Recent literature suggests that tetraspanin proteins (transmembrane 4 superfamily; TM4SF proteins) may associate with each other and with many other transmembrane proteins to form large complexes that sometimes may be found in lipid rafts. Here we show that prototype complexes of CD9 or CD81 (TM4SF proteins) with αβ1 (an integrin) and complexes of CD63 (a TM4SF protein) with phosphatidylinositol 4-kinase (PtdIns 4-K) may indeed localize within lipid raft-like microdomains, as seen by three different criteria. First, these complexes localize to low density light membrane fractions in sucrose gradients. Second, CD9 and α5 integrin colocized with ganglioside GM1 as seen by double staining of fixed cells. Third, CD9-α3β1 and CD81-α3β1 complexes were shifted to a higher density upon cholesterol depletion from intact cells or cell lysate. However, CD9-α3β1, CD81-α3β1, and CD63-PtdIns 4-K complex formation itself was not dependent on localization into raftlike lipid microdomains. These complexes did not require cholesterol for stabilization, were maintained within well solubilized dense fractions from sucrose gradients, were stable at 37 °C, and were small enough to be included within CL6B gel filtration columns. In summary, prototype TM4SF protein complexes (CD9-α3β1, CD81-α3β1, and CD63-PtdIns 4-K) can be solubilized as discrete units, independent of lipid microdomains, although they do associate with microdomains resembling lipid rafts.

Transmembrane 4 superfamily (TM4SF)1 proteins (also called tetraspanins) comprise a large group of ubiquitously expressed proteins that function in diverse contexts such as T- and B-cell activation, platelet aggregation, and cell fusion, motility, and proliferation (1–3). Although TM4SF molecules often associate with integrins, TM4SF proteins themselves have little influence on cell adhesion (4), and these proteins do not localize into focal adhesion complexes (5). The capability of TM4SF molecules to associate with each other and with a multitude of other molecules suggests that TM4SF proteins may serve as adaptors involved in the assembly of protein complexes in the membrane (2, 6). A particularly striking example of TM4SF protein complexes is the highly organized arrangement of uroplakins 1A and 1B with other proteins in the urothelial membrane (7).

TM4SF protein associations can be divided into three general categories. Level 1 consists of very robust associations, stable in Triton X-100 detergent, and likely to be direct. Perhaps the best example is the αβ1-CD151 complex (8, 9). Other possible direct associations include the αβ1-CD151, and αβ1-CD81 complexes (10). Level 2 associations are more numerous but nonetheless highly specific. These are disrupted by 1% Triton X-100 but are retained in 1% Brij 96 or Brij 97 detergent and other less hydrophobic detergents. These include complexes containing any of several different TM4SF proteins (such as CD9, CD63, CD81, CD151, or NAG-2), linked to each other, to a subset of integrins (αβ1, αβ1), or to other transmembrane proteins (11–19). In several experiments, antibodies to both TM4SF proteins and associated integrins could similarly inhibit cell motility or neurite outgrowth (8, 15, 16, 20). These results support the functional relevance of some level 1 and level 2 TM4SF complexes.

Level 3 associations are the most numerous and are least likely to be direct. These complexes are disrupted by Triton X-100, Brij 96, or Brij 97 but may be retained in 1% Brij 99, 1% CHAPS, or other less hydrophobic detergents. These include TM4SF associations with MHC class I and class II proteins and other Ig superfamily proteins (21–23), many additional integrins besides αβ1 and αβ1 (16, 24–26), proteoglycans (27), and various signaling molecules (28–32).

Because level 3 (and to a lesser extent level 2) complexes may contain large numbers of protein components and are resistant to mild detergents, it appeared possible that these complexes could be part of “raft” type plasma membrane microdomains (33–35). In this regard, association of integrin αβ2+CD47, a member of the immunoglobulin superfamily with multiple transmembrane domains, is cholesterol-dependent and occurs in a lipid raft environment (36). Likewise, β1 integrins (37), integrin αβ2 (38), and TM4SF protein CD9 (39) may localize in lipid raft domains. Furthermore, CD4 (40), CD36 (41), type II phosphatidylinositol 4-kinase (PtdIns 4-K) (42), various GPI-linked proteins (43, 44), and conventional protein kinase C isoforms (45) each have also been suggested to localize into raftlike domains. These same proteins including CD4 (46), CD36 (37), PtdIns 4-K (8, 30, 31), various GPI-linked proteins (47), and conventional protein kinase C (48) proteins associate with TM4SF proteins and/or integrins. Thus, we hypothesize that TM4SF-integrin plasma membrane complexes may, at least to some extent, resemble rafts.

Rafts differ from other parts of the plasma membrane by

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their high content of sphingolipids, cholesterol, and phospholipids with long, saturated fatty acid chains. This unique composition causes lipids in these microdomains to form a liquid-ordered phase rather than the conventional liquid-crystalline phase in the rest of the membrane (34). Thus, many raft-localized molecules are resistant to extraction with cold nonionic detergents. These insoluble membrane microdomains are also called detergent-resistant membranes or detergent-insoluble glycolipid-enriched membranes. The ratio of lipid to protein in these domains is higher than in surrounding parts of the membrane, enabling their segregation into the low density (light membrane) fractions of isopycnic sucrose gradients.

