Characterization of Novel Complexes on the Cell Surface between Integrins and Proteins with 4 Transmembrane Domains (TM4 proteins)

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Here we identified several new integrin/TM4 protein complexes on the cell surface. By immunoprecipitation using nonstringent conditions, and by reciprocal immunoprecipitation, we found that $\alpha^3\beta_1$ and $\alpha^6\beta_1$ integrins but not $\alpha^2\beta_1$, $\alpha^5\beta_1$, or $\alpha^6\beta_4$ integrins associated with CD9 and CD81 in $\alpha^3\beta_1$ /CD81, $\alpha^3\beta_1$ /CD9, $\alpha^6\beta_1$ /CD81, and $\alpha^6\beta_1$ /CD9 complexes. Also, cross-linking experiments established that $\alpha^3\beta_1$ /CD81, $\alpha^3\beta_1$ /CD9, and $\alpha^3\beta_1$ /CD63 associations occur on the surface of intact cells and suggested that a critical interaction site is located within extracellular domains. Cross-linking in conjunction with reimmunoprecipitation indicated that larger multi-component $\alpha^3\beta_1$ /TM4/TM4 complexes ($\alpha^3\beta_1$ /CD9/CD63, $\alpha^3\beta_1$ /CD81/CD63, and $\alpha^3\beta_1$ /CD9/CD81) also could be detected on the cell surface. Immunofluorescent staining showed redistribution of $\alpha^3\beta_1$ /TM4 complexes toward the periphery of cells plated on various extracellular matrix substrates and also showed that these complexes were localized in cell footprints. Staining of human tissues yielded additional results consistent with co-localization of $\alpha^3\beta_1$ and CD9, CD63, and CD81 proteins. In conclusion we suggest that the prevalence of integrin/TM4 complexes in diverse cellular environments is indicative of their general physiological importance.

INTRODUCTION

Integrins are membrane heterodimers (composed of α and β subunits), that directly mediate cell attachment to the extracellular matrix (ECM) and trigger distinct biochemical signals, including activation of focal adhesion kinase (FAK) and Rho protein, elevation of intracellular pH, and Ca^{+2} oscillations (Hynes, 1992; McNamee *et al.*, 1993). It is not yet clear how integrins, which do not exhibit apparent signaling properties by themselves, can initiate a diverse range of intracellular biochemical events. A prevailing hypothesis is that integrins can associate with signaling protein(s) to form an active complex following integrin-ligand interaction (Hynes, 1992).

A few integrin-associated proteins have been identified that could potentiate adhesion-dependent sig-

naling cascades. A membrane protein called IAP-50 was copurified with β_3 integrins from placenta and platelets (Brown *et al.*, 1990; Lindberg *et al.*, 1993). Anti-IAP-50 monoclonal antibody (mAb) blocked Ca^{+2} influx in endothelial cells plated on fibronectin or vitronectin, suggesting that IAP-50, a protein with multiple membrane-spanning domains, could possibly function as an integrin-associated ion channel (Schwartz *et al.*, 1993). Insulin receptor substrate-1 (IRS-1) associates with $\alpha^v\beta_3$ integrin from insulin-stimulated cells (Vuori and Ruoslahti, 1994), thus linking $\alpha^v\beta_3$, via IRS-1, to two other signaling proteins, Grb2 and PI-3 kinase. Thus, specific association between the $\alpha^v\beta_3$ integrin and IRS-1 may synergistically link growth factor- and integrin-mediated signaling pathways leading to cell proliferation. Several cytoplasmic proteins, including paxillin and FAK, bind to a synthetic peptide that mimics the cytoplasmic domain of the integrin β_1 subunit (Schaller *et al.*, 1995). These data are of particular interest because both pax-

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illin and FAK undergo rapid tyrosine phosphorylation upon cell adhesion (Burrige *et al.*, 1992; Kornberg *et al.*, 1992). Whether β_1 integrins in vivo are constitutively associated with these proteins or the association is induced by ligand binding requires further examination.

We and others have recently demonstrated that β_1 and β_3 integrins can associate with the membrane proteins CD9 and CD63 (Slupsky *et al.*, 1989; Rubinstein *et al.*, 1994; Berditchevski *et al.*, 1995; Nakamura *et al.*, 1995). Both CD9 and CD63 belong to the TM4 or tetraspan family of cell surface proteins with four putative transmembrane domains (Horejsi and Vilek, 1991; Wright and Tomlinson, 1994). Proteins in the TM4 family have been described as tumor-specific antigens (Hotta *et al.*, 1988; Szala *et al.*, 1990; Marken *et al.*, 1992; Takagi *et al.*, 1995), anti-proliferative antigen (Oren *et al.*, 1990), T cell activation antigens (Imai *et al.*, 1992; Nojima *et al.*, 1993), or lymphoid specific markers (Schwartz-Albiez *et al.*, 1988; Angelisova *et al.*, 1990). Although no direct evidence is yet available, numerous correlative data suggest a possible functional connection between integrins and TM4 proteins. For example, CD9, CD63, and CD81 were implicated in cell-cell adhesion, and also, CD9 and CD82 may facilitate cell migration and invasion into ECM (Toothill *et al.*, 1990; Bradbury *et al.*, 1993; Ikeyama *et al.*, 1993; Masellis-Smith and Shaw, 1994; Dong *et al.*, 1995). Each of these functions are also integrin-dependent biological phenomena. In addition, the involvement of TM4 proteins in transmembrane signaling was suggested in several studies (Bradbury *et al.*, 1993; Matsumoto *et al.*, 1993; Olweus *et al.*, 1993).

In the present study we provide evidence that CD63, CD9, and CD81/TAPA-1 combine with $\alpha^3\beta_1$ and $\alpha^6\beta_1$ integrins and with each other to form TM4/integrin and TM4/TM4 complexes on the cell surface. We have also found co-distribution of CD9, CD63, and CD81 with integrins in a variety of human tissues and cultured cell lines.

