Specific interactions among transmembrane 4 superfamily (TM4SF) proteins and phosphoinositide 4-kinase

Robert L. YAUCH¹ and Martin E. HEMLER²

Dana-Farber Cancer Institute, Rm D-1430, 44 Binney Street, Boston, MA 02115, U.S.A.

In earlier work we established that phosphoinositide 4-kinase (PI 4-kinase) may associate with transmembrane 4 superfamily (TM4SF, tetraspanin) proteins, but critical specificity issues were not addressed. Here we demonstrate that at least five different TM4SF proteins (CD9, CD63, CD81, CD151 and A15/TALLA1) can associate with a similar or identical 55 kDa type II PI 4-kinase. These associations were specific, since we found no evidence for other phosphoinositide kinases (e.g. phosphoinositide 3-kinase and phosphoinositide-4-phosphate 5-kinase) associating with TM4SF proteins, and many other TM4SF proteins (including CD82 and CD53) did not associate with PI 4-

kinase. CD63–PI 4-kinase complexes were almost entirely intracellular, and thus are distinct from other TM4SF–PI 4-kinase complexes (e.g. involving CD9), which are largely located in the plasma membrane. These results suggest that a specific subset of TM4SF proteins may recruit PI 4-kinase to specific membrane locations, and thereby influence phosphoinositide-dependent signalling.

Key words: CD9, CD53, CD63, CD81, CD82, CD151, tetraspanins.

INTRODUCTION

Mammalian transmembrane 4 superfamily (TM4SF) proteins (also known as tetraspans or tetraspanins) include at least 16 core members and a number of additional proteins with sequence similarities [1–3]. Nearly all mammalian cells contain large numbers of one or more TM4SF proteins. Several TM4SF proteins, including CD9, CD63, CD81, CD82 and CD151, may function in cell migration, proliferation and/or tumour cell metastasis [1,2,4]. Most TM4SF proteins can be found on the plasma membrane, and several are located in cell lamellipodia and filopodia, consistent with their role in cell motility [5,6]. Many TM4SF proteins, including CD9, CD37, CD53, CD63, CD81, CD82 and CD151, are also found in various intracellular granules and vesicles [7–12]. The CD63 protein, utilized as a marker for lysosomal-type vesicles, contains a ‘Gly-Tyr’ lysosomal-targeting motif [13] not found in other TM4SF proteins.

TM4SF proteins may associate with several other transmembrane proteins, including CD19, CD21, integrins, proteoglycans and various members of the immunoglobulin superfamily [1,2,4,14,15]. TM4SF protein complexes also can include the transmembrane-signalling enzyme γ -glutamyl transpeptidase [16], and the membrane enzyme phosphoinositide 4-kinase (PI 4-kinase). PI 4-kinase activity has been found in immunoprecipitates of CD63, CD81 and CD151 proteins [5,17]. Through TM4SF proteins, PI 4-kinase can be recruited into complexes with integrins [18], with the CD151 protein acting as a major link between PI 4-kinase and the $\alpha 3 \beta 1$ integrin [17]. We hypothesize that TM4SF protein-dependent recruitment of PI 4-kinase may result in the localized conversion of PtdIns into PtdIns4P. PtdIns4P would then be available for further conversion into PtdIns(3,4)P₂ [(by phosphoinositide 3-kinase (PI 3-kinase)] or PtdIns(4,5)P₂ [by phosphoinositide-4-phosphate 5-kinase (PI-4-P 5-kinase)]. These phosphoinositide products regulate many cellular processes, including growth, survival and vesicular trafficking [19]. At present, forms of PI 4-kinase called PI 4-

kinase α and PI 4-kinase β have been cloned and characterized [19]; however, the PI 4-kinase responsible for the majority of PI 4-kinase activity in many cell types has not yet been cloned. This latter enzyme has been purified and defined as a 55 kDa type II PI 4-kinase that is stimulated by non-ionic detergent, and inhibited by adenosine [20–22] and monoclonal antibody (mAb) 4C5G [23].

Since TM4SF proteins have the potential to associate with each other and with many other proteins, a major concern has been that PI 4-kinase activity and other activities may be recruited non-specifically into TM4SF protein complexes. Here we show that TM4SF–PI 4-kinase complexes are highly specific. First, we determined that only a single type of PtdIns kinase appears to associate with TM4SF proteins under the conditions utilized. Secondly, we found that only a subset of TM4SF proteins can associate with this kinase. Thirdly, we observed that TM4SF–PI 4-kinase association can occur at distinct cellular locations. To evaluate a variety of different TM4SF proteins with distinct expression patterns, and to ascertain the generality of our results, we have used a range of carcinoma, fibrosarcoma, erythroleukaemic, T- and B-lymphoid, and other cell lines in these studies.

MATERIALS AND METHODS

Cell lines, antibodies and reagents

B-, T- and erythroleukaemic cell lines (OCI-Ly8, Molt-4 and K562- $\alpha 2$) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. K562- $\alpha 2$ cells, transfected with human integrin $\alpha 2$, have been described elsewhere [24]. The epidermoid carcinoma line A431 and fibrosarcoma cell line HT1080 were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and antibiotics. HT1080-CD9 cells [25] were established by transfecting HT1080 cells with full-

Abbreviations used: PI 3-kinase, phosphoinositide 3-kinase; PI 4-kinase, phosphoinositide 4-kinase; PI-4-P 5-kinase, phosphoinositide-4-phosphate 5-kinase; TM4SF, transmembrane 4 superfamily; mAb, monoclonal antibody; PKC μ , protein kinase C μ .

¹ Present address: Sugen, Inc., 230 East Grand Avenue, South San Francisco, CA 94080, U.S.A.

² To whom correspondence should be addressed (e-mail martin_hemler@dfci.harvard.edu).

length CD9 cDNA cloned into the pZeoSV expression vector (Invitrogen, San Diego, CA, U.S.A.).

Anti-TM4SF protein mAbs were as follows: anti-CD9, DU-ALL (Sigma) and C9-BB [25]; anti-CD37, HD28 [7]; anti-CD53, HD77 [26]; anti-CD63, 6H1 [27] and NAG3 [28]; anti-CD81, M38 [29]; anti-CD82, M104 [29]; anti-CD151, 5C11 [17]; anti-NAG2, NAG2 [28]; and anti-TALLA1/A15, B2D [30]. Other antibodies used were: anti-CD98, 8A6; anti- $\alpha 2\beta 1$, A2-IIE10 [31]; anti-integrin $\alpha 4$ subunit, B5G10 [32]; anti-integrin $\beta 1$ subunit, TS2/16 [33]; anti-protein kinase $C\mu$ (PKC μ) (Santa Cruz Biotechnology); anti-PtdIns 4-kinase, 4C5G (Upstate Biotechnology, Lake Placid, NY, U.S.A.); and negative control antibodies P3 [34], J2A2 [35] and 187.1 [36]. An anti-PI 3-kinase-specific polyclonal antibody raised against a glutathione-S-transferase fusion protein containing the N-terminal SH2 domain of the rat p85 subunit of PI 3-kinase was kindly provided by Dr Tom Roberts (Dana-Farber Cancer Institute, Boston, MA, U.S.A.). Adenosine and wortmannin were purchased from Sigma.

