The Major CD9 and CD81 Molecular Partner

IDENTIFICATION AND CHARACTERIZATION OF THE COMPLEXES*

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By associating with specific partner molecules and with each other, the tetraspanins are thought to assemble multimolecular complexes that may be especially relevant with respect to metastasis. We have previously identified a 135-kDa molecule (CD9P-1) as a major molecular partner of CD9 in cancer cell lines. This molecule was identified, after immunofluorescence purification and mass spectrometry analysis, as the protein encoded by the KIAA1436 gene and the human ortholog of a rat protein known as FPRP. Cross-linking experiments detected a complex of the size of CD9 plus CD9P-1, showing that these glycoproteins directly associate with each other, probably in the absence of any other molecule. The use of chimeric CD9/CD82 molecules revealed the role of the second half of CD9, comprising the large extracellular loop and the fourth transmembrane domain. CD9P-1 was also shown to form separate complexes with CD81 and with an unidentified 175-kDa molecule. It also associated with other tetraspanins under conditions maintaining tetraspanin/tetraspanin interactions. The identification of a protein strongly linked to the tetraspanin web and the production of a specific monoclonal antibody will help to further characterize the role of this “web” under physiological and pathological conditions.

The tetraspanins (also called tetraspans or TM4SF molecules) are molecules with four transmembrane regions found in all cells but erythrocytes. They have been implicated in many cellular functions such as adhesion, migration, co-stimulation, signal transduction, and differentiation (1). These various effects may be explained by the organization by tetraspanins of a network of molecular interactions, the tetraspanin web (previously called the tetraspan web) (2–4). Among the molecules participating in this “web” are a subset of β1 integrins in most cell types, CD4, CD8, major histocompatibility complex molecules, and CD19 in lymphoid cells (1, 5). The effect of tetraspanins on cell function may partly reflect the engagement of associated molecules.

Increasing evidence shows the importance of tetraspanins in physiological and pathological situations. Recently, a relation between mutations of Tall1/TM4SF2 and certain cases of X-linked mental retardation was demonstrated (6). Moreover, the crucial role of CD9 in sperm-egg fusion has been shown (7–9). Importantly, CD9, like CD82, acts as a suppressor of metastasis since its transfection in melanoma cells was found to reduce their metastatic potential (10). An inverse correlation between the expression of CD9 in the primary tumor and the appearance of metastases in melanomas, colon, lung, and breast cancers has been reported (11–14). The expression of CD9 and CD82 is also frequently lower in metastatic cells compared with the primary tumor (12, 15, 16). Another tetraspanin that might play a role in cancer is CD81, which is a possible receptor for hepatitis C virus, a major cause of hepatocellular carcinoma (17).

The tetraspanin web model raises the question of the identification of web-associated molecules participating in the function of CD9. The implication in cell migration and metastasis is often proposed to be a consequence of its association with integrins (5, 18–21). However, recent data suggest that CD9 most likely interacts only indirectly with integrins, possibly through tetraspanin/tetraspanin interactions (4, 22).

Molecules interacting directly with CD9 may be more relevant with respect to CD9 function. We have recently shown that inside the tetraspanin web, specific tetraspanin-partner complexes could be identified based on the resistance of these complexes to digitonin treatment (4), and we reasoned that these primary complexes would actually correspond to direct interactions inside the tetraspanin web. In this previous study, the major molecular partner of CD9 in carcinoma cells was a 135-kDa molecule that we call CD9P-1 (CD9 partner 1). We now report on the production of an anti-CD9P-1 mAb† that was used to purify and identify CD9P-1 and to characterize its association with several tetraspaninas.

EXPERIMENTAL PROCEDURES

Monoclonal Antibodies—The previously described anti-tetraspanin mAbs used in this study were SYB-1 and ALB-6 (CD9) (2, 18), Z81 (CD81; provided by Dr. F. Lanza) (23), and 11B1G4 (CD151; provided by Dr. L. K. Ashman) (21). The new anti-tetraspanin mAbs TS63 (IgG1, CD63), TS81 (IgG2a, CD81), TS82 and TS82b (both IgG1, CD82), and TS151 (IgG1, CD81) is an unrelated anti-CD151 mAb, different from the previously described TS151r mAb (4)) as well as the anti-β1 integrin mAb β1-vjf have been produced in our laboratory. These new mAbs were used in most experiments except for immunoblotting, for which SYB-1, 11B1G4, and Z81 were used. Also, among the anti-CD82 mAbs, TS82 was used for immunoprecipitation and TS82b for immuno-

† The abbreviations used are: mAb, monoclonal antibody; CHO, Chinese hamster ovary; DSP, dithiobis(succinimidyl propionate); MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonic acid; PGF-2α, prostaglandin F-2α.
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noblotted. The anti-CD55 mAb 12A12 has been previously described (24).