MATERIALS AND METHODS

Cell Lines

B-, T- and promyelocytic cell lines (Ramos, OCI-LY8, Molt-4, Jurkat, K562, and K562-A3) were maintained in RPMI-1640 media with 10% fetal calf serum (FCS). The melanoma, sarcoma, and carcinoma cells (OCM-1, LOX, RD, HT1080, A431, A549, MDA-MB-231, Hela, and HepG2) were grown in DMEM media supplemented with 10% FCS. Transformed primary lung fibroblasts, VA-2, and mammary epithelial cells, MTSV 1-7, were maintained as previously described (Bartek *et al.*, 1991; Berditchevski *et al.*, 1995). Primary foreskin fibroblasts and smooth muscle cells were grown in DMEM media supplemented with 10% FCS. Human umbilical vein endothelial cells were generously provided by Dr. Peter Libby (Brigham and Women's Hospital, Boston, MA). HT1080-C9 and K562-A3CD9 cell lines were established by transfecting HT1080 and K562-A3 cells with full length CD9 cDNA (generated by reverse transcriptase-polymerase chain reaction) that was cloned into the expression plasmid pZeoSV (Invitrogen, San Diego, CA).

Monoclonal Antibodies

Anti-integrin mAbs used were as follows: anti- α^2 , A2-2E10 (Bergelson *et al.*, 1994); anti- α^3 , A3-X8 (Weitzman *et al.*, 1993), A3-IVA5 (Weitzman *et al.*, 1993), and A3-IIF5 (Weitzman *et al.*, 1993); anti- α^5 , A5-PUJ2 (Lee *et al.*, 1995); anti- α^6 , A6-ELE (Lee *et al.*, 1995); anti- β_1 , A1A5 (Hemler *et al.*, 1983). Anti-TM4 mAbs used were as follows: anti-CD63, 6H1 (Berditchevski *et al.*, 1995) and RUU.SP. 2.28 (Toothill *et al.*, 1990); anti-CD81, M38 (Fukudome *et al.*, 1992), and 5A6 (Oren *et al.*, 1990). New mouse mAbs C9-BB (anti-CD9) and NAG-3 (anti-CD63) were generated after the immunization of mice with purified TM-4 complexes as will be described in detail elsewhere. The anti-CD9 mAb, DU-ALL-1, was obtained from Sigma Co. (St. Louis, MO) and anti-CD9 mAb, Alb.6, and anti-CD81 mAb, JS64, were purchased from Immunotech Inc. (Westbrook, ME). New mouse mAb 7C6, anti-integrin α^2 , and 8E11, a new antibody to CD109 (a previously described 170/150-kDa cell surface protein (Sutherland *et al.*, 1991)) were generated after immunization of mice with A549 cells (Pasqualini *et al.*, 1993). Also, we used the anti-MHC class I mAb, W6/32 (Barnstable *et al.*, 1978).

Immunoprecipitation

Cells were surface labeled with NHS-LC-biotin (Pierce, Rockford, IL) or Na¹²⁵I according to established protocols and lysed in immunoprecipitation buffer (1% Brij 96, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES], pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 2 mM phenylmethylsulfonyl fluoride, 20 μ g/ml aprotinin, and 10 μ g/ml leupeptin) for 1 h at 4°C. Immune complexes were collected on protein A beads that were already pre-bound with mAb, followed by four washes with the immunoprecipitation buffer. For "stringent" conditions, the immunoprecipitation buffer was supplemented with 0.2% SDS. Immune complexes were eluted from protein A beads either with 0.1 M glycine, pH 2.7, or with Laemmli elution buffer and proteins were separated on SDS-PAGE. Dried gels were exposed with O-XAR film (Kodak, Rochester, NY) for 1-20 days at -70°C. When labeled with biotin, separated proteins were transferred to nitrocellulose membranes and visualized with HRPO-conjugated ExtrAvidin (Sigma) using Renaissance Chemiluminescent Reagents (NEN, Cambridge, MA).

Reprecipitation experiments were performed as described earlier (Berditchevski *et al.*, 1995) with minor modifications. Briefly, the TM4 or integrin complexes were immunoprecipitated from Brij 96 lysates prepared from surface-biotinylated MDA-MB-231 or HT1080-C9 cells. After five washes with the immunoprecipitation buffer, the protein complexes were dissociated for 30 min at 4°C with 0.5% SDS added to the immunoprecipitation buffer. The eluates were subsequently diluted 1:1 with the immunoprecipitation buffer and re-precipitated with the appropriate mAbs directly coupled to Sepharose beads. The samples were then processed for further analysis as above.

For cross-linking, MDA-MB-231 cells ($2-3 \times 10^7$) were treated with different concentrations of 3/3'-dithiobis(sulfo-succinimidyl propionate) (DTSSP) or dithiobis(succinimidyl propionate) (DSP) (Pierce), for 1 h at 4°C and subsequently quenched with 40 mM Tris, pH 8.0, for 15 min. After solubilization in immunoprecipitation buffer supplemented with 0.2% SDS for 2 h at 4°C, protein complexes were immunoprecipitated as above and analyzed on SDS-PAGE under reducing conditions.

For experiments involving reprecipitation after cross-linking, MDA-MB-231 cells ($1-2 \times 10^7$) were first treated with 0.5 mM DSP, then surface labeled with Na¹²⁵I, and subsequently lysed in immunoprecipitation buffer containing 0.2% SDS. Protein complexes were recovered with either mAb 6H1 (anti-CD63) or the mAb M38 (anti-CD81) directly conjugated to Sepharose beads. After three washes, protein complexes were eluted from the beads into lysis buffer at 94°C for 5 min and equal aliquots of the eluate were subjected to reprecipitation with anti-CD9 (C9-BB), anti-CD81 (M38), or anti- β_1 mAbs directly coupled to Sepharose beads. The mAbs used for reprecipitation were also tested in control experiments, in which cell

lysate prepared after cross-linking was pre-heated at 94°C for 5 min and used directly for immunoprecipitation.