The tendency of TM4SF proteins to associate with each other and with so many other proteins has created enormous problems in evaluating the specific biochemical properties of these proteins. The possible location of these complexes in raft-type microdomains, coupled with the appearance of level 2 and level 3 complexes only under mild detergent conditions, suggests that possibly these complexes may exist only in the context of large and poorly solubilized membrane vesicles. In this regard, the integrin $\alpha_{4}\beta_{2}$-CD63 complex was excluded from a Sepharse CL4B column, suggesting a size equal to or greater than 20 million daltons (49).

Here we examine TM4SF complexes in terms of their densities, relative sizes, and possible relationship to lipid rafts. As prototype level 2 complexes, we have analyzed CD9-$\alpha_{4}\beta_{2}$ (12, 14) and in some experiments CD81-$\alpha_{4}\beta_{2}$ (12, 15, 20) associations. As a prototype level 3 complex, we analyzed CD63-PtdIns 4-K association (30, 31). In each case, we asked whether these complexes only exist in the context of large raftlike vesicles or whether they may exist in a truly soluble form of a reasonable size. These studies have been carried out using A431 and HT1080 cell lines because they express significant levels of $\alpha_{4}$ association (3, 2, 10) complexes only exist in the context of large raftlike vesicles or that possibly these complexes may exist only in the context of large raftlike vesicles or whether they may exist in a truly soluble form of a reasonable size. These studies have been carried out using A431 and HT1080 cell lines because they express significant levels of $\alpha_{4}$ association.

**Experimental Procedures**

**Cell Lines and Antibodies**—Fibrosarcoma cell line HT1080 and epidermoid carcinoma line A431 were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics. Monoclonal antibodies used were anti-integrin $\alpha_{4}$, A2-H1E10 (50); anti-integrin $\alpha_{3}$, A3-IV5A (51); anti-integrin $\beta_{1}$, TS2/16 (52); anti-CD9, C9-BB (12); and anti-CD81, TS2/16 (52); anti-CD9, A3-IVA5 (51); anti-integrin $\beta_{1}$, TS2/16 (52); anti-CD9, C9-BB (12); anti-CD81, TS2/16 (52); and anti-E-cadherin, C20820 (Transduction Laboratories, Lexington, KY); and anti-caveolin-1, C13630 (Transduction Laboratories). Also utilized was a rabbit polyclonal antibody to the cytoplasmic domain of integrin $\alpha_{3}$A (54).

**Immunoprecipitation and Blotting**—Cells were labeled with sulfo-N-hydroxysuccinimide-CL-Biotin (Pierce) at 0.1 mg/ml for 30 min at 4 °C, washed with cold PBS containing 200 mM glycine, and lysed with buffer containing 1% detergent (Triton X-100, CHAPS, Brij 99, or Brij 96), 25 mM HEPES, 150 mM NaCl, 5 mM MgCl$_2$, 20 g/ml aprotinin, 10 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 2 mM NaF, 10 mM sodium pyrophosphate, and 10 mM Na$_2$VO$_4$. After 1 h at 4 °C, insoluble material was removed by centrifugation at 16,000 × g (10 min, 4 °C), and the supernatant was cleared with protein G-Sepharose (Amersham Pharmacia Biotech). The column was eluted with the respective buffer used for cell lysis and with so many other proteins has created enormous problems in evaluating the specific biochemical properties of these proteins. The possible location of these complexes in raft-type microdomains, coupled with the appearance of level 2 and level 3 complexes only under mild detergent conditions, suggests that possibly these complexes may exist only in the context of large raftlike vesicles or whether they may exist in a truly soluble form of a reasonable size. These studies have been carried out using A431 and HT1080 cell lines because they express significant levels of $\alpha_{4}$ association.

**Gel Chromatography**—Sephrose CL6B columns (Amersham Pharmacia Biotech; 1 × 18 cm) were equilibrated with lysis buffer at 4 °C (Brij 99-containing buffers) or at room temperature (Brij 96 lysis), and 0.7 ml of cell lysate (from ~5 × 10$^6$ cells) was applied to the top of the column. The column was eluted with the respective buffer used for cell lysis and with so many other proteins has created enormous problems in evaluating the specific biochemical properties of these proteins. The possible location of these complexes in raft-type microdomains, coupled with the appearance of level 2 and level 3 complexes only under mild detergent conditions, suggests that possibly these complexes may exist only in the context of large raftlike vesicles or whether they may exist in a truly soluble form of a reasonable size. These studies have been carried out using A431 and HT1080 cell lines because they express significant levels of $\alpha_{4}$ association.

**Membrane Depletion**—To specifically remove cholesterol, intact cells were washed three times in PBS to remove serum and then incubated in Dulbecco’s modified Eagle’s medium containing 10 or 20 mM methyl-$\beta$-cycloexodextrin (MCD) for 30–60 min at 37 °C. The sample was then successively centrifuged at 200, 2000, and 16,000 × g to remove cells and cellular debris from the sample. For treatment of immunoprecipitated samples, immune complexes on Sepharose beads were incubated in lysis buffer without detergent but containing 10 mM MCD for up to 60 min at 4 or 37 °C. Levels of cholesterol in lysates and cell-free supernatants of MCD-treated cells were determined using the “Cholesterol 20” kit (Sigma). This kit utilizes cholesterol oxidase to produce hydrogen peroxide, which is then detected in a coupled colorimetric peroxidase assay. Ganglioside GM1 was measured in a dot blot assay, utilizing cholera toxin B subunit, anti-cholera toxin polyclonal antibody, and HRP-conjugated anti-rabbit antibody (all reagents from Sigma).
RESULTS