Generation of mAbs—BALB/c mice were injected intraperitoneally twice with 106 HeLa cells, and a final boost was performed 3 weeks later with CD9-containing complexes collected from a Brij 97 lysate of 106 HeLa (21) and then one more week. The mAbs were purified from footpad draining lymph nodes. The cell monolayers (a 75-cm² tissue culture flask was used) were incubated with 5 ml of buffer containing 10 mM HEPES (pH 7.3), 150 mM NaCl, 0.2 mM CaCl2, and 0.2 mM MgCl2 immediately before use, yielding final DSP concentrations of 0.5 and 0.1 mM and a final Me2SO concentration of 1%. A reagent was raised to 0.5 mg/ml to increase sensitivity. Briefly, cells biotinylated horseradish peroxidase complex (Amersham Pharmacia Biotech, Arlington Heights, IL) and a FACS/Calibur flow cytometer (Becton Dickinson, San Jose, CA). Positive supernatants were then further characterized by immunoprecipitation. The anti-CD9P-1 mAb 1F11 is of the IgG1 subclass.

Plasmids and Transfection—The two chimeric molecules CD81x9 and CD81x81 have been previously described (25). The KIAA1436 cDNA (26) was obtained from the Kazusa DNA Research Institute (Chiba, Japan), and was subcloned in the pcDNA3 vector (Invitrogen, Groningen, The Netherlands). For expression of the KIAA1436 gene product, CHO cells (5 × 105 cells in 0.4 ml of RPMI 1640 medium) were electroporated at room temperature with 10 μg of cDNA using the Gene Pulser apparatus (Bio-Rad, Ivry, France). The conditions were 200 V and 500 nanofarads. To obtain cells stably expressing CD9P-1, G418 (Life Technologies, Inc., Cergy-Pontoise, France) was added 2 days later at 0.25 mg/ml. After 2 weeks, positive cells were selected by the immunomagnetic bead technique as recommended by the manufacturer (MACS, Miltenyi Biotec, Bergish Gladbach, Germany) using a combination of mAb 1F11 and anti-mouse antibody coupled to submicroscopic magnetic beads.

Cell Labeling and Immunoprecipitation—Surface labeling of cells with EZ-Link Sulfo-NHS-LC-biotin (Pierce) was performed as previously described (2, 4), except that the concentration of the labeling reagent was raised to 0.5 mg/ml to increase sensitivity. Briefly, cells were washed three times in Hank’s buffered saline and incubated in 10 mM HEPES (pH 7.3), 150 mM NaCl, 0.2 mM CaCl2, and 0.2 mM MgCl2 containing 0.5 mg/ml EZ-Link Sulfo-NHS-LC-biotin. After a 30-min incubation at 4 °C, the cells were washed three times in 20 ml Tris (pH 7.4), 157 mM NaCl, 0.2 mM CaCl2, and 0.2 mM MgCl2 to remove free biotin and to inhibit the reactive group. Cells labeled or not were lysed directly in the tissue culture flask (2 ml for a 75-cm² flask) in lysis buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 0.025% NaN3, 0.5% Triton X-100, 0.01% Tween 20, 10 mM EDTA, 150 mM NaCl, and 0.1% Triton X-100; and 5 volumes of 50 mM sodium phosphate buffer (pH 6.5) containing 500 mM NaCl and 0.1% Triton X-100 before elution with 50 mM glycine HCl (pH 2.5), 150 mM NaCl, and 0.1% Triton X-100. The fractions were analyzed by SDS-polyacrylamide gel electrophoresis and SYPRO ruby staining as described by the manufacturer (Molecular Probes, Inc., Eugene, OR).

For identification, the purified protein was concentrated using a Microcon YM-50 (Millipore Corp., Bedford, MA) and separated by SDS-polyacrylamide gel electrophoresis (7.5% gel) under nonreducing conditions. The gels were silver-stained by successive incubations of 0.2% sodium thiosulfate for 2 min and in 0.1% silver nitrate for 40 min. The gel was dried and exposed to X-ray film for 10 min. The autoradiograms were scanned with a computer scanner, and the band intensities were quantitated using the NIH Image program.