Immunofluorescence

The HT1080-C9 cells were grown on glass coverslips for 24–48 h or plated on coverslips pre-coated overnight with ECM proteins (collagen I, fibronectin, and laminin-1) and cell footprints were prepared and stained with mAbs as described earlier (Berdichevski *et al.*, 1995). For double-immunofluorescent staining of the HT1080-C9 cell footprints, NHS-fluorescein (Pierce) was directly conjugated to anti-CD9 (C9-BB), anti-CD81 (M38), and anti-CD109 (8E11) mAbs, and NHS-rhodamine (Pierce) was conjugated to anti- α^3 (A3-IVA5) mAb using the manufacturer's protocol. For surface staining, cells were resuspended in a buffer containing 150 mM NaCl, 2 mM MgCl₂, 0.5 mM CaCl₂, 5 mM KCl, 5 mM glucose, 20 mM HEPES, pH 7.4, and then plated for 1–2 h on glass coverslips pre-coated with the proteins (collagen I, fibronectin, and laminin-1) and fixed with 2% paraformaldehyde for 7 min. Coverslips coated with ECM protein containing laminin-5 were prepared essentially as described previously (Weitzman *et al.*, 1993). Briefly, human squamous A431 carcinoma cells were grown on glass coverslips for 2–4 days and sequentially treated with 1% Triton X-100, 1 M NaCl with 2 M urea, and then 8 M Urea. The coverslips were typically used within the next 24–48 h. After incubation with a blocking solution (phosphate-buffered saline [PBS] containing 20% heat-inactivated goat serum) cells were sequentially stained with primary mouse mAbs followed by fluorescein-conjugated goat anti-mouse polyclonal antibody. Coverslips were mounted on slides in FluorSave (Calbiochem, La Jolla, CA) and analyzed under a Zeiss Axioscop microscope equipped with optics for epifluorescence (Carl Zeiss, Thornwood, NY).

Adhesion Assay

A standard static cell-ECM adhesion assay (25–30 min) was performed as previously described (Lee *et al.*, 1995). In experiments studying the effect of mAbs on adhesion, BCECF-AM-labeled cells were preincubated with mAbs at 4°C for 30 min and then aliquoted into 96-well plates pre-coated with ECM substrates.

Immunohistochemistry

Fresh tissue was obtained from material submitted to the Surgical Pathology Division of the Department of Pathology, Washington University School of Medicine, St. Louis, MO. The tissue was embedded in OCT compound (Miles Laboratory, Elkart, IN), snap frozen in liquid nitrogen-cooled isopentane, and stored at –70°C. Frozen sections (6 μ m thick) were fixed briefly in acetone and held at –20°C before staining by the immunoperoxidase technique, using mouse mAbs against α^3 integrin subunit (A3-X8), CD9 (C9-BB), CD63 (NAG3), and CD81 (M38). Detection was achieved with the biotinylated anti-mouse IgG and avidin-biotin-peroxidase complex (Vector, Burlingame, CA), as previously described (Zutter, 1991). Sections were counterstained with methyl green.

RESULTS

$\alpha^3\beta_1$ Integrin and CD63 Associate with Similar Patterns of Additional Proteins

In the course of studying the association of CD63 protein with $\alpha^3\beta_1$ integrin (Berdichevski *et al.*, 1995), we found that other surface proteins could be consistently co-precipitated with both anti- α^3 and anti-CD63 mAbs. The results of three such experiments are shown in Figure 1. Human cell lines, K562-A3, HT1080, and MDA-MB-231, were surface labeled with

¹²⁵I, proteins were solubilized with mild detergent (1% Brij 96), and the $\alpha^3\beta_1$ -CD63 complex was immunoprecipitated using either anti- α^3 or anti-CD63 mAbs. Two characteristic integrin α and β bands of 150 and 130 kDa were readily detectable in all three anti-CD63 immunoprecipitates (Figure 1, lanes b, e, and h). On the other hand, the CD63 protein could not be easily identified in the anti- α^3 immunoprecipitates due to a low efficiency of surface labeling and a diffuse appearance on PAGE as a broad 45- to 55-kDa band (Figure 1, lanes a, d, and f). In addition to the $\alpha^3\beta_1$ integrin, both anti- α^3 and anti-CD63 mAbs co-immunoprecipitated several other proteins of 95, 75, 65, 37, and 22 kDa from the K562-A3 cell lysate (Figure 1, lanes a and b; small arrowheads). Similarly, anti- α^3 and anti-CD63 mAbs detected additional 95-, 80-, and 22-kDa proteins from the HT1080 cell lysate (Figure 1, lanes d and e), and 190-, 80-, 70-, 66-, 24-, 23-, and 22-kDa proteins from the MDA-MB231 cell lysate (Figure 1, lanes g and h). None of the co-precipitated proteins were seen in control P3 lanes (Figure 1, lanes c, f, and i). When cells were surface labeled with biotin, anti- α^3 and anti-CD63 mAbs again co-precipitated several apparently similar proteins (our unpublished results). These results suggest that the $\alpha^3\beta_1$ integrin and CD63 are either the components of a larger multiprotein complex or associated with the same subset of membrane proteins in separate complexes.

Integrins $\alpha^3\beta_1$ and $\alpha^6\beta_1$ but not $\alpha^6\beta_4$ Associate with CD9 and CD81

It has been shown that multiple TM4 proteins can combine in one multiprotein complex (Imai and Yoshie, 1993; Angelisova *et al.*, 1994). Here we observed small 22- to 25-kDa proteins co-precipitated with anti-CD63 or anti- α^3 mAbs, that resembled the sizes of CD9 and CD81. This led us to examine cells (MDA-MB-231) expressing high levels of CD9 and CD81 proteins, to determine whether these latter proteins could also associate with β_1 integrins and CD63. Under nonstringent conditions, anti-CD9 and anti-CD81 mAbs (Figure 2, lanes d and e) co-precipitated several proteins similar to those detected in anti- α^3 and anti-CD63 immunoprecipitates (Figure 2, lanes b and f). None of these putative CD9-, CD63-, CD81-, or $\alpha^3\beta_1$ -associated proteins were detected in anti- α^2 or P3 control immunoprecipitates (Figure 2, lanes a and g). With 0.2% SDS in the immunoprecipitation buffer, associated proteins largely disappeared from integrin- and TM4-immunoprecipitates (Figure 2, lanes i–m). This result emphasizes the noncovalent nature of associated protein interactions. Because the 150-kDa and 130-kDa proteins co-precipitated with CD63 were identified previously as the α^3 and β_1 integrin subunits (Berdichevski *et al.*, 1995), we assumed that both CD9 and CD81 could

Figure 1. Co-immunoprecipitation of additional surface proteins with CD63 protein and $\alpha^3\beta_1$ integrin. K562-A3 (lanes a–c), HT1080 (lanes d–f), and MDA-MB-231 cells (lanes g–i) were surface labeled with Na^{125}I , lysed in 1% Brij 96 buffer, and proteins from the lysate were immunoprecipitated with anti- α^3 , mAb A3-X8 (lanes a, d, and g); with anti-CD63, mAb 6H1 (lanes b, e, and h); or with negative control, mAb P3 (lanes c, f, and i). Proteins were resolved on 11% PAGE under nonreducing conditions. Small arrows indicate positions of protein bands detected in both α^3 and CD63 immunoprecipitates, and large arrows show α^3 and β_1 integrin bands.

associate with the components of the $\alpha^3\beta_1$ /CD63 complex.

