilized by the α3β1–tetraspanin protein complexes to manipulate actin cytoskeleton in the MDA-MB-231 cells? As a part of the current study, we specifically addressed the question as to whether or not activity of the α3β1–tetraspanin protein complexes is linked to the P13K signaling pathways. Our data clearly indicate that this link is possible. First, we found that LY 29004, a specific inhibitor of P13K, has completely abolished a morphogenetic effect of the mAb and attenuated mAb-induced invasiveness of the MDA-MB-231 cells. Second, we observed that clustering of the α3β1–intigrin protein complexes stimulates phosphorylation of c-Akt, a process that is tightly dependent on the activity of P13K (Downward, 1998). Although by itself phosphorylation of c-Akt may not be directly related to the rearrangement of the actin cytoskeleton, these data illustrate a functional connection between the α3β1–integrin protein complexes and P13K-dependent signaling. Earlier studies have shown that P13K activity can be stimulated by cell adhesion to ECM (King et al., 1997). Furthermore, at least two integrin receptors (e.g., α6β4 and α1β1β3) were specifically implicated in activation of P13K-dependent signaling in carcinoma cells and in platelets (Shaw et al., 1997; Banfic et al., 1998). However, the proximal events linking the ligation of these integrins to the P13K signaling pathways remain unknown. Thus, our data not only indicate that another integrin, α3β1, is involved in the P13K-dependent signaling, but also point to a specific type of the integrin accessory proteins that is required for this process (see also below). Theoretically, integrins can affect the P13K-dependent signaling either directly (by modulating enzymatic activity of P13K) or indirectly (by regulating activities and/or compartmentalization of other cellular proteins involved in the P13K signaling, e.g., other phosphatidylinositide kinases, phosphatidylinositide phosphatases, cytoskeletal proteins). In turn, activation of P13K may be linked to tyrosine phosphorylation of cellular proteins (Shaw et al., 1997), which either directly (through Src homology 2 [SH2] domain of p85 subunit of the class I P13K) or indirectly (through Ras-dependent activation of p110 subunit of P13K) recruit and activate the enzyme (Rodriguez-Viciana et al., 1996; Fruman et al., 1998). The fact that a variety of the P13K coimmunoprecipitated with pTyr-containing cellular proteins was not affected in cells attached to the antitetraspanin mAbs argues against the former possibility, and suggests alternative mechanisms. For example, it is possible that the function of α3β1–tetraspanin protein complexes is linked to the activation of the class II or class III P13Ks, the enzymes that are regulated in different fashion from well-characterized mechanisms of activation of the class I P13Ks. A similar pathway has been shown to operate in platelets after the activation of the α1β1β3 integrin (Banfic et al., 1998). Alternatively, it is possible that the complexes can influence the P13K-dependent signaling without directly affecting the activity of the enzyme. For example, it is feasible that the α3β1–tetraspanin protein complexes can regulate local concentration of phosphatidylinositol (PtdIns)-4-P, a potential substrate for P13K, using associated phosphatidylinositol 4-kinase (Berditchevski et al., 1997b; Y auch et al., 1998). This may subsequently increase production of PtdIns-3,4-P2. Not only does this lipid specifically target various cellular proteins whose function may be linked to actin cytoskeleton (e.g., gelsolin, profilin, some isoforms of protein kinase C [Martín, 1998]), but when further converted to PtdIns-3,4,5-P3, it may facilitate additional recruitment (through the SH2 domain of its regulatory subunit) of P13K and subsequent allosterical activation of the enzyme (Fruman et al., 1998). Conversely, we cannot exclude a possibility that α3β1–tetraspanin protein complexes modulate activity of phosphoinositide phosphatases (e.g., SH2 domain–containing inositol 5’-phosphatase, PTE N), thus affecting local concentrations of D-3 phosphoinositides available for binding to their protein targets (including c-Akt). Distinguishing between these various possibilities represents an important challenge for future studies.
An important aspect for consideration in invasive migration is the balance between the protruding forces generated by the cell and the surrounding extracellular matrix, a mechanical barrier that confronts them. Although different groups of ECM-degrading enzymes may be potentially involved, our data indicate that MMPs have a major role in both supporting protrusive activity and invasive migration of MDA-MB-231 cells. The involvement of integrins in MMP-dependent degradation of ECM occurs at various levels, including regulation of production of the enzymes (see Introduction), their activation (Stanton et al., 1998), and site-specific targeting (Brooks et al., 1996).