Immunofluorescence Staining—mAb to CD55 (mAb 12A12) and anti peptides were extracted twice with 50 μl of 60% acetonitrile and 1% trifluoroacetic acid. Peptides were selected in the mass range of 800–4000 Da. Analyses were performed using a PerSeptive Biosystems matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) Voyager-DE mass spectrometer operated in the delayed extraction mode. Peptide mixtures were analyzed using a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in acetone containing 1% trifluoroacetic acid. Peptides were selected in the mass range of 800–4000 Da. Spectra were calibrated using Calibration Mixture 2 of the Sequazyme peptide mass standards kit (PerSeptive Biosystems). The search program MS-Fit, developed by the University of California at San Francisco, was used for searching the NCBI Protein Database. Typical search parameters were as follows: maximum allowed peptide mass error 800 ppm, consideration of one incomplete cleavage per peptide, and full molecular mass and pH range.

Immunofluorescence Staining—Serial sections (4 μm thick) of frozen samples from a human tissue bank maintained at −80 °C were prepared at −20 °C in a temperature-controlled microscope. They were incubated with mAb to either CD9 or CD9P-1 for 1 h at 4 °C and stained in 0.1% Triton X-100. Following incubation at 37 °C, the sections were washed three times in phosphate-buffered saline and mounted in phosphate-buffered saline/glycerol (3:7) and examined within 4 h with a fluorescence microscope (Olympus BH-2).
cells were transfected with a CD9 cDNA with or without KIAA1436 cDNA, and we examined whether the two molecules could interact in digitonin. As shown in Fig. 2B, CD9 co-immunoprecipitated with the KIAA1436 protein from cells transfected with both cDNAs and reciprocally.

**CD9P-1 Is Both a CD9 and a CD81 Partner—**Due to their association, the patterns of surface proteins that co-immunoprecipitate with the different tetraspanins from Brij 97 or CHAPS extracts are identical in a given cell line (2, 3, 4, 28) (exemplified by CD9 in Fig. 3A). A similar pattern, although with quantitative differences, was observed for CD9P-1 (Fig. 3A), which is concordant with the strong interaction of this molecule with CD9. In digitonin, the tetraspanin/tetraspanin interactions were no longer observed, and accordingly, the patterns of molecules that co-immunoprecipitated with different tetraspanins were distinct (4). Under these conditions, CD9P-1 clearly co-immunoprecipitated with CD9 and a lower fraction of CD81. Several molecules, particularly one at 63 kDa (under nonreducing conditions), present in both the CD9 and CD81 immunoprecipitates were absent from the CD9P-1 immunoprecipitate (Fig. 3A). If these molecules were barely detectable in our previous study, they are now clearly visible because the sensitivity of the experiments has been increased. A 175-kDa molecule (under nonreducing conditions) was present in the CD9P-1 immunoprecipitate, but not in the CD9 or CD81 immunoprecipitates. Molecules comigrating with CD9P-1 were clearly present in the CD9, CD81, and CD151 immunoprecipitates from digitonin lysates (Fig. 3 and our previous study (4)).

To determine the relationship between these molecules and the KIAA1436 protein, cells were surface-labeled with biotin before lysis with digitonin and immunoprecipitation with anti-tetraspanins mAbs. After elution, the co-immunoprecipitated proteins were identified by a second round of immunoprecipitations using mAb 1F11 or an anti-β, mAb (Fig. 3B). CD9P-1 was present in both the CD9 and CD81 immunoprecipitates, but not in the CD151 immunoprecipitate. As previously described (4), the 135-kDa molecule (125 kDa under nonreducing conditions) present in the CD151 immunoprecipitate is the β, integrin subunit, which is not present in the CD9 or CD81 immunoprecipitates.

**CD9P-1 Associates with Multiple Tetraspanins under Conditions Allowing Tetraspanin/Tetraspanin Interactions—**To study the interaction of CD9P-1 with additional tetraspanins and to confirm the preceding data, Western blot analysis of immunoprecipitates was performed (Fig. 4A). After lysis of HeLa cells in Brij 97, a fraction of CD9P-1 was found to associate with CD63, CD82, and CD151 in addition to CD9 and CD81. The association of CD151 with CD9P-1 in Brij 97 extracts was confirmed by two-step immunoprecipitation (Fig. 3C). No tetraspanin other than CD9 and CD81 remained associated with CD9P-1 in digitonin.