Flow cytometry

Cells were incubated with negative control or specific mAb at 4 °C in the presence of NaN₃ for 1 h, washed three times, and then incubated with a secondary FITC-conjugated goat anti-mouse immunoglobulin (Calbiochem). Stained cells were analysed using a EPICS XL flow cytometer (Coulter Co., Miami, FL, U.S.A.). Specific mean fluorescence intensity values were determined by subtracting fluorescence obtained using negative control antibodies.

Immunoprecipitation and Western blotting

Cell lines were lysed for 1 h at 4 °C in immunoprecipitation buffer (150 mM NaCl, 5 mM MgCl₂, 25 mM Hepes, pH 7.5) supplemented with 1 % Brij 99 detergent (Acros, Pittsburgh, PA, U.S.A.), 10 mM Na₂P₃O₇, 1 mM PMSF, 20 μ g/ml aprotinin, 10 μ g/ml leupeptin, 2 mM NaF and 100 μ M Na₃VO₄. Insoluble material was cleared by centrifugation at 16000 g for 15 min, and the lysate was precleared with Protein A-Sepharose (Pharmacia Biotech) precoupled with 187.1 mAb for 1 h at 4 °C to eliminate non-specific binding material. For CD63 immunodepletion experiments, lysates were incubated four times for 1 h at 4 °C with anti-CD63 mAb, 6H1, covalently coupled to CNBr-activated Sepharose (Pharmacia Biotech) or with Sepharose alone (mock immunodepletion). After preclearance, lysates were incubated with specific mAbs coupled to 187.1-Protein A-Sepharose for 1 h at 4 °C. Immunoprecipitates were extensively washed (four times) in immunoprecipitation buffer supplemented with 1 % Brij 99. In the PKC μ immunoblotting experiment, immunoprecipitations were carried out using Protein G-Sepharose (Pharmacia Biotech). In some experiments, intact cells were treated with antibodies to cell-surface proteins in the presence of sodium azide at 4 °C to prevent internalization of antigens. Then excess antibodies were removed, cells were lysed, and immune complexes were captured by incubation with 187.1-Protein A-Sepharose, as described above.

For Western blotting, immune complexes were resolved by SDS/PAGE, electrophoretically transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH, U.S.A.) and membranes were blocked with PBS containing 0.05 % Tween 20 and 5 % dry milk. Membranes were probed with primary antibodies (either 1:500 of mouse anti-CD81, M38 ascites or 1 μ g/ml rabbit anti-PKC μ), washed 4 times with PBS containing 0.05 % Tween 20, and incubated for 1 h with the appropriate secondary antibody [anti-mouse IgG (Fc specific)-peroxidase

conjugate or anti-rabbit IgG-peroxidase conjugate (Sigma)]. $\beta 1$ integrin immunoblotting was carried out as previously described [17]. After final washes, proteins were visualized using Renaissance Chemiluminescent Reagent (DuPont NEN, Boston, MA, U.S.A.).

Measurement of PtdIns-specific lipid kinase activity

Lipid kinase assays were carried out as previously described with a few modifications [17]. Immunoprecipitated complexes were washed an additional time with 10 mM Hepes + 5 mM MgCl₂, and kinase assays were carried out directly on Sepharose beads. To analyse PtdIns 4-kinase activity, reactions were carried out in 20 mM Hepes (pH 7.5) buffer containing 5 mM MgCl₂, 20 μ M ATP (Pharmacia Biotech), 0.3 % Triton X-100, 10–15 μ Ci of [γ -³²P]ATP (DuPont NEN) and 200 μ g/ml sonicated L- α -phosphatidylinositol (Avanti Polar Lipids, Inc., Alabaster, AL, U.S.A.) as a substrate. For some experiments, specific inhibitors of PI 4-kinase or PI 3-kinase were added to reactions. These included 5 μ g of mAb 4C5G [37], 200 μ M adenosine, 50 nM wortmannin, 10 μ M wortmannin or vehicle control. Reactions were carried out for 5–10 min at room temperature (while enzyme activity was still linear with time) and stopped with 2 M HCl prior to extracting lipids with chloroform/methanol (1:1, v/v). The organic layer was resolved by TLC using either a chloroform- or propanol-based solvent system on potassium oxalate-treated silica gel 60 aluminum sheets (EM Science, Darmstadt, Germany). TLC plates were exposed to O-Xar film (Kodak, Rochester, NY, U.S.A.) and/or beta-emitting radioactivity incorporated into [³²P]PtdIns was quantified using a Betascope 603 Blot Analyzer (Betagen Corp., Waltham, MA, U.S.A.) or by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Lipid kinase activity is expressed either as c.p.m. for Betascope analysis, or as pixel intensity for phosphorimager analysis, within a defined area representing the [³²P]PtdIns spot. Specific lipid kinase activity was determined by subtracting c.p.m. measured in control IgG immunoprecipitates (P3 or J2A2 mAb).

Measurement of other lipid kinase activities

Reactions were carried out as above, except that the 0.3 % Triton X-100 detergent was omitted (0.006 % Nonidet P40 used in place) and substrates differed. To measure PI 3-kinase activity exclusively, phosphatidylserine (40 %)/L- α -phosphatidylinositol 4,5-diphosphate (60 %) (Sigma) was used as a substrate. To measure PI 4-P 5-kinase and PI 3-kinase activities at the same time, a combination of phosphatidylserine/L- α -phosphatidylinositol/L- α -phosphatidylinositol 4-monophosphate (Sigma)/L- α -phosphatidylinositol 4,5 diphosphate (2/1/1/1, by vol.) was utilized as a substrate. In these reactions, phosphatidylserine (Avanti Polar Lipids) was used as a carrier to distribute negative charges of the phosphates. Lipid products were resolved by TLC, as above, using a propanol-based solvent system. Standard PtdIns4P, PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ reaction products were generated from an anti-PI 3-kinase immunoprecipitate using crude bovine brain phosphoinositides (Sigma) as a substrate.

Determination of PI 4-kinase subunit size

HT1080-CD9 cells were lysed with 1 % Brij 99, and the indicated TM4SF proteins were immunoprecipitated as described above. Immune complexes were solubilized in 62 mM Tris/HCl, 3 % SDS, 2 mM dithiothreitol, 10 % (v/v) glycerol, and run on a 10 % polyacrylamide gel at 4 °C. The gel was washed in 50 mM

Tris/HCl (pH 7.2), 0.25% Triton X-100, 2 mM dithiothreitol, and lanes were cut into 0.25 cm segments. Approximate molecular mass values were determined by comparison with standards run in a parallel lane. Segments were subsequently homogenized in 10 mM Hepes/5 mM MgCl₂ overnight at 4 °C, and lipid kinase activity was measured as described above.