Adding to the specificity for CD9- and CD81-interactions, anti- α^6 mAb failed to co-precipitate the TM4 proteins and, conversely, the characteristic 210-kDa protein band corresponding to the β_4 integrin subunit was absent from the anti-TM4 immunoprecipitates (Figure 2, lanes c–f). Thus, under these conditions the $\alpha^6\beta_4$ integrin did not associate with CD9, CD63, and CD81 proteins. To strengthen this conclusion, we performed experiments in which the TM4 complexes purified from MDA-MB-231 cells were then dissociated with SDS and subjected to re-immunoprecipitation with either anti- α^3 or anti- α^6 mAbs. As shown (Figure 3D) the $\alpha^3\beta_1$ integrin but not the $\alpha^6\beta_4$ integrin was reprecipitated from the TM4 immunoprecipitates (Figure 3D, compare lanes a, c, and e with b, d, and f).

Because expression of $\alpha^6\beta_1$ integrin on MDA-MB-231 cells was essentially undetectable, it was not feasible to test its association with CD9 and CD81. Thus to address this question we carried out a reprecipitation experiment using HT1080-C9 cells, which lack the $\alpha^6\beta_4$ integrin, but express high levels of CD9, CD81, and several β_1 integrins, including $\alpha^2\beta_1$, $\alpha^3\beta_1$, $\alpha^5\beta_1$, and $\alpha^6\beta_1$ on their surface (Figure 3A). Both $\alpha^3\beta_1$ and $\alpha^6\beta_1$ integrins, but not $\alpha^2\beta_1$ or $\alpha^5\beta_1$ integrins could be recovered from the CD9 and CD81 immunoprecipitates (Figure 3B). In reciprocal experiments, CD9 and CD81 could be reprecipitated

only from $\alpha^3\beta_1$ and $\alpha^6\beta_1$, but not $\alpha^2\beta_1$ or $\alpha^5\beta_1$ immunoprecipitates (Figure 3C). Together these reprecipitation results not only demonstrated that CD9 and CD81 could associate with the $\alpha^3\beta_1$ and $\alpha^6\beta_1$ integrins but also suggested that the integrin-TM4 interactions could be affected by both α and β integrin subunits.

Integrin/TM4 Complexes Are Present on the Cell Surface

To demonstrate that association of TM4 proteins with integrins occurs on the cell surface, we performed cross-linking experiments. Intact MDA-MB-231 cells were first pre-treated with a cleavable cross-linker, DSP, then surface-labeled with ^{125}I , and subsequent immunoprecipitation was carried out under stringent conditions to disrupt the noncovalent association between the integrins and the TM4 proteins (e.g. as in Figure 2). After immunoprecipitation was completed, protein complexes were dissociated under reduced conditions to break covalent cross-linker bonds. As a result of cross-linking, additional proteins, including 135-kDa integrin subunits, were detected in the CD9, CD63, and CD81 immunoprecipitates even in high stringency conditions (Figure 4A, lanes c–e). Notably, a protein band of 22–23 kDa, similar in size to CD9 and/or CD81, could be co-precipitated with anti- α^3 (Figure 4, lanes a and g) but not with anti- α^5 (Figure 4,

Figure 2. Co-immunoprecipitation of additional surface proteins with CD9, CD63, CD81, and $\alpha^3\beta_1$ integrin. MDA-MB-231 cells were surface labeled with Na^{125}I , lysed either in buffer containing 1% Brij 96 or 1% Brij 96 with 0.2% SDS, and then proteins were precipitated with mAbs against indicated integrin subunits (lanes a–c and h–j) or TM4 proteins (lanes d–f and k–m). Precipitated proteins were resolved on 11% PAGE under nonreducing conditions. The mAbs were as follows: A2-2E10, anti- α_2 ; A3-X8, anti- α_3 ; A6-ELE, anti- α_6 ; C9-BB, anti-CD9; 6H1, anti-CD63; M38, anti-CD81; and P3, negative control. Thick arrows indicate positions of integrin and TM4 protein bands. Thin arrows indicate positions of unknown protein bands detected in both TM4 and α^3 immunoprecipitates.

lane b) or negative control mAbs (Figure 4, lane f). When used at a concentration of 100 μM , DSP did not efficiently cross-link CD63 to 22- to 23-kDa proteins (Figure 4A, lane d). However, the CD63-immunoprecipitate did contain these proteins when cells were pre-treated with 1 mM DSP (Figure 4A, lane i). From earlier immunoprecipitation and reprecipitation experiments we concluded that the 135-kDa protein band corresponds to the $\alpha^3\beta_1$ integrin while the 22- to 23-kDa protein may be a combination of CD9 and CD81.

To verify whether extracellular regions of $\alpha^3\beta_1$ and TM4 proteins might interact directly, we carried out an experiment using DTSSP, a membrane impermeable cross-linker. Upon treatment of intact MDA-MB-231 cells with 1 mM DTSSP, all three anti-TM4 mAbs co-precipitated a 135-kDa protein resembling $\alpha^3\beta_1$ integrin (Figure 4B, lanes b–d) and the anti- α^3 mAb co-precipitated 22- to 23-kDa protein(s) resembling CD9 and CD81 (Figure 4B, lane a). These cross-linking experiments demonstrate that protein complex(es) combining CD9, CD63, and CD81 proteins with β_1 integrins may occur on the cell surface, with at least one of the putative TM4-integrin interaction sites possibly located within extracellular domains.