CD9-α3β1 Complexes under Different Detergent Conditions—A key feature of lipid rafts is their relative resistance to detergents (33, 34). To examine the detergent resistance of TM4SF-integrin protein complexes, A431 human carcinoma cells were surface labeled with biotin and lysed using four different detergent conditions, and then immunoprecipitations were carried out. In 1% Brij 99 detergent, immunoprecipitation of α3β1 integrin yielded a protein comigrating with CD9, and immunoprecipitation of CD9 yielded α3β1 integrin. Both proteins appeared at nearly the same level regardless of which antibody was utilized, consistent with a high stoichiometry of complex formation. In 1% CHAPS, similar results were obtained, except that less α3β1 was associated with CD9 and less CD9 was associated with the immunoprecipitated α3β1. In contrast, under conditions of 1% Triton X-100 or RIPA (1% Triton X-100, 0.5% deoxycholate, 0.1% SDS), essentially no CD9-α3β1 complex was observed. The identity of α3β1 in CD9 immunoprecipitations and CD9 in α3 integrin immunoprecipitations has been demonstrated numerous times by reimmunoprecipitation and/or by immunoblotting (Refs. 12 and 14 and data not shown).

Several additional proteins, not identified here, were also present in both the CD9 and α3β1 immunoprecipitated complexes. The levels of these other proteins progressively diminished as conditions were shifted from 1% Brij 99 to 1% CHAPS to 1% Triton X-100 to RIPA. The prominent ~85-kDa protein and more weakly labeled 70-kDa protein seen in α3 immunoprecipitations (especially in Triton and RIPA conditions) probably arise from α3 proteolysis as characterized elsewhere (57). In control experiments, the α3β1 integrin was immunoprecipitated in the absence of any apparent CD9 association, and E-cadherin immunoprecipitates showed no evidence for either CD9 or integrin association, regardless of detergent conditions.

CD9 and α3 Integrin Appear in Raftlike Membrane Microdomains—Because of the types of proteins sometimes found to associate with TM4SF proteins and integrins (see Introduction), and from results such as those shown in Fig. 1, we hypothesized that α3β1–CD9 complexes might occur in raftlike microdomains that would be maintained in Brij or CHAPS conditions but disrupted in 1% Triton. Indeed, sucrose density gradient analysis of a 1% Triton lysate revealed that only a trace of CD9 or α3 integrin (~1%) was present in the low density, lipid-enriched, light membrane fractions (Fig. 2A, lanes 3–5). As expected, the majority of total cell surface biotin-labeled proteins (top panel) and E-cadherin (bottom panel) were in the dense fractions (lanes 7–12), while caveolin-1 (well known to appear in Triton-insoluble vesicular microdomains (41)) served as a positive control for the light membrane fraction.

In contrast, in 1% CHAPS, substantial amounts of α3 integrin and CD9 appeared in the light membrane fractions, as did the positive control protein, caveolin-1 (Fig. 2B). Again, the majority of E-cadherin and total biotin-labeled proteins were in the dense fractions. Similarly in 1% Brij 99, large amounts of CD9, α3 integrin, and caveolin were present in the light membrane fractions (Fig. 2C), while larger proportions of total biotin-labeled proteins and E-cadherin also appeared in the light membrane fractions (Fig. 2C). Despite the increased proportion of E-cadherin in light membrane fractions in Brij 99 conditions, it did not show any coimmunoprecipitation with α3 or CD9 (Fig. 1). Also, α3 integrin was present in light membrane fractions (not shown) although not associated with CD9 or α3 integrin. Thus, as many others have noted, components can appear together in light membrane fractions without necessarily being associated.

Because detergents such as CHAPS or Brij 99 could artificially induce cell surface protein complex formation in the light membrane fractions, we utilized an alternative detergent-free isolation method developed by Song et al. (56). Results obtained using this high pH “carbonate” method (Fig. 2D) were similar to those obtained using 1% CHAPS (Fig. 2B). Substantial amounts of CD9 and α3 integrin appeared in the light membrane fractions, while most of the E-cadherin and total biotin-labeled protein were present in the dense fractions.

To further address the raftlike nature of CD9 and α3 integrin complexes, GM1 ganglioside colocalization studies were carried out, using cholera toxin as a probe for GM1. A431 cells were first incubated with biotinylated CT-B, and then after cells were fixed, CT-B was visualized (Fig. 3A, right panels). In the same experiment, fixed cells were also double-stained for CD9, α3 integrin, or CD71 (transferrin receptor) (Fig. 3A, left panels). As indicated, CD9 and α3 integrin each showed substantial colocalization with CT-B-stained GM1 ganglioside, whereas CD71 staining was largely nonoverlapping with the CT-B staining. A reciprocal double staining experiment was also carried out (Fig. 3B). Spread A431 cells were first incubated with anti-CD9, anti-α3, or anti-CD71 mAb, and then after fixation, those proteins were visualized (Fig. 3B, left panels). Double staining was then carried out using biotinylated CT-B (Fig. 3B, right panels). Again, staining of CD9 or α3 integrin showed substantial overlap with CT-B staining, whereas staining for CD71, a nonraft protein (44), was quite distinct. Colocalization of CD9 and α3 integrin was readily observed (Fig. 3, A and B) when cells were fixed immediately after staining with primary antibody; it was not necessary to induce further clustering of the antigens by adding second antibody or multivalent anti-CT-B reagents.