RESULTS

Specificity of TM4SF protein–PtdIns kinase association

It was previously shown that the TM4SF proteins CD63, CD81 and CD151 form complexes with PI 4-kinase [5,17]. Here we have tested whether other lipid kinases might associate with the TM4SF proteins. To assess PI 3-kinase and PI 4-P 5-kinase activities, reactions were carried out using either a combination of phosphoinositide substrates [PtdIns, PtdIns4P, PtdIns(4,5)P₂] or PtdIns(4,5)P₂ alone. For these experiments, 0.3% Triton X-100 (which inhibits PI 3-kinase) was omitted. As shown in Figure 1, we failed to detect any PI 3-kinase and PI 4-P 5-kinase activities in CD53, CD63, CD81 or CD82 immunoprecipitates from Molt-4 cells. In a positive control experiment, immunoprecipitated PI 3-kinase did yield the expected PtdIns4P, PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ products. From additional cell lines, including A431 carcinoma cells, Jurkat T cells and RD rhabdomyosarcoma cells (results not shown), we again failed to detect PI 3-kinase or PI 4-P 5-kinase activities associated with any TM4SF protein examined (i.e. CD9, CD53, CD63, CD81 or CD82).

We next asked whether TM4SF proteins other than CD63, CD81 and CD151 could associate with PI 4-kinase. To address this issue, we immunoprecipitated multiple TM4SF proteins, and then carried out PI 4-kinase assays (with 0.3% Triton X-100 included). PI 4-kinase activity (measured as ³²P incorporation into PtdIns4P) was clearly present in CD9, CD63 and CD81 immunoprecipitates from CD9-transfected HT1080 cells. Neg-

Figure 1 Analysis of lipid kinase activities co-immunoprecipitating with TM4SF proteins from Molt-4 cells

Molt-4 cells were lysed with 1% Brij 99 and immunoprecipitated with control Ig, or antibodies to TM4SF proteins (CD53, CD63, CD81, CD82), $\alpha 4$ integrin or PtdIns 3-kinase. Immunoprecipitates (I.P.) were assayed for lipid kinase activity using a pool of purified phospholipids, PI, PIP and PIP₂ (left panel) or purified PIP₂ alone (right panel) as substrates, as described in the Materials and methods section. Previously it was shown that comparatively large amounts of CD53, CD63, CD81, CD82 and $\alpha 4$ integrin complexes could be immunoprecipitated from Molt-4 cells [65]. Abbreviations used and in this and subsequent Figures: PI, phosphoinositide; PIP, PtdIns4P; PIP₂, PtdIns(4,5)P₂; PIP₃, PtdIns(3,4,5)P₃.

Figure 2 Analysis of PtdIns-specific kinase activity associated with various TM4SF proteins

HT1080-CD9 fibrosarcoma cells were lysed with 1% Brij 99 and immunoprecipitated with the indicated antibodies. Immunoprecipitates were assayed for lipid kinase activity in the presence of 0.3% Triton X-100 using purified PI as a substrate.

ligible PI 4-kinase activity was detected in immunoprecipitates of the abundantly expressed control proteins, CD98 and integrin $\alpha 2\beta 1$, and in control immunoglobulin immunoprecipitates (Figure 2). Again we saw no evidence for the formation of PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ products in any of the immunoprecipitates.

Quantification of TM4SF-associated PtdIns4P production allowed comparison of results for several different TM4SF proteins, as five different cell lines were used to obtain results for nine different TM4SF proteins (Table 1). To facilitate comparisons among the different TM4SF proteins on different cell lines, each TM4SF-associated kinase activity is presented as the fold increase above the kinase activity non-specifically associated with the very abundant CD98 protein. As indicated, TM4SF proteins could be separated into roughly three groups. The CD63, CD9 and A15/TALLA1 proteins showed relatively high levels of associated PI 4-kinase activity; CD151 and CD81 showed low to moderate levels of activity; and NAG2/TM4SF7, CD37, CD82 and CD53 all showed minimal associated PI 4-kinase activity, with activity levels at or less than negative control levels (Table 1). CD63-associated PI 4-kinase activity was high, despite there being relatively low levels of CD63 on the surface of the cells analysed. Indeed, as discussed below (see Figure 7), much of the CD63-associated PI 4-kinase activity is associated with intracellular CD63. Notably, CD81- and CD151-associated kinase levels were relatively low, considering the high levels of expression of these proteins on the cells analysed. The CD53 and CD82 TM4SF proteins were present at intermediate to high levels on the cells tested. However, NAG2 and CD37 were not highly expressed on the cells analysed (Table 1), and this could at least partially explain the low associated kinase levels.

When the PI 4-kinase activity associated with the same TM4SF protein is compared in different cell types, major variations can occur. For example, a similar amount of CD81, as detected by immunoblotting, yielded approx. 40-fold more associated PI 4-kinase from HT1080 cells compared with OCI-Ly8 cells (Figure 3, compare first and last columns). Even when the levels of immunoprecipitated CD81 from OCI-Ly8 cells were much higher than levels from HT1080, there was still less associated PI 4-kinase activity (Figure 3, middle columns).

TM4SF proteins associate with a type II PI 4-kinase of approx. 55 kDa

At least three different types of PI 4-kinase have been described [19,38]. Here we have utilized various enzyme stimulators and

Table 1 PI 4-kinase activity associated with TM4SF proteins

The indicated TM4SF proteins were immunoprecipitated from different cell lines and assayed for PI 4-kinase activity. MFI represents mean fluorescence intensity for a TM4SF protein on a given cell line. *n*, number of replicate PI 4-kinase assays. Stimulation index is the fold increase in PI 4-kinase activity for a TM4SF protein relative to the activity associated with CD98. CD98 is highly expressed on all five cell lines utilized (MFI = 150–373). Actual values for [³²P]PtdIns production ranged from 5–40 c.p.m. (for unreactive negative control mAb), 20–160 c.p.m. (for anti-CD98 control mAb) and from 600–10 000 c.p.m. for strong positives (CD9, CD63 and A15). Mean SI, mean stimulation index for each TM4SF protein, based on all available cells tested.

TM4SF protein	Cell line	MFI	<i>n</i>	Stimulation index	Mean SI
CD63	HT1080	52	13	164 ± 66	105
	A431	82	3	252 ± 112	
	K562	34	8	68 ± 18	
	OCI-Ly8	7	1	30	
	Molt-4	10	1	9.5	
CD9	HT1080-CD9	178	8	137 ± 43	56
	A431	113	3	29 ± 13	
	OCI-Ly8	55	1	2.5	
A15/TALLA1	Molt-4	77	1	172	172
CD81	HT1080	143	7	20 ± 8	8.7
	K562	46	7	6.5 ± 2.2	
	OCI-Ly8	372	5	5.8 ± 2.4	
	Molt-4	242	1	2.5	
CD151	HT1080	259	6	16 ± 7	9.5
	K562	30	3	3.0 ± 1.0	
CD53	K562	39	6	1.3 ± 0.5	1.3
CD37	OCI-Ly8	11	1	0.3	0.3
CD82	Molt-4	176	1	0.3	0.3
NAG2/TM4SF7	HT1080	18	1	0.2	0.2

Figure 3 Comparison of CD81-associated PtdIns4-K activity from different cell lines

CD81 was immunoprecipitated from various amounts of cell lysate from either OCI-Ly8 (5, 15 and 45×10^6 cells) or HT1080 (5 and 15×10^6 cells). Immunoprecipitates were split and either analysed for PtdIns-specific lipid kinase activity (upper panel), or immunoblotted for CD81 (lower panel). Specific lipid kinase activity was obtained as described in the Materials and methods section. The larger amount of PtdIns4P radioactivity seen in this experiment (compared with Table 1) is likely due to higher specific radioactivity of [³²P]PtdIns product at the time of analysis.

inhibitors to characterize the PI 4-kinase associated with five different TM4SF proteins, analysed in three different cell lines. As indicated in Figure 4, all five activities were stimulated by

0.3 % Triton X-100, and were strongly inhibited by the anti-PI 4-kinase mAb 4C5G or by 200 μ M adenosine. Notably, these PI 4-kinase activities were not inhibited by low or high doses of wortmannin, and thus differ from the PI kinase α and PI kinase β forms of PI 4-kinase [19,39].