Here we have shown that signaling through the α3β1 integrin, but not through α2β1 or α6β4 integrins, specifically regulates production of MMP-2. These results add to recent data that have implicated α3β1 integrin in the regulation of MMP-2 production and invasiveness of rhabdomyosarcoma and glioblastoma cells (Chintala et al., 1996; Kubota et al., 1997). Interestingly, in contrast to these earlier studies, treatment of MDA-MB-231 cells with anti-α3 or antitetraspanin mAbs did not induce activation of MMP-2. Furthermore, the expression level and cellular distribution of MT1-MMP, a membrane-type MMP known to be crucial for activation of MMP-2 (Birkedal-Hansen, 1995), were not affected in the MAb-treated cells (Sugiura, T., and F. Berditchevski, unpublished results). Thus, it is possible that either α3β1-dependent activation of MMP-2 in rhabdomyosarcoma cells involves a different type of α3β1-containing protein complex(es) (that is not expressed on MDA-MB-231 cells) or the α3β1-tetraspanin-induced signaling pathway is partly deficient in the breast carcinoma cells.

Although production of MMP-2 is clearly regulated in both normal and cancer tissues, surprisingly little is known about the signaling pathways that control this process. Prostaglandin E2, phorbol esters, and cAMPP were shown to stimulate transcription of MMP-2 in glomerular mesangial cells and fibrosarcoma cells (Arti et al., 1993; Tanaka et al., 1995). Notably, we have found that the MAb-induced production of MMP-2 does not involve the activation of cAMP-dependent protein kinase, but instead, requires the activity of PI3K. This suggests that D-3 phosphoinositides may be signaling mediators in this process. Interestingly, an inhibitor of protein tyrosine phosphatases did not affect the α3β1-tetraspanin-mediated production of MMP-2. This latter observation leads to two important conclusions: (a) production of MMP-2 by itself is not sufficient to stimulate protrusive activity of tumor cells; and (b) signaling pathways leading to the rearrangement of the actin cytoskeleton and formation of long invasive protrusions bifurcate after activation of PI3K.

Finally, an important conclusion that can be drawn from our study is that there is a functional diversity associated with various cell surface pools of the α3β1 receptors. Indeed, although in MDA-MB-231 cells α3β1 integrin is associated with both tetraspanin and emmprin, only the tetraspanin-containing complexes were implicated in the formation of invasive protrusions and the production of MMP-2. These observations provide strong evidence for the idea that the associated protein partners dictate signaling specificity of integrins. On the other hand, our data clearly indicate that there is a signaling redundancy between various α3β1-tetraspanin complexes, as all tested antitetraspanin mAbs induced similar morphological and biochemical responses. It has been postulated that tetraspanins form a network of various interconnected cell surface complexes, a tetraspan web, which, in fact, may be considered as one signaling entity (Lagaudriere-Gesbert et al., 1997; Yáñez-Mó et al., 1998). Thus, our results not only support this notion, but also highlight a specific signaling pathway involving the α3β1-tetraspanin protein web. It should be noted, that in spite of the apparent phenomenological similarities, there were quantitative differences (both at the morphological and biochemical levels) in cellular responses to various antitetraspanin mAbs. This may arise from the unique structural features of a particular tetraspanin (for example, CD63 possesses a lysosomal targeting signal that may specifically affect a postclustering internalization of the protein and its most proximal interacting partners) and, consequently, signaling asymmetry of the web (e.g., there may be differences in spatial proximity of a particular tetraspanin to a specific signaling protein associated with the web [for example, PI4K]).

Cellular invasion is a complex process controlled by multiple interconnected signaling pathways (observed effect of various pharmacological inhibitors in this study), and may involve different members of the integrin family of adhesion receptors. Indeed, we have shown here that not only α3β1 integrin, but also α2β1 integrin may contribute to the invasive phenotype of the MDA-MB-231 cells. Importantly, in preliminary experiments we have established that although it stimulates cell invasion, the anti-α2 mAb has minimal effect on chemotactic migration of the MDA-MB-231 cells towards absorbed Matrigel (Sugiura, T., and F. Berditchevski, unpublished results). This observation implies that α2β1 integrin is specifically engaged during migration through the three-dimensional environment. Although this issue remains open for further investigation, one possibility is that the anti-α2 mAb may affect transient adhesive interactions between the embedded cells and polymerized Matrigel. As discussed above, in the absence of strong attachment points (such as focal adhesions that are assembled in cells migrating on planar surfaces), these highly dynamic interactions would have a dominating role in determining a migratory potential of the cells.

Production of ECM-degrading enzymes (including MMPs), reorganization of the actin cytoskeleton (including destabilization of the cortical cytoskeleton), and the ability to generate traction forces are interdependent yet distinct cellular events that are critical for migration within three-dimensional ECM. Here we show that α3β1-tetraspanin protein complexes may play a crucial role in controlling all three constituents of the invasive process. Thus, further dissecting the signaling events associated with activation of the complexes may prove to be important for better understanding of the molecular mechanisms of tumor cell invasion and metastasis.

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