Evidence for TM4/TM4/Integrin Complexes on the Cell Surface

To determine whether $\alpha^3\beta_1$ integrin is combined with multiple TM4 proteins in one complex, we performed re-immunoprecipitation experiments after cross-linking. MDA-MB-231 cells were first treated with 1 mM DSP, surface labeled with ^{125}I , and then either CD63 or CD81 complex(es) were immunopurified from the cell lysate under stringent conditions. Protein complex(es) were then eluted from the antibody beads in lysis buffer at 94°C and re-precipitated with anti- β_1 , anti-CD9, or anti-CD81 mAbs. As shown in Figure 5, the $\alpha^3\beta_1$ integrin (135 kDa) and TM4 proteins (22–23 kDa) that were each cross-linked to CD63, were also associated with each other. For example, from the pool of CD63-associated proteins, anti-CD9 (Figure 5, lane b) and anti-CD81 (Figure 5c), both could co-precipitate $\alpha^3\beta_1$, and anti- α^3 mAb co-precipitated proteins resembling CD9/CD81 (Figure 5a). These results strongly suggest that CD63/CD9/ $\alpha^3\beta_1$ and CD63/CD81/ $\alpha^3\beta_1$ protein complexes can exist on the cell surface. Interestingly, the intensity of the 22- to 23-kDa protein band detected in the CD9 reprecipitate exceeded that observed in the β_1 or CD81 immunoprecipitates (Figure 5, compare lanes b, a, and c), suggesting that cell

Figure 3. TM4 proteins associated with $\alpha^3\beta_1$ and $\alpha^6\beta_1$ integrins but not with $\alpha^2\beta_1$, $\alpha^5\beta_1$, or $\alpha^6\beta_4$ integrins. HT1080-C9 (A–C) or MDA-MB-231 (D) cells were surface labeled with biotin and lysed in 1% Brij 96 buffer and cell lysates were then immunoprecipitated (Step 1) with mAbs against indicated integrins (C) or TM4 proteins (B and D). The immunoprecipitated protein complexes were dissociated with 0.2% SDS at 4°C and eluates were subjected to reprecipitation (Step 2) with anti-integrin (B and D) or anti-TM4 (C) mAbs directly coupled to Sepharose beads. In control experiments integrins were directly immunoprecipitated from the HT1080-C9 (A) and MDA-MB-231 (D, lanes g and h) cell lysates under stringent conditions (1% Brij 96 with 0.2% SDS). Precipitated proteins were resolved on 11% PAGE under reduced conditions. The mAbs were as follows: A2-2E10, anti- α^2 ; A3-X8, anti- α^3 ; PUJ2-A5, anti- α^5 ; A6-ELE, anti- α^6 ; C9-BB, anti-CD9; 6H1, anti-CD63; and M38, anti-CD81.

surface CD63 and CD9 proteins may be cross-linked more easily. Alternatively, it is possible that these proteins also associate with one another in a separate complex.

Similarly, both $\alpha^3\beta_1$ integrin and CD9 could be reprecipitated from immunopurified CD81-complexes (Figure 5, lanes e and f). The amount of $\alpha^3\beta_1$ integrin reprecipitated with anti-CD9 mAb was lower than that obtained with anti- β_1 mAb (Figure 5B, compare lanes e and f). Also, the intensity of CD9/CD81 protein bands in the β_1 reprecipitation lane was significantly lower than in the CD9 reprecipitation lane. These data suggest that a CD9/CD81/ $\alpha^3\beta_1$ complex may be less abundant on the cell surface than either CD81/ $\alpha^3\beta_1$ or CD81/CD9 complexes. Alternatively, bimolecular cross-linking may simply be more preva-

lent, and is not necessarily reflective of the overall architecture of the complexes.

The $\alpha^3\beta_1$ and TM4 Proteins Are Co-localized in Cells

We used indirect immunofluorescence staining to examine the distribution of $\alpha^3\beta_1$ integrin and TM4 proteins in HT1080-C9 cells spread on various ECM substrates. In nonpermeabilized HT1080-C9 cells spread on collagen I, strong staining along the cell perimeter was detected for all TM4 proteins (Figure 6, c–e) and for the $\alpha^3\beta_1$ integrin (Figure 6a) but not for CD109 (Figure 6b). In addition, we observed small clusters of the $\alpha^3\beta_1$, CD9, and CD81 evenly distributed on the apical surface of cells (Figure 6, a, c, and e). Similarly, co-distribution of the $\alpha^3\beta_1$ integrin and TM4 proteins

Figure 4. Detection of $\alpha^3\beta_1$ /TM4 complexes on the cell surface. Intact MDA-MB-231 cells were first treated with membrane-permeable DSP (A), or membrane-impermeable DTSSP (B) cross-linkers, then surface labeled with Na^{125}I and lysed in buffer containing 1% Brij 96 with 0.2% SDS. Protein complexes were immunoprecipitated from cell lysates with antibodies to α^3 (A, lanes a and g; B, lane a), α^5 (A, lane b), and the indicated TM4 proteins (A, lanes c–e and h–j; B, lanes b–d). Samples were boiled in Laemmli-loading buffer containing 5% β -mercaptoethanol to reduce the cross-linker bonds and analyzed on 11% PAGE. The mAbs were as follows: A3-X8, anti- α^3 ; PUJ2-A5, anti- α^5 ; C9-BB, anti-CD9; 6H1, anti-CD63; and M38, anti-CD81.

on the cell periphery was also seen when cells were plated on fibronectin, laminin-1, or crude ECM containing laminin-5 (our unpublished results). However, when cells were permeabilized with 0.1% Triton X-100 before incubation with primary mAbs, the peripheral staining observed for $\alpha^3\beta_1$ and TM4 proteins was no longer detectable, probably because the TM4 proteins are easily extractable (our unpublished results). In addition, when HT1080-C9 cells were plated on laminin-1 or on crude matrix containing laminin-5 and permeabilized in a similar fashion, we were unable to detect the TM4 proteins in focal contacts (our unpublished results).

Finally, we utilized two-color direct immunofluorescent staining to analyze localization of $\alpha^3\beta_1$ and TM-4 proteins within cell footprints. As illustrated (Figure 7), anti- α^3 , anti-CD9, and anti-CD81 mAbs co-stained cell footprints left by HT1080-C9 cells on uncoated coverslips (Figure 7, a, c, and e). These results are consistent with previous findings that the $\alpha^3\beta_1$ /CD63 complex could be detected in cell footprints (Berdichevski *et al.*, 1995). In contrast to $\alpha^3\beta_1$ and TM4 proteins, CD109 antigen and $\alpha^2\beta_1$ integrin did not localize to cell footprints even though both

were present at high levels on HT1080-C9 cells (Figure 7b and our unpublished results).