We were unable to reliably immunoprecipitate or immunoblot TM4SF-associated PI 4-kinase protein with either the 4C5G mAb or anti-PI kinase α polyclonal antisera described previously [5]. Thus to ascertain the size of the PI 4-kinase associating with TM4SF proteins, CD9 and CD63 immunoprecipitates were subjected to SDS/PAGE, and PI 4-kinase activities were then recovered from gel slices. As indicated in Figures 5(A) and 5(B), peak activities in both cases migrated in the 55–60 kDa size region. No kinase activity was recovered from a control immunoprecipitate (Figure 5C). Together, the results in Figures 4 and 5 indicate that diverse TM4SF proteins associate with a very similar, if not identical type II PI 4-kinase of approx. 55 kDa, that is distinct from PI 4-kinase α (100 and 230 kDa) and PI 4-kinase β (90 kDa) [19].

Evaluation of TM4SF–PI 4-kinase complexes

The TM4SF protein CD63 is present in nearly all cells, often shows more associated PI 4-kinase activity than other TM4SF proteins (e.g. Table 1), and itself associates with other TM4SF proteins [11,25]. Thus we considered that PI 4-kinase might primarily be associated with CD63, and through CD63 might be recruited indirectly into complexes with other TM4SF proteins. To address this issue, we carried out CD63 immunodepletion experiments, and then subsequently assessed PI 4-kinase association with CD9 in A431 cells (Figure 6A) and CD81 in HT1080 cells (Figure 6B). As indicated, immunodepletion of CD63 did not alter levels of CD9- and CD81-associated PI 4-kinase activity. Control experiments indicated that CD63-associated PI 4-kinase activity was almost completely removed upon CD63 immunodepletion, whereas the $\alpha 2 \beta 1$ integrin (VLA-2)

Figure 4 TM4SF proteins associate with a type II PI 4-kinase

The respective TM4SF proteins were immunoprecipitated from either A431 cells (CD9), HT1080-CD9 cells (CD63, CD81 and CD151) or Molt-4 cells (A15/TALLA1) and immunoprecipitates were assayed for lipid kinase activity under the indicated conditions. The data are presented as a percentage of the total activity in the presence of 0.3% Triton X-100. In the presence of Triton X-100 (100% activity), actual c.p.m. values corresponding to the [32 P]PtdIns spot are 2790 (CD9), 10500 (CD63), 87 (CD81), 1060 (CD151) and 3020 (A15/TALLA1).

showed minimal associated activity, regardless of CD63 immunodepletion. From these results, carried out in two different cellular environments, we conclude that CD63–PI 4-kinase complexes are separate from CD9–PI 4-kinase or CD81–PI 4-kinase complexes.

CD63–PI 4-kinase complexes were not only separate within cell lysates, but also showed a distinct subcellular distribution. Immunoprecipitation of total cellular CD9 or CD63 from

Figure 5 Determination of TM4SF-associated PI 4-kinase protein size

CD9 (A), CD63 (B) or control IgG (C) immunoprecipitates from HT1080-CD9 cells, lysed in 1% Brij 99, were subjected to SDS/PAGE. Gel-slice fractions were assayed for PtdIns-specific kinase activity, as described in the Materials and methods section. Protein standards were electrophoresed in a parallel lane and their positions relative to the gel-slice fractions are indicated by the arrows. Data are expressed as arbitrary pixel intensity units corresponding to the [32 P]PtdIns spot, as quantified by PhosphorImager. The data are representative of three separate experiments for CD9 and two separate experiments for CD63.

HT1080-CD9 cells yielded similar levels of associated PI 4-kinase activity (Figure 7, black bars), in agreement with results seen in Figure 2. However, when only the cell-surface subsets of CD9 and CD63 were immunoprecipitated, major differences emerged (Figure 7, grey bars). Whereas the level of PI 4-kinase associated with cell-surface CD9 was very similar to the level associated with total CD9, there was minimal PI 4-kinase activity associated with cell-surface CD63 in two separate experiments. These results indicate that PI 4-kinase associates mostly with CD9 that is at the cell surface, whereas CD63–PI 4-kinase complexes are largely

Figure 6 Effect of CD63 immunodepletion on the PI 4-kinase activity associated with CD9 and CD81

A431 cell (A) or HT1080 cell (B) lysates were extensively immunodepleted using anti-CD63-conjugated Sepharose beads (shaded bars) or unconjugated Sepharose beads (solid bars) prior to immunoprecipitating the indicated proteins. Immunoprecipitates were assayed for specific PI 4-kinase activity and data are presented as radioactivity (c.p.m.) incorporated into the corresponding [³²P]PtdIns spot. A431 cells expressed approx. 180, 128 and 77 mean fluorescence intensity units of $\alpha 2$ integrin, CD9 and CD63 respectively.

Figure 7 PI 4-kinase activities associated with CD9 and CD63 differ in cell distribution

Specific PI 4-kinase activity was measured in immunoprecipitates from the total pool of the indicated proteins present in HT1080-CD9 cell lysates (black bars) and was compared with activity present in immunoprecipitates of only the cell-surface fraction of the same proteins (grey bars). Data are presented as radioactivity (c.p.m.) incorporated into the corresponding [³²P]PtdIns spot from two separate experiments.

intracellular. Furthermore, these results indicate that there is minimal exchange of PI 4-kinase activity between CD63 and CD9 complexes occurring after cell lysis.

Figure 8 Association of the TM4SF proteins CD9 and CD63 with PI 4-kinase is independent of association with PKC μ

HT1080-CD9 cells were lysed in 1% Brij 99 and immunoprecipitated with the respective antibodies. The resultant immune complexes or lysates were resolved by SDS/PAGE, immunoblotted with antibodies to PKC μ (upper panel) or $\beta 1$ integrin (lower panel), and developed by chemiluminescence. Results are representative of two separate experiments.

It was recently shown that PKC μ associates with a type II PI 4-kinase distinct from PI 4-kinase α or PI 4-kinase β [40]. To determine whether PKC μ is present in our TM4SF-PI 4-kinase complexes, we immunoprecipitated CD9 and CD63 from HT1080-CD9 cells, and then looked for PKC μ by Western blotting. As indicated in Figure 8, PKC μ was readily detected from the whole cell lysate, whereas no PKC μ was detected in CD9, CD63 or control immunoprecipitates derived from approx. 25-fold more cell equivalents than used in the first lane. Notably, CD9 and CD63 immunoprecipitations were carried out using conditions (1% Brij 99 lysates), in which associations with $\beta 1$ integrins (Figure 8, bottom panel) and with PI 4-kinase (see Figures 2–7) are readily maintained.