CD9-α3β1 Complexes Are Perturbed upon Cholesterol Depletion but Do Not Require Cholesterol for Association—Partial depletion of cholesterol typically leads to a loss of protein localization into rafts (44). β-Cycloextrins are a class of heptasaccharides commonly used to selectively remove cholesterol from cellular membranes (58). By treating intact A431 cells with MβCD, we typically removed 40–60% of total cholesterol, as measured following cell lysis. After MβCD treatment of intact cells, we then analyzed CD9 and α3 integrin appearance...
in light membrane fractions, following isopycnic sucrose centrifugation using the detergent-free carbonate lysis method. As indicated in Fig. 4, the amounts of CD9 and α3 in light membrane fractions were greatly diminished compared with untreated control samples run in parallel. As expected, levels of caveolin in light membrane fractions were also diminished upon cholesterol depletion. Results in Fig. 4 are again consistent with CD9-α3 integrin complexes being present in raftlike microdomains.

The disappearance of CD9 and α3 integrin from light membrane fractions (lanes 3–6) was not accompanied by a large increase in these proteins in the dense fractions (Fig. 4, lanes 8–12). Thus, we looked for shed proteins in the MβCD-treated cell supernatant. Immunoprecipitations were carried out from cell-free supernatants of MβCD-treated A431 cells in the presence of 1% Brij 99. As indicated (Fig. 5A), shed complexes immunoprecipitated by antibodies to α3β1, CD9, or CD151 (a TM4SF protein tightly associated with α3β1 (9)) resembled complexes directly immunoprecipitated from whole cell lysates (e.g. Figs. 1 and 6) and thus appeared to remain intact. Control protein CD71 was present in the cell-free supernatant but was not part of the TM4SF-α3β1 complex (Fig. 5A). Control proteins caveolin and E-cadherin were not present in the shed fraction (not shown). In the absence of MβCD treatment, little protein was found in the shed fraction (Fig. 5B), thus establishing that shedding was indeed induced by MβCD, as seen previously for other protein complexes (59). Also, as seen previously (59), the ganglioside GM1 was present in shed membrane vesicles. As determined by an immunochemical assay involving cholera
M
ty between material isolated from cell-free supernatants of M
toxin B subunit, the amount of GM1 shed into the cell-free supernatant of MβCD-treated A431 cells was about 8 times higher than that of untreated samples (data not shown). In contrast, cholesterol was not present in shed membrane vesicles. After MβCD treatment, shed vesicles were isolated by centrifugation (200,000 × g for 1 h), and the pellet was washed once with PBS. Cholesterol measurements showed no difference between material isolated from cell-free supernatants of MβCD-treated or -untreated cells. In fact, all cholesterol present in MβCD-treated cell supernatants could be completely removed by dialysis, as expected for cholesterol-cyclodextrin complexes (data not shown). Sucrose gradient analysis of shed fractions confirmed that after cholesterol depletion, CD9 and α3 integrin complexes were no longer present in light membrane fractions (Fig. 5C, bottom panels, lanes 3–5), although they were perhaps slightly less dense than the total biotin-labeled protein (Fig. 5C, top panel). Thus, cholesterol depletion perturbed CD9–α3β1 complexes insofar as inducing shedding and increasing their density, but the protein complexes themselves remained intact.

To further test the effect of cholesterol depletion on TM4SF protein and integrin complexes, cell surface biotinylated proteins from A431 cells were immunoprecipitated, and then half of each complex was incubated with 20 mM MβCD (Fig. 6, + lanes). As indicated, treatment with MβCD clearly did not alter the levels of α3β1 associated with CD9, or CD9 associated with α3β1. Likewise, levels of all other associated proteins were not obviously altered, except for an unknown protein of ~18 kDa that was substantially diminished upon cholesterol depletion. Antibodies to another TM4SF protein, CD63, also precipitated complexes (containing α3β1 and CD9) that were not affected by MβCD treatment. E-cadherin (not shown) and α2 integrin and complexes were likewise unaltered. Treatment of cell lysates with MβCD or 10 μg/ml filipin (another cholesterol-disrupting agent) prior to immunoprecipitation again failed to alter the association of CD9 or CD63 with α3 integrins and other proteins (not shown).

CD9–α3β1 complexes readily occur outside of raftlike membrane vesicles—We next asked whether, in the absence of cholesterol depletion, CD9–α3β1 complexes would preferentially occur in the light membrane fractions from sucrose gradients. As indicated in Fig. 7A (left panel), both α3 integrin and CD9 from 1% Brij 99 lysates were present in light membrane fractions (lanes 2–5) as well as in dense fractions (lanes 9–12). As indicated by immunoprecipitation analysis of sucrose gradient fractions, CD9–α3 complexes were present in both the dense fractions (fractions 9–12) and the light membrane fractions (fractions 9–12) and appeared similar in each location (Fig. 7B, lanes a–c).

To demonstrate further that CD9–α3β1 complexes may occur outside of raftlike domains, we devised more stringent cell lysis conditions in which these molecules would be absent from the...
light membrane fractions but would remain associated. As seen in Fig. 7A (right panel), the inclusion of 0.2% Triton X-100 with 1% Brij 99 caused CD9 and α3, but not caveolin, to essentially disappear from the incompletely solubilized light membrane fractions of a sucrose gradient and appear exclusively in the dense fractions. Nevertheless, immunoprecipitation revealed that the CD9-α3 complex was fully maintained (Fig. 7B, lanes f and g) and was indistinguishable from the complexes seen in 1% Brij 99 (lanes a–c). In control experiments, anti-α3 integrin did not coimmunoprecipitate CD9, and anti-CD71 did not coimmunoprecipitate integrins or CD9 (Fig. 7B, lanes d, e, h, and i).