Cell and Tissue Distribution of the Integrin/TM4 Complexes

Expression of integrins and the TM4 proteins on a variety of cultured cells was analyzed by flow cytometry. Table 1 shows that for every cell type expressing either $\alpha^3\beta_1$ and/or $\alpha^6\beta_1$, two or more of the listed TM4 proteins were also present. Among the TM4 proteins, CD63 was present on all cells tested, CD81 was present on all cells except for the HepG2 cell line, and CD9 was present on all except HT1080 cells and three of the five nonadherent cell lines. In comparison, $\alpha^3\beta_1$ and $\alpha^6\beta_1$ were generally lacking from the nonadherent cell lines. However $\alpha^3\beta_1$ was present on all of the adherent cells (except for HepG2), and $\alpha^6\beta_1$ was also present on all adherent cell lines, except that in some cells (MDA-MB-231, MTSV 1–7, HepG2, and A431) α^6 was predominantly associated with the β_4 integrin subunit, with $\alpha^6\beta_1$ expressed only at a lower level (Table 1).

Figure 5. Evidence for $\alpha^3\beta_1$ /TM4/TM4 complexes on the cell surface. Intact MDA-MB-231 cells were treated with DSP (Step 1), then surface labeled with Na^{125}I , and lysed in buffer containing 1% Brij 96 and 0.2% SDS. Sepharose beads coupled with antibodies to CD63 (lanes a–d) or CD81 (e–g) were used to collect cross-linked protein complexes from cell lysates (Step 2). After subsequent washes, CD63 and CD81 complexes were eluted from the beads at 94°C into lysis buffer. The eluates were then subjected to re-immunoprecipitation (Step 3) with mAb to β_1 (lanes a and e), CD9 (lanes b and f), CD81 (lane c), or control CD109 (lanes d and g). Reprecipitated complexes were boiled in Laemmli-loading buffer containing 5% β -mercaptoethanol to reduce the cross-linker bonds and analyzed on 11% PAGE. The mAbs were as follows: A3-X8, anti- α^3 ; C9-BB, anti-CD9; 6H1, anti-CD63; M38, anti-CD81; and 8E11, anti-CD109.

Also, tissue staining was carried out to compare further the localization of $\alpha^3\beta_1$ integrin with CD9, CD63, and CD81. We stained sections of skin, breast, salivary gland, tonsils, and colon. As previously described (Carter *et al.*, 1990), high levels of the α^3 integrin subunit were expressed by the basal layers of the epidermis in the skin, with the most intense staining in the deepest basal cell layer (Figure 8A). Staining with antibodies to CD9 (Figure 8B), CD63 (Figure 8C), and CD81 (Figure 8D) showed a comparable high level of staining in the basal cell layer. However, the expression of CD9, CD63, and CD81 differed from $\alpha^3\beta_1$ in that they were not restricted to the basal cell layer. In

addition to the stratified squamous epithelium of the epidermis, the stratified cuboidal epithelium of the sweat glands and the endothelial cells of the small vessels within the dermis expressed all four proteins at intermediate to high levels. In contrast, lymphocytes within the dermis and fibroblasts with the dermis and subcutaneous adipose tissue expressed only TM4 proteins but not $\alpha^3\beta_1$ integrin.

In normal breast tissue (Figure 9), the $\alpha^3\beta_1$ integrin was expressed at high levels in the ducts and ductules and at intermediate levels on endothelial cells (Figure 9A). Antibodies to CD9 (Figure 9B), CD63 (Figure 9C), and CD81 (Figure 9D) showed remarkably similar staining of breast ducts and ductules. However, as seen in the skin, these antigens were additionally found on fibroblasts where $\alpha^3\beta_1$ was absent. The expression pattern of CD9, CD63, CD81, and $\alpha^3\beta_1$ integrin in the salivary gland resembled that in the breast (our unpublished results).

In tonsils the $\alpha^3\beta_1$ integrin was expressed most intensely by the basal cell layer of the nonkeratinizing stratified squamous epithelium in a manner similar to that described for the keratinizing stratified squamous epithelium of the skin (our unpublished results). The more superficial and differentiated layers were either weakly stained with anti- α^3 mAb or were completely negative. On the other hand, the CD9, CD63, and CD81 proteins were expressed not only by basal cell layers but also by higher and more superficial layers of the epithelium. The small and large vessels all stained with all three anti-TM4, as well as anti- α^3 mAbs. On the other hand, all the lymphocytes in the reactive follicles and the interfollicular zones failed to express α^3 at detectable levels, but did express relatively high levels of CD9, CD63, and CD81 (our unpublished results).

Sections of colonic wall containing colonic mucosa, smooth muscle bundles, and the ganglion cells of the myenteric plexus were also analyzed. The tall columnar epithelium of the colonic mucosa, the smooth muscle bundles making up the muscularis mucosa, muscularis externa, the ganglion cells of Auerbach's plexus within the colonic wall, and the endothelial cells of small and large vessels were all strongly positive for CD9, CD63, CD81, and $\alpha^3\beta_1$ integrin (our unpublished results). The mesenchymal cells, including fibroblasts and lymphocytes, were again positive for the TM4 proteins and negative for $\alpha^3\beta_1$ (our unpublished results).

DISCUSSION

In the present report we show that $\alpha^3\beta_1$ and $\alpha^6\beta_1$ integrins, but not $\alpha^2\beta_1$, $\alpha^5\beta_1$, or $\alpha^6\beta_4$ integrins can associate with multiple members of the TM4 protein family (CD9, CD63, and CD81). Importantly, integrin-TM4 interactions may occur in the context of larger

Figure 6. Codistribution of $\alpha^3\beta_1$ integrin and TM4 proteins in cells spread on collagen. HT1080-C9 cells were plated on collagen I-coated glass coverslips for 1 h, fixed with 2% paraformaldehyde, and stained with anti- α^3 mAb, A3-IVA5 (a), anti-CD109, 8E11 (b), anti-CD9, C9-BB (c), anti-CD63, 6H1 (d), and anti-CD81, M38 (e) mAbs. Fluorescein-conjugated goat anti-mouse Ig was used to visualize the staining.

multiprotein complexes, as evident from cross-linking of the $\alpha^3\beta_1$ integrin with multiple TM4 proteins. We have also demonstrated that for every cell line or tissue cell type expressing the $\alpha^3\beta_1$ integrin, multiple TM4 proteins were also present, thus providing ample opportunity for $\alpha^3\beta_1$ /TM4 complexes to be formed *in vivo*.