The PtdIns4P lipid and PI 4-kinase enzyme activity may sometimes appear within detergent-insoluble lipid raft complexes [41,42]. Thus we considered that TM4SF-PI 4-kinase complex formation could simply be a non-specific by-product of incomplete detergent solubilization. To address this issue, A431 cell lysate in 1% Brij 99 detergent was fractionated using a standard sucrose gradient centrifugation protocol, designed to separate lipid rafts [41]. Immunoprecipitation analysis revealed that a fraction of CD63 and CD9 (10–30%) was present at the 5/35% sucrose interface, where lipid raft components are typically found. However, the majority of CD63-associated PI 4-kinase (91%) was present in the dense sucrose gradient fractions, near the 35/45% sucrose interface, whereas only 9% was present in the light membrane fractions. Furthermore, in the dense fractions, the level of CD63-associated PI 4-kinase activity (174000 c.p.m.) was approx. 35-fold greater than the level of PI 4-kinase associated with negative control proteins (CD71, $\alpha 2$ integrin) also present in the dense fractions. Thus TM4SF-PI 4-kinase complex formation occurs in detergent-soluble complexes, separate from lipid rafts.

DISCUSSION

TM4SF specificity

Previously we demonstrated that CD63, CD81 and CD151 associated with a 55 kDa type II PI 4-kinase [5,17]. However, concerns about the specificity of this interaction arise from the ability of TM4SF proteins to form networks of associations with each other, integrins, transmembrane Ig superfamily proteins, proteoglycans and with various signalling enzymes (see the Introduction). Here we show that, despite the tendency of TM4SF proteins to have so many partners, interactions with PI 4-kinase are highly specific. It is now clear that at least five different TM4SF proteins (CD63, CD9, A15/TALLA1, CD81 and CD151) associate with apparently the same 55 kDa type II PI 4-kinase. Strongest activity was seen with the TM4SF proteins CD63, A15/TALLA1 and CD9, whereas weaker activity was seen with CD81 and CD151. Interestingly, those five TM4SF proteins fall into two distinct subgroups with respect to primary sequence similarities, as indicated by the dendrogram in Figure 9. Other TM4SF proteins (including CD82 and CD53) failed to associate with PI 4-kinase. Importantly, these are part of a separate subgroup of TM4SF proteins (Figure 9). The TM4SF proteins CD37 and NAG2 are also part of this subgroup, and indeed they also did not associate with PI 4-kinase. However, these latter results will need to be confirmed in cells showing higher CD37 and NAG2 expression levels.

The results summarized in Figure 9 have been obtained using multiple cell types (carcinoma, fibrosarcoma, erythroid and lymphoid) and thus appear to be generally applicable. However, while a high level of specificity among TM4SF proteins is indicated, we caution against over-extrapolating these results. Indeed, we have discovered a degree of cell-type-specific variability in the amount of PI 4-kinase associated with TM4SF proteins. We postulate that the level of PI 4-kinase activity associating with a particular TM4SF protein could be influenced strongly by the presence of other TM4SF proteins that may compete for association, by the amount of PI 4-kinase protein available in the cell or by unknown factors that could regulate PI 4-kinase activity.

Figure 9 PI 4-kinase association correlates with TM4SF protein sequence similarities

Similarity values among TM4SF protein sequences were obtained using the OLD DISTANCES program in the GCG sequence analysis package. These values were then used to generate a TM4SF sequence dendrogram. ND, not done.

Is there a common site within TM4SF proteins that specifies PI 4-kinase association? Thus far we have failed in several attempts to obtain conclusive results using CD9/CD82 chimeras obtained from E. Rubinstein and co-workers [43]. At present, we suspect that PI 4-kinase association may require multiple interdependent TM4SF sites. Association of the epidermal growth factor receptor with PI 4-kinase has been mapped to an epidermal growth factor receptor region containing the amino acid sequence YLRRRHIVRKRTLRLQLQERELVE [44]. However, this sequence is not similar to conserved sequences found within CD9, CD63, CD81, CD151 and A15/TALLA1, suggesting that epidermal growth factor receptor association might occur by a distinct mechanism.

PI 4-kinase specificity

Given the tendency of TM4SF proteins to form so many associations, the issue of PI 4-kinase specificity is also critical. Utilizing conditions designed to allow detection of other PtdIns kinases (e.g. PI 3-kinase or PI-4-P 5-kinase), we failed to observe any other PtdIns activity associated with five different TM4SF proteins, analysed in four different cellular environments (including lymphoid, carcinoma and rhabdomyosarcoma cell types). The PI 4-kinase activity associated with each TM4SF immunoprecipitate was stimulated by Triton X-100, inhibited by adenosine, and inhibited by mAb 4C5G. Also, in the absence of any suitable antibodies, we utilized SDS/PAGE gel-slice analysis to show that the PI 4-kinase associated with both CD9 and CD63 was approx. 55 kDa in size. Together these properties are entirely consistent with TM4SF proteins associating with the 55 kDa type II PI 4-kinase that has been previously characterized [19] but not yet cloned. Other known PI 4-kinases are PI 4-kinase α and PI 4-kinase β . Neither of these enzymes is 55 kDa, and neither is prominent at the plasma membrane [19,38]. Also, both may be inhibited by wortmannin, whereas 55 kDa type II PI 4-kinase is not inhibited by wortmannin.

Elsewhere, PKC μ was found to associate with a PI 4-kinase, with properties consistent with the uncloned 55 kDa type II enzyme. At the same time, PKC μ also associated with a PI-4-P 5-kinase [40]. However, under conditions in which TM4SF–PI 4-kinase association and TM4SF–integrin association were maintained, we did not observe any associated PKC μ . Also, as mentioned above, TM4SF proteins associated with PI 4-kinase in the absence of any obvious PI-4-P 5-kinase activity. Thus PKC μ must associate with PI 4-kinase and PI-4-P 5-kinase in a complex distinct from those involving TM4SF proteins.

How distinct are TM4SF–PI 4-kinase complexes?

Throughout these studies, a major concern has been that non-specific TM4SF protein associations with each other, and with other proteins, might lead to non-specific association with PI 4-kinase. Indeed, it would be especially problematic if TM4SF proteins and PI 4-kinase were present in large, poorly solubilized, lipid raft-type vesicles. However, the high level of TM4SF–PI 4-kinase complexes present in solubilized, non-raft dense fractions of a sucrose gradient helps to alleviate some of this concern. In addition, the highly distinct properties of the various TM4SF proteins (Figure 9) further establish that PI 4-kinase association is highly specific. Finally, we immunodepleted nearly all CD63-associated PI 4-kinase activity, without altering CD9- or CD81-associated PI 4-kinase activity, and we readily isolated cell-surface CD9–PI 4-kinase complexes separate from intra-

cellular CD63–PI 4-kinase complexes. These results further indicate that the high levels of PI 4-kinase activity associated with CD63 do not carry over into other TM4SF complexes, and also they argue strongly against post-lysis exchange of PI 4-kinase among detergent protein micelles.