Although caveolin-1 colocalized with α3β1 and CD9 in the light membrane fractions of some sucrose gradients (Figs. 2, 4, and 7A) and interactions between caveolin-1 and β1 integrins have been suggested (60, 61), we could not coprecipitate caveolin-1 with either CD9 or α3 integrin in Brij 99 conditions. Furthermore, we have previously observed α3β1-CD9 complexes in the K562 cell line (12), which does not express caveolin-1.

Besides cholesterol, the actin cytoskeleton may also markedly influence the localization of proteins into rafts (62) and the distribution of TM4SF protein complexes (5). However, when cells were treated with latrunculin B (10 μM) before lysis to disrupt actin filaments, we observed no change in the association of CD9 with α3β1 (not shown). Another key feature of detergent-resistant raft complexes in vitro is their sensitivity to increased temperature (33, 34). In this regard, our TM4SF-α3β1 complexes (such as seen in Figs. 6 and 7B) were completely stable despite incubation of cell lysates for 30 min at 37°C prior to immunoprecipitation (not shown).

**CD81-α3β1 Complexes Also Occur Independent of Raftlike Microdomains**—To expand the generality of our findings, we next analyzed TM4SF complexes using a different cell line (HT1080), a different TM4SF protein (CD81), and a detergent (Brij 96) that is a little more stringent than Brij 99 or CHAPS with respect to TM4SF complexes. After sucrose density gradient fractionation, anti-CD81 immunoprecipitation revealed the presence of CD81-α3β1 complexes in both light membrane fractions (Fig. 8, middle panel, lanes 1–3) and in dense fractions (lanes 4–10). The identity of α3β1 in CD81 complexes was confirmed many times by reimmunoprecipitation or by immunoblotting (Refs. 12 and 20 and not shown). Upon treatment of the cell lysate with Mg/CD to deplete cholesterol prior to sucrose gradient fractionation and immunoprecipitation.

**Comparison of CD81 Immunoprecipitates** —Fig. 8, middle and bottom panels) with total biotin-labeled cell surface protein (top panel) revealed that only a minority of total labeled protein was in the CD81 complexes. Also, with the aid of additional sucrose layers to increase resolution, we observed that CD81-α3β1 complexes (bottom panel) were slightly less dense than the bulk of the total labeled proteins (top panel). Distinct complexes between CD81 and unknown proteins of ~60 and 200 kDa were also resolved. The CD81–200-kDa complex (peak, lanes 10–14) was more dense than CD81-α3β1 (peak, lanes 7–10), whereas the CD81–60-kDa complex (peak, lanes 5 and 6) was less dense.

**How Big Are TM4SF-α3β1 Complexes** —It was shown elsewhere that TM4SF-integrin complexes could be rather large (~20 x 10^6 daltons (49)), thus raising concerns regarding specificity and complexity. To address the issue of size, gel filtration was carried out using Sepharose CL6B columns and 1% Brij 96 (level 2) cell lysis conditions. As indicated, surface-biotinylated CD9-α3β1 complexes from A431 cells were readily retained within the included volume of the column, where they comi-
CD9 was immunoprecipitated from 50 µl of each fraction with mAb DU-ALL-1, and associated proteins were revealed after nonreducing SDS-PAGE and blotting with ExtrAvidin-HRP. The distribution of total protein (as determined in a Bradford assay) is shown in the top panel. B, a Brij 96 extract of surface-biotinylated HT1080 cells (∼5 × 10⁶ cells in 500 µl of lysate) was fractionated as in A, and 14 fractions of ∼600 µl were collected. CD81 was immunoprecipitated from 500 µl of each fraction with mAb M38, and CD81 complexes were resolved by SDS-PAGE and visualized with ExtrAvidin-HRP.

Graded with the bulk of the total cellular protein (Fig. 9A). In contrast, a subpopulation of surface-biotinylated CD81-αβ₃ complexes from HT1080 cells was present in the void volume (Fig. 9B), perhaps due to association with lipid raftlike domains. However, the majority of CD81-αβ₃ complexes were retained within the column (Fig. 9B, lanes 3–11). An additional population of CD81 (lanes 11–14) appeared not to be associated with other large cell surface proteins. Due to the presence of 1% Brij 96 in these experiments, size cannot be determined accurately. Nonetheless, the results suggest that nearly all of the CD9 complexes and most of the CD81 complexes may be considerably smaller than 4 × 10⁶ Da.

Analysis Of CD63-PtdIns 4-K as a Prototype Level 3 Complex—Because TM4SF “level 3” interactions may include so many different molecules, we hypothesized that many of these interactions were especially likely to result from indirect associations within large lipid microdomains. Sensitivity to Brij 96 diminished. However, the majority of CD81-αβ₃ complexes may be considerably smaller than 4 × 10⁶ Da.

FIG. 9. Gel filtration of CD9 and CD81 complexes. A, A431 cells were surface-biotinylated and lysed in 1% Brij 96, and the extract was fractionated by size exclusion chromatography on Sepharose CL6B. CD9 was immunoprecipitated from 50 µl of each fraction with mAb DU-ALL-1, and associated proteins were revealed after nonreducing SDS-PAGE and blotting with ExtrAvidin-HRP. The distribution of total protein (as determined in a Bradford assay) is shown in the top panel. B, a Brij 96 extract of surface-biotinylated HT1080 cells (∼5 × 10⁶ cells in 500 µl of lysate) was fractionated as in A, and 14 fractions of ∼600 µl were collected. CD81 was immunoprecipitated from 500 µl of each fraction with mAb M38, and CD81 complexes were resolved by SDS-PAGE and visualized with ExtrAvidin-HRP.