Our data regarding interactions of CD81 with integrins are novel. To our knowledge this is the first report describing the association of CD81 with any other molecules in adherent cells. CD81, like CD9 and CD63, can specifically interact with $\alpha^3\beta_1$ and $\alpha^6\beta_1$ integrins, as demonstrated in reciprocal re-immunoprecipitation and cross-linking experiments. In addition, the results showing the interaction of CD9 with the $\alpha^6\beta_1$ integrin complement earlier reports describing the associations of CD9 with $\alpha^3\beta_1$ (Nakamura *et al.*,

1995), $\alpha^4\beta_1$ (Rubinstein *et al.*, 1994), and $\alpha^{IIb}\beta_3$ (Slusky *et al.*, 1989). Although not observed in the present study, an association between CD9 and $\alpha^5\beta_1$ integrin was previously found in B-cells (Rubinstein *et al.*, 1994). Thus, interaction of CD9 with $\alpha^5\beta_1$ integrin may require a specialized cellular environment, which perhaps was not present in cells used in our study. Alternatively, it is possible that detergent conditions used elsewhere (Rubinstein *et al.*, 1994) were better able to maintain CD9/ $\alpha^5\beta_1$ association. However, in cross-linking experiments, even before any detergents were added, we still saw no evidence for the presence of $\alpha^5\beta_1$ /TM4 complexes on the cell surface.

The specificity for the integrin-TM4 association was further extended by the observation that $\alpha^6\beta_1$ integrin but not $\alpha^6\beta_4$ integrin could associate with the TM4 proteins, suggesting that β as well as α integrin sub-

Figure 7. Colocalization of $\alpha^3\beta_1$ integrin and TM4 proteins within cell footprints. HT1080-C9 cells grown on glass coverslips for 24 h, were preincubated in PBS for 25 min at room temperature and cells were detached from the coverslips during three successive washes with PBS. Cell footprints were simultaneously stained with rhodamine-conjugated anti- α^3 mAb, A3-IVA5 (a, c, and e) and with fluorescein-conjugated anti-CD9, C9-BB (d), anti-CD81, M38 (f), and anti-CD109, 8E11 (b) mAbs. The arrows show co-localization of $\alpha^3\beta_1$ integrin and TM4 proteins within footprints.

units are important for the interaction. It is presently unknown what region(s) of the integrin heterodimer is/are engaged in binding with the TM4 proteins. The deletion of the cytoplasmic domain or replacement of the transmembrane and the cytoplasmic domains of the α^3 integrin subunit with the appropriate regions from the α^2 or α^5 subunit did not diminish association of the integrin with CD9, CD63, or CD81 (Berditchevski *et al.*, 1995; our unpublished results). In agreement with this data, cross-linking by the membrane impermeable agent DTSSP showed that the extracellular portions of TM4 proteins and integrin might be proximal to each other and therefore could be involved in a direct interaction. Interestingly, in another study the site of CD81 interaction with CD19 was mapped to the extracellular domain of the latter (Matsumoto *et al.*, 1993). However, from our results, we cannot exclude that cytoplasmic and/or transmembrane domains of both α and β integrin subunits could modulate integrin-TM4 interactions. The importance of intracytoplasmic and extracellular interactions between the TM4 and "non-TM4" membrane proteins has been recently established (Imai *et al.*, 1995). Specifically, the

intracellular portion of CD4 was both necessary and sufficient to confer its association with CD81, whereas the CD82-CD4 interaction involved both cytoplasmic and extracellular domains of CD4 (Imai *et al.*, 1995). Furthermore, the site for the CD4-CD81 interaction was the basic, -RCRHRRR-, membrane-proximal region of the CD4 cytoplasmic domain. Notably, cytoplasmic domains of several integrin β subunits, including β_1 , β_3 , β_5 , and β_6 but not β_4 , have a stretch of four charged amino acids, -HDRR/K-, immediately adjacent to their transmembrane domains (Hemler *et al.*, 1995). Experiments with mutagenized integrins will be required to verify whether this region of the β_1 (and other integrin β subunits) could mediate the interaction with CD81.

Although prior studies described interactions of some integrins with single TM4 proteins (see above), this is the first report where one integrin ($\alpha^3\beta_1$) is found to associate with at least two different TM4 proteins in higher order complexes (CD9/CD63/ $\alpha^3\beta_1$, CD9/CD81/ $\alpha^3\beta_1$, and CD81/CD63/ $\alpha^3\beta_1$). It was recently demonstrated that four members of the TM4 family (CD37, CD53, CD81, and CD82) could be com-

Table 1. Expression of $\alpha^3\beta_1$, $\alpha^6\beta_1$, and TM4 proteins in cultured cells^a

Cells	$\alpha^3\beta_1$	$\alpha^6\beta_1$	CD9	CD63	CD81
B-cells					
Ramos	—	—	—	+	+
OCI-LY8	—	—	+	+	+
T-cells					
Molt-4	—	—	—	+	+
Jurkat	±	±	+	+	+
Promyelocytic cells					
k562	—	—	—	+	+
Melanomas					
OCM	+	+	+	+	+
LOX	+	+	+	+	+
Sarcomas					
RD	±	+	±	+	+
HT1080	+	+	—	+	+
HT1080-C9	+	+	+	+	+
Carcinomas					
A431	+	$\alpha^6\beta_4^b$	+	+	+
MDA-MB-231	+	$\alpha^6\beta_4$	+	+	+
Hela	+	+	+	+	+
HepG2	+	$\alpha^6\beta_4$	+	+	—
Transformed primary cells					
Lung fibroblasts (VA2)	+	+	+	+	+
mam. epithel. (MTSV 1-7)	+	$\alpha^6\beta_4$	+	+	+
Primary cells					
HUVEC	+	+	+	+	+
smooth muscle cells	+	±	+	+	+
foreskin fibroblasts	+	+	+	+	+

^a Cells were incubated with saturating concentrations of primary anti-integrin and anti-TM4 mAbs, followed by fluorescein-conjugated goat anti-mouse Ig. Staining was analyzed by flow cytometry using the FACScan program. Staining was scored as “—”, “±”, or “+” if fluorescence intensity was respectively < twofold, two to fivefold, or > fivefold higher than staining observed for the P3 control mAb. The mAbs were as follows: A3-X8, anti- α^3 ; A6-ELE, anti- α^6 ; C9-BB, anti-CD9; 6H1, anti-CD63; and M38, anti-CD81.