Further evidence for distinct TM4SF–PI 4-kinase complexes is that CD9–PI 4-kinase association was elevated in a cell (HT1080-CD9) where CD81- and CD151-associated activity was low, and A15/TALLA1 was absent. Conversely, CD81 activity in OCI-Ly8 cells was elevated relative to CD9 activity, while CD151 was absent; and A15/TALLA1 activity was elevated in Molt-4 cells, where CD81 activity was low and CD151 was absent. Finally, we previously demonstrated that $\alpha 3\beta 1$ integrin-associated PI 4-kinase activity was almost entirely due to association with CD151, and not CD81 or CD63 [17]. All of these results strongly suggest that each type of TM4SF–PI 4-kinase complex can be formed independent of other TM4SF proteins.

Nearly all TM4SF proteins yet analysed associate with integrins, with the $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins showing perhaps the strongest association [18,45]. Notably, the K562, OCI-Ly8 and Molt-4 cells analysed here do not express $\alpha 3$ or $\alpha 6$ integrins, yet still show substantial TM4SF–PI 4-kinase association. Whereas the TM4SF protein site for PI 4-kinase association should be on the intracellular side of the molecule, the TM4SF protein site for integrin interactions is in the extracellular large loop [46]. Together these results indicate that TM4SF–PI 4-kinase association is independent of integrins. Interestingly, another molecule (CD98) that associates with integrins [47] consistently showed minimal associated PI 4-kinase activity, despite being very highly expressed in the plasma membrane of the cell lines studied here.

Functional Implications

The TM4SF proteins CD9 [48–50], CD63 [51], CD81 [52,53] and CD151 [17,53] may all contribute to the regulation of cell migration. We hypothesize that these molecules may recruit PI 4-kinase to key sites, where it could then influence cell migration due to effects on phosphoinositide signalling, and cytoskeletal protein interactions. Studies elsewhere have shown that CD9 is present mostly at the cell surface, as seen for example on endothelial cells [12], oocytes [54,55], kidney epithelial cells [56] and trophoblasts [57]. Consistent with this, our CD9–PI 4-kinase complexes were found largely at the cell surface, and thus CD9 may play a role in PI 4-kinase recruitment to that site. In contrast, CD63–PI 4-kinase complexes were largely intracellular, suggesting that CD63 could retain PI 4-kinase within intracellular compartments. In this regard, CD63 is diminished at the cell surface, and instead is present in various intracellular compartments such as B-cell MHC class II-enriched vesicles [10], lysosomes [58], platelet-dense granules [59] and endothelial cell Weibel–Palade bodies [60]. It remains to be determined whether TM4SF proteins may be associated with or responsible for PI 4-kinase activity being present in chromaffin granules [61], synaptosomal vesicles [62], coated vesicles [63] and glucose transporter 4 vesicles [64].

In conclusion, we have found that a 55 kDa type II PI 4-kinase, but not other PtdIns kinases, specifically associates with select members of the TM4SF family in multiple cell types. Two new strongly associating TM4SF proteins have been identified (CD9 and A15/TALLA1), as well as at least two non-associating TM4SF proteins (e.g. CD53 and CD82). Furthermore, largely non-overlapping complexes were observed at the plasma membrane (CD9–PI 4-kinase), and within intracellular compartments (CD63–PI 4-kinase). Together these results indicate that although

TM4SF proteins have a reputation for associating with each other and with many other proteins, complexes formed with PI 4-kinase are highly specific. Thus a subset of TM4SF proteins has the potential to exert a strong influence on phosphoinositide signalling by determining the localized production of PtdIns4P. PtdIns4P may then be available for further processing to the multipotent phosphoinositides PtdIns(4,5) P_2 , PtdIns(3,4) P_2 and PtdIns(3,4,5) P_3 that regulate a host of cellular signalling and cytoskeletal processes.

We acknowledge Eric Rubinstein for helpful comments, and for CD82/CD9 chimaeric molecules. We thank Isao Tachibana for preparation of the TM4SF dendrogram, and Christoph Claas for sucrose gradient analyses. This work is supported by a grant from the National Institutes of Health (GM38903).

REFERENCES

- Wright, M. D. and Tomlinson, M. G. (1994) The ins and outs of the transmembrane 4 superfamily. *Immunol. Today* **15**, 588–594
- Maecker, H. T., Todd, S. C. and Levy, S. (1997) The tetraspanin superfamily: molecular facilitators. *FASEB J.* **11**, 428–442
- Todd, S. C., Doctor, V. S. and Levy, S. (1998) Sequences and expression of six new members of the tetraspanin/TM4SF family. *Biochim. Biophys. Acta* **1399**, 101–104
- Hemler, M. E., Mannion, B. A. and Berditchevski, F. (1996) Association of TM4SF proteins with integrins: relevance to cancer. *Biochim. Biophys. Acta* **1287**, 67–71
- Berditchevski, F., Tolias, K. F., Wong, K., Carpenter, C. L. and Hemler, M. E. (1997) A novel link between integrins, TM4SF proteins (CD63, CD81) and phosphatidylinositol 4-kinase. *J. Biol. Chem.* **272**, 2595–2598
- Sugiura, T. and Berditchevski, F. (1999) Function of $\alpha 3\beta 1$ -tetraspanin protein complexes in tumor cell invasion. Evidence for the role of the complexes in production of matrix metalloproteinase 2 (MMP-2). *J. Cell Biol.* **146**, 1375–1389
- Schwartz-Albiez, R., Dorken, B., Hofmann, W. and Moldenhauer, G. (1988) The B cell-associated CD37 antigen (gp40–52). Structure and subcellular expression of an extensively glycosylated glycoprotein. *J. Immunol.* **140**, 905–914
- Berger, G., Masse, J. M. and Cramer, E. M. (1996) Alpha-granule membrane mirrors the platelet plasma membrane and contains the glycoproteins Ib, IX, and V. *Blood* **87**, 1385–1395
- Cramer, E. M., Berger, G. and Berndt, M. (1994) Platelet alpha-granule and plasma membrane share two new components: CD9 and PECAM-1. *Blood* **84**, 1722–1730
- Escola, J. M., Kleijmeer, M. J., Stoorvogel, W., Griffith, J. M., Yoshie, O. and Geuze, H. J. (1998) Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J. Biol. Chem.* **273**, 20121–20127
- Hammond, C., Denzin, L. K., Pan, M., Griffith, J. M., Geuze, H. J. and Cresswell, P. (1998) The tetraspan protein CD82 is a resident of MHC class II compartments where it associates with HLA-DR, -DM, and -DO molecules. *J. Immunol.* **161**, 3282–3291
- Sincock, P. M., Fitter, S., Parton, R. G., Berndt, M. C., Gamble, J. R. and Ashman, L. K. (1999) PETA-3/CD151, a member of the transmembrane 4 superfamily, is localised to the plasma membrane and endocytic system of endothelial cells, associates with multiple integrins and modulates cell function. *J. Cell Sci.* **112**, 833–844
- Metzelaar, M. J., Wijngaard, P. L. J., Peters, P. J., Sixma, J. J., Nieuwenhuis, H. K. and Clevers, H. C. (1991) CD63 antigen: a novel lysosomal membrane glycoprotein, cloned by a screening procedure for intracellular antigens in eukaryotic cells. *J. Biol. Chem.* **266**, 3239–3245
- Horejsi, V. and Vilek, C. (1991) Novel structurally distinct family of leucocyte surface glycoproteins including CD9, CD37, CD53 and CD63. *FEBS Lett.* **288**, 1–4
- Jones, P. H., Bishop, L. A. and Watt, F. M. (1996) Functional significance of CD9 association with $\beta 1$ integrins in human epidermal keratinocytes. *Cell Adhes. Commun.* **4**, 297–305
- Nichols, T. C., Guthridge, J. M., Karp, D. R., Molina, H., Fletcher, D. R. and Holers, V. M. (1998) Gamma-glutamyl transpeptidase, an ecto-enzyme regulator of intracellular redox potential, is a component of TM4 signal transduction complexes. *Eur. J. Immunol.* **28**, 4123–4129
- Yauch, R. L., Berditchevski, F., Harler, M. B., Reichner, J. and Hemler, M. E. (1998) Highly stoichiometric, stable and specific association of integrin $\alpha 3\beta 1$ with CD151 provides a major link to phosphatidylinositol 4-kinase and may regulate cell migration. *Mol. Biol. Cell* **9**, 2751–2765
- Hemler, M. E. (1998) Integrin-associated proteins. *Curr. Opin. Cell Biol.* **10**, 578–585
- Fruman, D. A., Meyers, R. E. and Cantley, L. C. (1998) Phosphoinositide kinases. *Annu. Rev. Biochem.* **67**, 481–507
- Endemann, G., Dunn, S. N. and Cantley, L. C. (1987) Bovine brain contains two types of phosphatidylinositol kinase. *Biochemistry* **26**, 6845–6852