**Stability of the CD9-CD9P-1 and CD81-CD9P-1 Complexes—**With the exception of the association of integrin αβ, with CD151, the majority of surface proteins described to associate with tetraspanins are loosely linked to these molecules, and the association can be seen only in mild detergents. In contrast, a fraction of CD9P-1 still remained associated with CD9 or CD81 after lysis in Triton X-100 (Fig. 4B). Surprisingly, although more CD9P-1 associated with CD9 than with CD81 in Brij 97 and digitonin extracts, the CD81/CD9P-1 association was more stable since it was still observed in the presence of Triton X-100 + 0.2% SDS in both A431 (Fig. 4B) and HeLa (data not shown) cells. No CD151/CD9P-1 association could be detected under these conditions, which disrupt tetraspanin/tetraspanin interactions.

**Size of the CD9- and CD9P-1-containing Complexes Deter-
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Analysis was by MALDI-TOF mass spectrometry of the peptide masses, followed by searching the NCBI Protein Database. The matching of peptide masses labeled * or ** was compatible with oxidation of 2 or 1 methionine, respectively.

| m/z submitted | MH* matched | Δ | Residues | Peptide sequence |
|---------------|-------------|---|----------|-----------------|
| 888.6470      | 888.0613    | 659.518 | 400–406  | SYHLLYR         |
| 888.6470      | 889.0572    | −461.389| 918–924  | LMSMEMD*        |
| 923.2821      | 922.9733    | 334.6116| 678–684  | VQDEFIR         |
| 977.4097      | 977.0653    | 352.5191| 206–213  | KEGPEFELR       |
| 1068.7664     | 1068.1351   | 609.7344| 282–291  | ALSDADGGYR      |
| 1083.5254     | 1083.2371   | 266.1538| 240–249  | SVLALTHEGR      |
| 1098.3134     | 1098.3391   | −23.3657| 132–140  | LQREGILLR       |
| 1122.9283     | 1122.2727   | 584.1798| 488–495  | FTGWSYKR        |
| 1167.9454     | 1167.2271   | 615.3742| 538–546  | EKTDTFNP       |
| 1533.8159     | 1532.7212   | 714.2014| 687–699  | MYTQYVSDAGLYR |
| 1549.8725     | 1548.7206   | 743.8055| 687–699  | MYTQYVSDAGLYR* |
| 1667.0918     | 1665.8089   | 770.1550| 351–364  | ADDVRPEVTWSFR  |
| 1746.7735     | 1746.9924   | −125.2936| 556–569  | GNYYCVSANTQR   |
| 1913.5461     | 1912.0325   | 791.6655| 260–276  | YHSDGVRLDTVGSDAYR |
| 1929.5079     | 1928.1222   | 718.6655| 164–184  | CSTPSDTAVQGNYEDTVQVR |
| 2314.2154     | 2314.2154   | 777.7036| 382–399  | DSVHSHPVHSLVHSDAR |
| 2314.2154     | 2314.2154   | −547.3253| 1098.3134| 1098.3391  |
| 2314.2154     | 2314.2154   | 716.1136| 379–399  | LDRSLSVHSSHPHVHSLVHSDAR |

**Fig. 2. CD9P-1 is the product of the KIAA1436 gene.** A, CHO cells were transiently transfected with a CD9 cDNA alone or with a KIAA1436 cDNA and analyzed 48 h later by flow cytometry for the expression of CD9P-1 and CD9. B, transfected CHO cells were lysed with digitonin before immunoprecipitation with the anti-CD9 or anti-CD9P-1 mAb as indicated. The immunoprecipitates were analyzed by Western blot analysis using biotin-labeled anti-CD9P-1 or anti-CD9 mAb.

**TABLE I**
Assignment of peptide masses to the human KIAA1436 sequence

A weak 170-kDa complex was immunoprecipitated by the anti-CD151 mAb and revealed by the anti-CD9 mAb. That only high molecular mass complexes containing both CD9 and CD151 could be detected indicates that the association of these complexes containing CD9 or CD9P-1 were visualized by Western blotting using the anti-CD9 mAb SYB-1 or the anti-CD9P-1 mAb 1F11. After cross-linking, the presence of complexes recognized by the anti-CD9P-1 mAb in the CD9 immunoprecipitate (Fig. 5, upper left panel) and, reciprocally, the presence of complexes recognized by the anti-CD9 mAb in the CD9P-1 immunoprecipitate (Fig. 5, lower middle panel) clearly indicate the existence of direct CD9-CD9P-1 complexes in the cell.