FIG. 10. Distribution of PtdIns 4-K in isopycnic sucrose gradients. Brij 99, CHAPS, and Triton lysates of A431 cells were fractionated on sucrose gradients as shown in Fig. 2, and then 50-µl aliquots of each fraction were used to quantitate PtdIns 4-K activity (using 20 µCi of [γ-32P]ATP, for 30 min, at 37°C).

Substantial fraction of CD63 appeared in light membrane fractions (Fig. 7A, left panel). Substantial PtdIns 4-K activity from 1% Brij 99 or 1% CHAPS lysates was also present in the light membrane fractions, whereas 1% Triton lysates yielded essentially no PtdIns 4-K activity in the light membrane fractions (Fig. 10). Immunoprecipitation analysis of 1% Brij 99 sucrose gradient fractions revealed that PtdIns 4-K activity was associated with CD63 in both light membrane and dense fractions (Fig. 11, A and B). Furthermore, under conditions (1% Brij 99 plus 0.2% Triton X-100) in which CD63 was almost entirely present in dense fractions (Fig. 7A, right panel), PtdIns 4-K association was again well maintained (Fig. 11C). Little PtdIns 4-K association with negative control proteins (α₃ integrin, transferrin receptor) was observed in Fig. 11, especially in the dense fractions (Fig. 11, B and C). PtdIns 4-K association with CD9 was also maintained in both the dense and light fractions from a 1% Brij 99 gradient and from the dense fractions of a 1% Brij 99 plus 0.2% Triton gradient (not shown). Thus, TM4SF-PtdIns 4-K complexes remained intact, even when completely solubilized and removed from light membrane lipid vesicle fractions.

TM4SF-PtdIns 4-K association did not depend on cholesterol. Treatment of immunoprecipitated CD63, CD9, and α₃ integrin complexes with MβCD did not result in loss of PtdIns 4-K activity (Fig. 11D). Instead, the activity was elevated for unknown reasons. As expected, the most PtdIns 4-K activity was associated with CD63, less was associated with CD9 or α₃ integrin, and little was associated with negative control proteins E-cadherin and α₂ integrin. Control experiments indicated that MβCD itself had no effect on PtdIns 4-K activity when added directly to PtdIns 4-K assay mixtures (not shown). Treatment of intact cells with MβCD did not induce PtdIns 4-K shedding (not shown), probably because PtdIns 4-K is associated with the inner leaflet of the plasma membrane. Disruption of cholesterol-containing complexes from A431 cell lysates by the addition of filipin (10 µg/ml) had no impact on PtdIns 4-K activity associated with CD63 or CD9 (not shown). In other experiments, A431 cell lysates (before immunoprecipitation of CD63-PtdIns 4-K complexes) or immobilized CD63-PtdIns 4-K complexes (after immunoprecipitation) were incubated for 30 min at 37°C. In neither case was complex formation diminished.
FIG. 11. CD63-PtdIns 4-K complexes occur in dense fractions and do not depend on cholesterol. A431 cells were lysed in 1% Brij 99 (A and B) or 1% Brij 99 plus 0.2% Triton X-100 (C), and then sucrose density gradients were carried out as described in the legend to Fig. 2. (Note that mixtures of detergents were preincubated overnight at 4 °C to form mixed micelles.) Aliquots of pooled light membrane fractions (LMF, 2–5) or dense fractions (DF, 9–12) were then immunoprecipitated using mAb to integrin α2 (A2-HE10), CD71 (OKT9), or CD63 (6H1). Following immunoprecipitation, immune complexes were analyzed for PtdIns 4-K activity (using 5 μCi of [γ-32P]ATP for 30 min at 37 °C). D, A431 cells were lysed in 1% Brij 99, and then immunoprecipitations were carried out using mAbs indicated in Fig. 6. Immune complexes on beads were then treated with buffer that either did (+) or did not (−) contain 10 mM MjCD, prior to PtdIns 4-K analysis (using 5 μCi of [γ-32P]ATP for 10 min at room temperature). Note that the addition of 1 mM MjCD directly to the PtdIns 4-K assay mixture did not alter the enzyme activity (not shown).

Finally, CD63-PtdIns 4-K complexes were fractionated using a CL6B gel filtration column to allow a rough estimate of complex size. From a Brij 99 lysate of A431 cells, a portion of the CD63-associated PtdIns 4-K activity was partly excluded from the column (Fig. 12, fractions 1 and 2), whereas another portion was included well within the column (Fig. 12A, fractions 5–10). If 0.2% Triton X-100 was included with the Brij 99, the yield of CD63-associated PtdIns 4-K was diminished, but the majority of the remaining activity migrated well within the included volume of the column (Fig. 12B). In both detergent conditions, a substantial fraction of the total PtdIns 4-K activity migrated in fractions 10–18, indicating that it was smaller in size and mostly dissociated from CD63. From these results, we conclude that CD63-PtdIns 4-K complexes can exist at a size considerably less than 4 × 10^6 Da.