- 21 Graziani, A., Ling, L. E., Endemann, G., Carpenter, C. L. and Cantley, L. C. (1992) Purification and characterization of human erythrocyte phosphatidylinositol 4-kinase. Phosphatidylinositol 4-kinase and phosphatidylinositol 3-monophosphate 4-kinase are distinct enzymes. *Biochem. J.* **284**, 39–45
- 22 Walker, D. H., Dougherty, N. and Pike, L. J. (1988) Purification and characterization of a phosphatidylinositol kinase from A431 cells. *Biochemistry* **27**, 6504–6511
- 23 Ling, L. E., Schulz, J. T. and Cantley, L. C. (1989) Characterization and purification of membrane-associated phosphatidylinositol-4-phosphate kinase from human red blood cells. *J. Biol. Chem.* **264**, 5080–5088
- 24 Chan, B. M. C. and Hemler, M. E. (1993) Multiple functional forms of the integrin VLA-2 derived from a single α^2 cDNA clone: interconversion of forms induced by an anti- β_1 antibody. *J. Cell Biol.* **120**, 537–543
- 25 Berditchevski, F., Zutter, M. M. and Hemler, M. E. (1996) Characterization of novel complexes on the cell surface between integrins and proteins with 4 transmembranes (TM4 proteins). *Mol. Biol. Cell* **7**, 193–207
- 26 Hadam, M. R. (1989). In *Leukocyte Typing. IV White Cell Differentiation Antigens* (Knapp, W., Dorken, B., Gilks W., R. Rieber, E. P., Schmidt, R. E., Stein, H. and von dem Borne, A. E. G. Kr., eds.), Cluster report: CD53. pp. 674–678, Oxford University Press, New York
- 27 Berditchevski, F., Bazzoni, G. and Hemler, M. E. (1995) Specific association of CD63 with the VLA-3 and VLA-6 integrins. *J. Biol. Chem.* **270**, 17784–17790
- 28 Tachibana, I., Bodorova, J., Berditchevski, F., Zutter, M. M. and Hemler, M. E. (1997) NAG-2, a novel transmembrane-4 superfamily (TM4SF) protein that complexes with integrins and other TM4SF proteins. *J. Biol. Chem.* **272**, 29181–29189
- 29 Fukudome, K., Furuse, M., Imai, T., Nishimura, M., Takagi, S., Hinuma, Y. and Yoshie, O. (1992) Identification of membrane antigen C33 recognized by monoclonal antibodies inhibitory to human T-cell leukemia virus type 1 (HTLV-1)-induced syncytium formation: altered glycosylation of C33 antigen in HTLV-1-positive T cells. *J. Virol.* **66**, 1394–1401
- 30 Takagi, S., Fujikawa, K., Imai, T., Fukuhara, N., Fukudome, K., Minegishi, M., Tsuchiya, S., Konno, T., Hinuma, Y. and Yoshie, O. (1995) Identification of highly specific surface marker of T-cell acute lymphoblastic leukemia and neuroblastoma as a new member of the transmembrane four superfamily. *Int. J. Cancer* **61**, 706–715
- 31 Bergelson, J. M., St. John, N., Kawaguchi, S., Pasqualini, R., Berditchevski, F., Hemler, M. E. and Finberg, R. W. (1994) The I domain is essential for echovirus 1 interaction with VLA-2. *Cell Adhes. Commun.* **2**, 455–464
- 32 Hemler, M. E., Huang, C., Takada, Y., Schwarz, L., Strominger, J. L. and Clabby, M. L. (1987) Characterization of the cell surface heterodimer VLA-4 and related peptides. *J. Biol. Chem.* **262**, 11478–11485
- 33 Hemler, M. E., Sánchez-Madrid, F., Flotte, T. J., Krensky, A. M., Burakoff, S. J., Bhan, A. K., Springer, T. A. and Strominger, J. L. (1984) Glycoproteins of 210,000 and 130,000 m.w. on activated T cells: cell distribution and antigenic relation to components on resting cells and T cell lines. *J. Immunol.* **132**, 3011–3018
- 34 Lemke, H., Hammerling, G. J., Hohmann, C. and Rajewsky, K. (1978) Hybrid cell lines secreting monoclonal antibody specific for major histocompatibility antigens of the mouse. *Nature (London)* **271**, 249–251
- 35 Hemler, M. E. and Strominger, J. L. (1982) Monoclonal antibodies reacting with immunogenic mycoplasma proteins present in human hematopoietic cell lines. *J. Immunol.* **129**, 2734–2738
- 36 Yelton, D. E., Desaymard, C. and Scharff, M. D. (1981) Use of monoclonal anti-mouse immunoglobulin to detect mouse antibodies. *Hybridoma* **1**, 5–11
- 37 Endemann, G. C., Graziani, A. and Cantley, L. C. (1991) A monoclonal antibody distinguishes two types of phosphatidylinositol 4-kinase. *Biochem. J.* **273**, 63–66
- 38 Wong, K., Meyers, R. and Cantley, L. C. (1998) Subcellular locations of phosphatidylinositol 4-kinase isoforms. *J. Biol. Chem.* **272**, 13236–13241
- 39 Balla, T., Downing, G. J., Jaffe, H., Kim, S., Zolyomi, A. and Catt, K. J. (1997) Isolation and molecular cloning of wortmannin-sensitive bovine type III phosphatidylinositol 4-kinases. *J. Biol. Chem.* **272**, 18358–18366
- 40 Nishikawa, K., Toker, A., Wong, K., Marignani, P. A., Johannes, F. J. and Cantley, L. C. (1998) Association of protein kinase C μ with type II phosphatidylinositol 4-kinase and type I phosphatidylinositol-4-phosphate 5-kinase. *J. Biol. Chem.* **273**, 23126–23133
- 41 Waugh, M. G., Lawson, D., Tan, S. K. and Hsuan, J. J. (1998) Phosphatidylinositol 4-phosphate synthesis in immunisolated caveolae-like vesicles and low buoyant density non-caveolar membranes. *J. Biol. Chem.* **273**, 17115–17121
- 42 Pike, L. J. and Miller, J. M. (1998) Cholesterol depletion delocalizes phosphatidylinositol bisphosphate and inhibits hormone-stimulated phosphatidylinositol turnover. *J. Biol. Chem.* **273**, 22298–22304