^b A431, MDA-MB-231, MTSV 1-7, and HepG2 cells predominantly express the $\alpha^6\beta_4$ integrin and only low levels of the $\alpha^6\beta_1$ integrin.

bined with the MHC class II, CD19 and CD21 proteins in a large oligomeric complex (Angelisova *et al.*, 1994). Also, CD81 and CD82 were suggested to jointly associate with CD4 or CD8 proteins (Imai and Yoshie, 1993). Thus, the ability to associate with each other and with other membrane proteins within larger complexes could be a general feature of all TM4 proteins. Notably, because CD63 could be cross-linked with CD9 and CD81 only when cells were treated with the membrane permeable cross-linker, our data suggest that at least one of the putative sites for TM4-TM4 interaction may be located within the transmembrane and/or cytoplasmic regions.

Due to the technical limitations of cross-linking experiments it is difficult to determine precisely the relative amounts of integrin/TM4/TM4 compared with integrin/TM4 and TM4/TM4 complexes on the cell

surface. Although our data showed that the latter complexes were more prevalent (Figure 5), this may simply be because two cross-linking events within the same complex are less likely to occur. Thus, further studies will be necessary to determine a composition and precise stoichiometry of integrin/TM4 complexes. In preliminary experiments, we detected TM4 proteins in high molecular weight fractions (> 440 kDa) from a continuous sucrose gradient. A protein stoichiometry of 1:1:1 for the CD9/CD63/ $\alpha^3\beta_1$ and CD81/CD63/ $\alpha^3\beta_1$ complexes would yield a molecular mass of 330–360 kDa, implying that the complexes either have unequal representation of different TM4 proteins or could contain additional protein(s). Unknown protein bands with similar electrophoretic mobility observed in $\alpha^3\beta_1$ and TM4 immunoprecipitates (Figures 1 and 2) are possible candidates for the “missing” components of the complexes.

Immunofluorescent staining showed that in cells spread on different ECM substrates, TM4 proteins redistributed together with the $\alpha^3\beta_1$ integrin toward the cell perimeter and could be also found together in cell footprints (Figures 6 and 7). Close proximity of the TM4/integrin complexes to the sites of contacts of cells with the ECM substrates suggests that the functions of TM4 proteins could be related to cell adhesion. However, in agreement with previous reports (Ikeyama *et al.*, 1993; Hadjiargyrou and Patterson, 1995), we found no evidence indicating that TM4 proteins could directly modulate integrin-mediated cell attachment (our unpublished results). On the other hand, recent data clearly demonstrated that CD9 and CD82 could play an important role in cell motility (Ikeyama *et al.*, 1993; Dong *et al.*, 1995). Furthermore, a co-localization of $\alpha^3\beta_1$ and TM4 proteins at the cell periphery suggests that the $\alpha^3\beta_1$ /TM4 complexes might be functionally associated with lamellipodia, flattened extensions of the cell likely to be important for cell migration. Whether in this case TM4 proteins directly affect integrin-ligand binding or regulate post-adhesion signaling events remains to be seen.

From our analysis of a variety of cell lines, it is clear that every cell expressing $\alpha^3\beta_1$ or $\alpha^6\beta_1$ also expressed two or more members of the TM4 family. Similarly, tissue staining results again showed that all cells expressing $\alpha^3\beta_1$ may also express multiple TM4 proteins. In this regard, we have now co-precipitated TM4 proteins with $\alpha^3\beta_1$ integrins from every one of five different cell lines tested, including promyelocytic cells, sarcomas, and carcinomas (see also Berditchevski *et al.*, 1995). Thus, it seems very possible that the $\alpha^3\beta_1$ and $\alpha^6\beta_1$ integrins, wherever expressed, may have a capability to associate with TM4 family members. Notably, TM4 proteins were also detected on cells that do not have either $\alpha^3\beta_1$ or $\alpha^6\beta_1$. This finding is perhaps consistent with the ability of TM4 proteins to associate

Figure 8. Localization of the $\alpha^3\beta_1$ integrin and TM4 proteins in cryostat sections of human skin. Frozen skin sections (6 μm) were reacted with (A) anti- α^3 , mAb A3-X8, (B) anti-CD9, C9-BB, (C) anti-CD63, NAG-3, and (D) anti-CD81, M38 mAbs. The detection was as described in MATERIALS AND METHODS.

with several other types of transmembrane proteins including other integrins.

The biochemical functions of TM4 proteins are presently unknown. One earlier report proposed the direct association of CD9 with G-proteins (Seehafer and Shaw, 1991), thus suggesting that TM4 proteins could serve as membrane adapters linking integrins to signaling molecules. Also it was hypothesized that TM4

proteins could function as ion channels (Wright and Tomlinson, 1994), as suggested by their four putative membrane spanning domains, that are relatively abundant in hydrophilic amino acids and contain conservative charged amino acids. The fact that different TM4 proteins could associate with each other on the cell membrane (Imai and Yoshie, 1993; Angelisova *et al.*, 1994; present study) is perhaps consistent with this

Figure 9. Localization of the $\alpha^3\beta_1$ integrin and TM4 proteins in cryostat sections of human breast. Cryostat sections (6 μm) of normal resting mammary gland were reacted with (A) anti- α^3 , (B) anti-CD9, (C) anti-CD63, and (D) anti-CD81 mAbs as in Figure 8.

idea. In this regard, the best studied example of a ligand-gated ion channel, the acetylcholine receptor, is an oligomer composed of up to five homologous subunits each containing four hydrophobic α helical transmembrane regions (Unwin, 1993).

In conclusion, our results make it increasingly apparent that multiple members of the TM4 superfamily can specifically associate with multiple β_1 integrins. Given the overlapping functional attributes of these different types of proteins, it seems highly likely that

these associations have a general physiological importance.

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