**DISCUSSION**

**Prototype TM4SF Protein Complexes Occur as Discrete Units**—TM4SF proteins have been reported to associate with each other, with integrins, and with many other types of transmembrane proteins and other proteins, thus potentially forming an extensive network (1, 3, 6, 63). Many of these associations are best seen when weak (i.e. less hydrophobic) detergents are utilized. Thus, a major concern regarding many TM4SF protein complexes has been that they may represent incompletely solubilized vesicular material. These complexes may sometimes be very large (>20 million Da (49)) and not very dense (37, 39), consistent with incomplete solubilization and a high lipid/protein ratio. Indeed, our initial results showed that the detergent conditions most permissive for TM4SF associations with other proteins (e.g. 1% Brij 99, 1%}

**CHAPS** were also most likely to yield a large fraction of TM4SF proteins and associated proteins within the incompletely solubilized light membrane fractions of a sucrose gradient.

However, our studies of a few prototype TM4SF protein complexes now demonstrate that (a) these complexes are well maintained within the detergent solubilized dense fractions of sucrose gradients, (b) they are not dependent on cholesterol for maintenance of association, and (c) they can occur well within the included volume of a Sepharose CL6B gel filtration column. Also we have confirmed that these complexes are indeed highly specific in terms of the components present in the complex. Together, these results emphasize that these complexes are not artifacts of incomplete solubilization but instead represent discrete units that are of moderate size and capable of being fully solubilized.

Association of α3β1 with TM4SF proteins CD9, CD81, or CD63 was observed in the dense fractions of sucrose gradients carried out using three different detergent conditions (1% Brij 99, 1% Brij 99 plus 0.2% Triton X-100, 1% Brij 96) and two different cell types (A431 carcinoma and HT1080 fibrosarcoma). Likewise, CD63 association with PtdIns 4-K was maintained in dense fractions using multiple detergent conditions (1% Brij 99, 1% Brij 99 plus 0.2% Triton X-100). None of these associations were altered by the addition of actin or microtubule-disrupting agents (not shown). Thus, the density of these complexes did not appear to be influenced by association with cytoskeletal proteins. Results shown here for CD9, CD81, CD63, α3β1, and PtdIns 4-K complexes are in striking contrast to results seen elsewhere for other TM4SF protein and/or integrin complexes. For example, associations of β1 integrins with CD36 (37), α1β3 with CD47 (36), α1β3 with CD63 (49), and β1 integrins with CD98 

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Neither CD9-α3β1, CD63-α3β1, CD81-α3β1 nor CD63-PtdIns 4-K complexes were dissociated upon cholesterol depletion with MβCD, regardless of whether added to intact cells, to lysates prior to sucrose gradients, to lysates prior to immunoprecipitation, or to immune complexes after immunoprecipitation. In this regard, αβ2 integrin association with TM4SF proteins again is in contrast to αβ2 integrin association with CD47/iAP. The latter association occurred largely within the light membrane fractions from a sucrose gradient and was disrupted upon cholesterol depletion (36). It is not clear why CD63-associated PtdIns 4-K activity actually increased upon MβCD treatment. Possibly, the enzyme activity could be inhibited by cholesterol itself or by a cholesterol-dependent associated protein not yet identified. In this regard, TM4SF proteins associate with at least one unidentified protein in a cholesterol-dependent manner (e.g. Fig. 6 and data not shown). Upon treatment of intact cells with MβCD, CD9-αβ2 complexes were not dissociated but were shed, consistent with previous demonstrations that MβCD may induce shedding of intact, raft-derived vesicles from the outer leaflet of the plasma membrane (59, 64). Treatment with MβCD did not cause shedding of PtdIns 4-K complexes, consistent with their not being present on the plasma membrane outer leaflet.

Incompletely solubilized low density fractions from sucrose gradients typically contain large vesicles of 0.05–1 μm in diameter (65, 66). However, complexes of CD9-αβ1, CD81-αβ1, and CD63-PtdIns 4-K each migrated well into the included volume of Sepharose CL6B columns, indicating sizes of substantially less than 4 million Da. This result again is consistent with these complexes occurring outside of the context of an ordered lipid microdomain. In contrast, CD63-αβ2 complexes seen elsewhere were excluded from a Sepharose CL4B column, suggesting a size of >20 million Da (49). Our results also confirm the highly specific nature of the TM4SF complexes. Of the total surface labeled protein, only a few proteins were present in association with CD9 or CD81, whereas the majority of other proteins (including αβ1 integrin, CD71, and E-cadherin) were not. Also, the selective association of PtdIns 4-K with CD63, CD9, and α3 integrin, but not α2 integrin, CD71, or E-cadherin, is consistent with the high degree of TM4SF-PtdIns 4-K selectivity seen elsewhere (8, 31). Furthermore, proteins colocalizing in the light membrane fractions of sucrose gradients (i.e. cavinolin, α2 integrin) did not associate with CD9, CD81, or PtdIns 4-K simply because they were in that fraction.

Prototype TM4SF Protein Complexes in Lipid Raftlike Domains—Although not dependent on a lipid raftlike environment for association, nonetheless we establish here that prototypic TM4SF protein complexes may localize into raft-type microdomains. First, under multiple detergent lysis conditions, CD9-αβ1, CD81-αβ1, or CD63-PtdIns 4-K complexes were evident in the light membrane fractions of sucrose gradients, where detergent-resistant lipid-protein microdomains are typically found. Second, upon cholesterol depletion, the CD9-αβ1 and CD81-αβ1 complexes shifted out of the light membrane fractions and into the dense fractions. This result was obtained either upon treatment of intact cells or treatment of a Brij 96 cell lysate. This dependence on cholesterol is typical of raft-type microdomains (35, 34). Third, CD9-αβ2 complexes colocalized with the ganglioside GM1 on the surface of intact cells. Again, this is typical of raft-type microdomains (33, 34).