- 43 Rubinstein, E., Poindessous-Jazat, V., Le Naour, F., Billard, M. and Boucheix, C. (1997) CD9, but not other tetraspans, associates with the β_1 integrin precursor. *Eur. J. Immunol.* **27**, 1919–1927
- 44 Prasad, K. V., Kapeller, R., Janssen, O., Repke, H., Duke-Cohan, J. S., Cantley, L. C. and Rudd, C. E. (1993) Phosphatidylinositol (PI) 3-kinase and PI 4-kinase binding to the CD4-p56lck complex: the p56lck SH3 domain binds to PI 3-kinase but not PI 4-kinase. *Mol. Cell Biol.* **13**, 7708–7717
- 45 Serru, V., Naour, F. L., Billard, M., Azorsa, D. O., Lanza, F., Boucheix, C. and Rubinstein, E. (1999) Selective tetraspan-integrin complexes (CD81/ α 4 β 1, CD151/ α 3 β 1, CD151/ α 6 β 1) under conditions disrupting tetraspan interactions. *Biochem. J.* **340**, 103–111
- 46 Yauch, R. L., Kazarov, A. R., Desai, B., Lee, R. T. and Hemler, M. E. (2000) Direct extracellular contact between integrin α 3 β 1 and TM4SF protein CD151. *J. Biol. Chem.* **275**, 9230–9238
- 47 Zent, R., Fenczik, C. A., Calderwood, D. A., Liu, S., Dellos, M. and Ginsberg, M. H. (2000) Class- and splice variant-specific association of CD98 with integrin beta cytoplasmic domains. *J. Biol. Chem.* **275**, 5059–5064
- 48 Miyake, M., Koyama, M., Seno, M. and Ikegama, S. (1991) Identification of the motility-related protein (MRP-1), recognized by monoclonal antibody M31–15, which inhibits cell motility. *J. Exp. Med.* **174**, 1347–1354
- 49 Anton, E. S., Hadjiargyrou, M., Patterson, P. H. and Matthew, W. D. (1995) CD9 plays a role in Schwann cell migration *in vitro*. *J. Neurosci.* **15**, 584–595
- 50 Shaw, A. R. E., Domanska, A., Mak, A., Gilchrist, A., Dobler, K., Visser, L., Poppema, S., Fliegel, L., Letarte, M. and Willett, B. J. (1995) Ectopic expression of human and feline CD9 in a human B cell line confers β 1 integrin-dependent motility on fibronectin and laminin substrates and enhanced tyrosine phosphorylation. *J. Biol. Chem.* **270**, 24092–24099
- 51 Radford, K. J., Thorne, R. F. and Hersey, P. (1997) Regulation of tumor cell motility and migration by CD63 in a human melanoma cell line. *J. Immunol.* **158**, 3353–3358
- 52 Domanico, S. Z., Pelletier, A. J., Havran, W. L. and Quaranta, V. (1997) Integrin α 6 β 1 induces CD81-dependent cell motility without engaging the extracellular matrix migration substrate. *Mol. Biol. Cell* **8**, 2253–2265
- 53 Yáñez-Mó, M., Alfranca, A., Cabañas, C., Marazuela, M., Tejedoro, R., Ursa, M. A., Ashman, L. K., De Landázuri, M. O. and Sánchez-Madrid, F. (1998) Regulation of endothelial cell motility by complexes of tetraspan molecules CD81/TAPA-1 and CD151/PETA-3 with α 3 β 1 integrin localized at endothelial lateral junctions. *J. Cell Biol.* **141**, 791–804
- 54 Le Naour, F., Rubinstein, E., Jasmin, C., Prenant, M. and Boucheix, C. (2000) Severely reduced female fertility in CD9-deficient mice. *Science* **287**, 319–321
- 55 Miyado, K., Yamada, G., Yamada, S., Hasuwa, H., Nakamura, Y., Ryu, F., Suzuki, K., Kosai, K., Inoue, K. and Ogura, A. et al. (2000) Requirement of CD9 on the egg plasma membrane for fertilization. *Science* **287**, 321–324
- 56 Nakamura, K., Iwamoto, R. and Mekada, E. (1995) Membrane-anchored heparin-binding EGF-like growth factor (HB-EGF) and diphtheria toxin receptor-associated protein (DRAP27)/CD9 form a complex with integrin α 3 β 1 at cell-cell contact sites. *J. Cell Biol.* **129**, 1691–1705
- 57 Hirano, T., Higuchi, T., Ueda, M., Inoue, T., Kataoka, N., Maeda, M., Fujiwara, H. and Fujii, S. (1999) CD9 is expressed in extravillous trophoblasts in association with integrin α 3 and integrin α 5. *Mol. Hum. Reprod.* **5**, 162–167
- 58 Peters, P. J., Borst, J., Oorschot, V., Fukuda, M., Krahenbuhl, O., Tschopp, J., Slot, J. W. and Geuze, H. J. (1991) Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. *J. Exp. Med.* **173**, 1099–1109
- 59 Nishibori, M., Cham, B., McNicol, A., Shalev, A., Jain, N. and Gerrard, J. M. (1993) The protein CD63 is in platelet dense granules, is deficient in a patient with Hermansky-Pudlak syndrome, and appears identical to granulophysin. *J. Clin. Invest.* **91**, 1775–1782
- 60 Vischer, U. M. and Wagner, D. D. (1993) CD63 is a component of Weibel-Palade bodies of human endothelial cells. *Blood* **82**, 1184–1191
- 61 Wiedemann, C., Schafer, T. and Burger, M. M. (1996) Chromaffin granule-associated phosphatidylinositol 4-kinase activity is required for stimulated secretion. *EMBO J.* **15**, 2094–2101
- 62 Wiedemann, C., Schafer, T., Burger, M. M. and Sihra, T. S. (1998) An essential role for a small synaptic vesicle-associated phosphatidylinositol 4-kinase in neurotransmitter release. *J. Neurosci.* **18**, 5594–5602
- 63 Campbell, C. R., Fishman, J. B. and Fine, R. E. (1985) Coated vesicles contain a phosphatidylinositol kinase. *J. Biol. Chem.* **260**, 10948–10951
- 64 Kristiansen, S., Ramlal, T. and Klip, A. (1998) Phosphatidylinositol 4-kinase, but not phosphatidylinositol 3-kinase, is present in GLUT4-containing vesicles isolated from rat skeletal muscle. *Biochem. J.* **335**, 351–356
- 65 Mannion, B. A., Berditchevski, F., Kraeft, S.-K., Chen, L. B. and Hemler, M. E. (1996) TM4SF proteins CD81 (TAPA-1), CD82, CD63 and CD53 specifically associate with α 4 β 1 integrin. *J. Immunol.* **157**, 2039–2047