The major complex stained by the anti-CD9 mAb in both the CD9 and CD9P-1 immunoprecipitates, using a low concentration DSP, has an apparent molecular mass of ~150 kDa under nonreducing conditions (Fig. 5, lower left and middle panels). This band is likely to correspond to one molecule of CD9 (~24 kDa) linked to one molecule of CD9P-1 (~125 kDa under nonreducing conditions). It was also detected in the CD9 immunoprecipitates probed by mAb 1F11 (Fig. 5, upper left panel). This band is difficult to observe in the CD9P-1 immunoprecipitates probed by mAb 1F11 because of the proximity of non-cross-linked CD9P-1 (Fig. 5, upper middle panel).

A high molecular mass band (>400 kDa) stained by both the anti-CD9P-1 and anti-CD9 mAbs also appeared with higher concentrations of DSP in both the CD9 and CD9P-1 immunoprecipitates. This indicates that the CD9-CD9P-1 complexes can engage in higher order complexes. An ~300-kDa complex present in the 1F11 immunoprecipitate was not readily immunoprecipitated (Fig. 5, upper left panel) or labeled (Fig. 5, lower middle panel) by the anti-CD9 mAbs. This suggests that CD9 might not be present in this complex or only as a minor component. It probably corresponds to CD9P-1 associated with the 175-kDa molecule that co-immunoprecipitated with CD9P-1 in digitonin (Fig. 3A). Several bands appeared after cross-linking in the CD9 immunoprecipitates probed by the anti-CD9 mAb, but not in the CD9P-1 immunoprecipitates, showing that CD9 directly associates with other molecules. This is concordant with the higher expression of CD9 compared with CD9P-1 (Table II) and the presence in CD9 immunoprecipitates collected from digitonin lysates of molecules not present in the CD9P-1 immunoprecipitates (Fig. 3A).
two molecules principally takes place in multimolecular complexes.

The Second Half of CD9 Is Involved in CD9P-1 Interaction—We then tested whether chimeric CD9/CD82 molecules could associate with CD9P-1 in digitonin. The first construction, CD9x82, consists of the first three transmembrane domains of CD9 joined to the second half of CD82, comprising its large extracellular loop and the fourth transmembrane region. CD82x9 is the reciprocal construction. CD9x82 was recognized by anti-CD82 mAbs, and CD82x9 by anti-CD9 mAbs. The two chimeric molecules were transiently transfected in CHO cells stably expressing CD9P-1, and their ability to associate with CD9P-1 was studied by co-immunoprecipitation. CD9P-1 was shown to associate with CD82x9, but not with CD9x82 (Fig. 6). These data show that the large extracellular domain and/or the fourth transmembrane domain of CD9 is involved in the interaction with CD9P-1.

Cell and Tissue Distribution of CD9P-1 and CD9—The expression of CD9P-1 and CD9 in a variety of cultured

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**Fig. 3. Analysis of tetraspanin/CD9P-1 interactions after surface labeling.** A, biotin-labeled HeLa cells were lysed in Brij 97 or digitonin, and immunoprecipitations with the anti-tetraspanin, anti-CD9P-1, or anti-integrin (Int.) mAbs were performed. The pattern of proteins co-immunoprecipitated with CD9P-1 is similar to that with CD9 (and other tetraspanins) in Brij 97, including molecules comigrating with CD9 and CD81. In digitonin, CD9P-1 was present in both the CD9 and CD81 immunoprecipitates and reciprocally. The black dots indicate bands specifically found in the CD9 and CD81 immunoprecipitates collected from Brij 97 lysates, and the arrowhead indicates the 175-kDa molecule found in the CD9P-1 immunoprecipitate. B, biotin-labeled HeLa cells were lysed in digitonin before immunoprecipitation (IP) of CD9, CD81, and CD151 complexes. The proteins that co-immunoprecipitated were eluted with 1% Triton X-100 or anti-tetraspanin mAbs. Low amounts of CD81 and CD151 in the CD63 immunoprecipitate collected from Brij 97 lysates and low amounts of CD81 in the CD9P-1 immunoprecipitate collected from digitonin extracts were detected and may not be visible. C, the same experiment was carried out, except that lysis was with Brij 97. CD9P-1 was found in all three tetraspanin immunoprecipitates, but not in the control CD55 immunoprecipitate.
cells was analyzed by flow cytometry. All cell lines expressing CD9P-1 also expressed CD9 and, with the exception of HepG2, CD81. The highest expression of CD9P-1 was observed in three colon carcinoma cell lines and in A431, a squamous carcinoma cell line. Normal lung embryonic fibroblastic cells were the only adherent cells tested that did not express CD9P-1. Moreover, only in fibrosarcoma and hepatocyte carcinoma cell lines was the expression of CD9 lower than that of CD9P-1. Few hematopoietic cell types expressed CD9P-1. Besides the pre-B cell line Hoon, the other three positive cell lines (K562, HEL, and DAMI) are of the erythromegakaryocytic lineage, and it will be interesting to determine whether CD9P-1 constitutes a new member for this lineage. We have determined that in both HEL and K562 cells, CD9P-1 had the expected molecular mass and associated with multiple tetraspanins (data not shown).