Previously, CD9 was suggested to be present in T cell rafts (in Triton X-100 conditions), where it could perhaps facilitate T cell costimulation (39). Here we have confirmed the raftlike association of CD9 (and CD9 complexes), although in our hands this was not very obvious using Triton X-100 conditions. Instead, we needed to utilize Brij 99, CHAPS, or detergent-free conditions to see appreciable CD9 in the light membrane fractions of sucrose gradients. Also, our CD63-PtdIns 4-K raft-type localization results are consistent with previous reports that the PtdIns 4-K enzyme, as well as PtdIns 4-K lipid substrate (PtdIns) and product (PtdIns 4-P) are in rafts (42, 67). The αβ1 integrin had not been shown previously to localize into raftlike microdomains, although results elsewhere did suggest that β1 integrins (37, 38), αβ2 integrins (36) may sometimes associate with raftlike microdomains. The association of integrins with gangliosides (69) is also consistent with raftlike colocalization. With the increasing realization that integrin adhesive function may depend on integrin lateral diffusion (70, 71), it will be interesting to determine whether raft-type domains contribute to that process.

Different Types of Rafts—An abundance of evidence now suggests the existence of distinct types of lipid microdomains, each with specific components. For example, microdomains containing caveolin are distinct from other types of microdomains (42, 66, 72, 73). Also, microdomains may differ in terms of types of gangliosides that are present (72), the presence of particular GPI-linked proteins (65), and the presence or absence of other specific cell surface proteins (74). Integrin association with caveolin (60, 61) and the GPI-linked protein uPAR (60) might suggest that these known raft-associated molecules could facilitate the recruitment of integrin complexes into raftlike domains. However, the TM4SF protein complexes studied here are present in a distinct type of domain, not containing caveolin, uPAR, or other GPI-linked proteins. Using conditions in which we see TM4SF-αβ1, and TM4SF-PtdIns 4-K complexes, we have failed to coimmunoprecipitate caveolin or uPAR with αβ integrin or TM4SF proteins from multiple cell types. Also, our TM4SF complexes are readily observed in cell types (e.g. K562 erythroleukemia cells) that do not contain caveolin or uPAR. In addition, extensive analysis of numerous additional TM4SF-associated proteins has failed to yield any GPI-linked proteins.3 The absence of caveolin from our TM4SF-PtdIns 4-K complexes is consistent with a previous demonstration that the majority of PtdIns 4-K from A431 cells is present in noncaveolar light membrane fractions (42).

Another means of subdividing rafts is by detergent solubility (65, 74). Rafts were originally defined as being Triton-insoluble (33), but subsequent data have emphasized that distinct types of rafts may differ markedly in terms of detergent solubility. In our studies, detergents such as Brij 96 and Brij 99 were better than Triton X-100 in maintaining TM4SF complexes in raftlike domains. Notably, microdomains containing β1 and β2 integrins as seen elsewhere (37, 38) were insoluble in Triton X-100 and thus may differ from the TM4SF-αβ1 complexes analyzed here.

According to one model, different types of rafts may be distinct and nonadjacent (74). Alternatively, there may be a continuum of highly ordered detergent insoluble rafts, in proximity to semiderided domains that are more soluble in detergent (65). Such a continuum of detergent-insoluble rafts adjacent to less ordered rafts could explain our observation that CD9 and αβ1 integrin are found in the same structures of the plasma membrane as GM1, while at the same time GM1 can be a molecule highly enriched in caveolae of A431 cells (75).

Functional Relevance—Rafts have broad functional relevance with respect to signaling and protein sorting (33, 34, 76). However, in the absence of transmembrane proteins, it has been difficult to visualize how rafts can act as concentrated signaling loci. Although TM4SF proteins are not very tightly associated with the cytoskeleton (consistent with their ease of

3 C. Claas, C. S. Stipp, and M. E. Hemler, unpublished results.
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extration by detergents), the actin cytoskeleton does appear to influence TM4SF protein distribution (5). We propose that TM4SF-containing microdomains, by being in proximity to highly ordered raftlike domains, could help regulate the distribution and function of rafts. For example, TM4SF protein localization into cell filopodia or microvilli (77, 78) could potentially influence the distribution of rafts to these same locations. Conversely, TM4SF protein complexes may also help to recruit signaling enzymes into the proximity of rafts. For example, raft-associated TM4SF complexes may come into proximity with raft signaling enzymes into the proximity of rafts. For example, TM4SF-containing microdomains, by being in proximity to highly ordered raftlike domains, could help regulate the distribution and function of rafts. For example, TM4SF complexes in rafts may come into proximity with raft signaling components, such as Src family kinases and G proteins (44, 73).

Studies elsewhere point to a potential functional role for TM4SF protein complexes in rafts. For example, costimulation of T lymphocytes clearly involves reorganization of rafts (79), and CD81 and CD9 both play a role in T cell costimulation (6). TM4SF protein complexes in rafts. For example, costimulation of T lymphocytes clearly involves reorganization of rafts (79), and CD81 and CD9 both play a role in T cell costimulation (6). TM4SF protein complexes in rafts. For example, costimulation of T lymphocytes clearly involves reorganization of rafts (79), and CD81 and CD9 both play a role in T cell costimulation (6).

In conclusion, we have utilized a few prototype TM4SF complexes to establish that TM4SF complexes may exist as well (17). Nichols, T. C., Guthridge, J. M., Karp, D. R., Molina, H., Fletcher, D. R., and Horejsi, V. M. (1998) Euro. J. Immunol. 28, 4123–4129.
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