Tissue distribution analysis was performed to compare further the patterns of expression of CD9P-1 and CD9. It has been reported that CD151 co-distributes with the integrin $\alpha_\beta_2$ in the basal layer of the epidermis, whereas CD9 expression is not restricted to this layer (3, 22, 29). As shown in Fig. 7, CD9P-1 was expressed by keratinocytes, with the labeling being more intense in basal layers than in upper strata. A faint labeling of CD9P-1 was observed in salivary glands at the basal surface of acini and in interglandular spaces, which strongly stained for CD9. No detectable staining could be observed in tonsils, heart, kidneys, colon, bronchi, lungs, thyroid, and liver, in which the anti-CD9 mAb stained epithelial cells, blood vessels, and/or fibroblasts (data not shown). The high level expression of CD9P-1 in certain cancer cell lines, such as those derived from the colon or fibrosarcoma, contrasts with the lack of detection in their normal counterparts, raising the question of the possible up-regulation of this molecule during tumorigenesis.

**DISCUSSION**

The current view of the function of tetraspanins is that they are organizers, facilitators, or adaptors that assemble various molecular complexes on the cell surface and that they can participate in the signaling activity of associated molecules (1, 2, 5). So far, the $\beta_1$ integrins are the major molecules identified in these complexes, and CD151 has been shown to form a direct complex with integrins (24, 30). In contrast, the interaction of CD9 with integrins is most likely indirect (4, 22). The identification of molecules that can interact directly with CD9 would provide important clues to understand its function and to further characterize the tetraspanin web. We demonstrate here that CD9P-1, a 135-kDa molecule previously observed in CD9 and CD81 immunoprecipitates collected from digitonin lysates (4), is such a molecule.

To identify CD9P-1, mAbs were first produced by immunizing mice with HeLa cells, followed by a final boost consisting of CD9-containing complexes. One mAb, 1F11, was shown to recognize a 135-kDa CD9-associated molecule, and it was used to purify the protein. Mass spectrometry analysis indicated that the best match was the product of a recently cloned human gene, KIAA1436 (26), a protein with six putative immunoglobulin domains. The computed molecular mass (97 kDa after removal of the signal sequence) is compatible with the mass of CD9P-1 after removal of N-glycans. The product of KIAA1436 was indeed specifically recognized by mAb 1F11 and associated with CD9 after transfection. We conclude that CD9P-1 is the product of the KIAA1436 gene and the human ortholog of the rat protein FPRP (the PGF-2$\alpha$ receptor regulatory protein) (31). To avoid confusion with other KIAA genes and because there is no evidence that this molecule can regulate prostaglandin receptors in human, we prefer to continue calling this protein CD9P-1.

The CD9/CD9P-1 interaction is likely to be meaningful. Indeed, this interaction is quite stable since it was observed under stringent conditions in the presence of Triton X-100. Moreover, $\sim$70% of CD9P-1 was co-immunoprecipitated with CD9. In the cell lines studied here, a lower fraction of CD9 ($\sim$30%) was co-immunoprecipitated with CD9P-1, and this is consistent with the higher expression of CD9 in these cell lines. In HEL cells, nearly all CD9P-1 molecules were found to be associated with CD9 (data not shown). In CHO cells transfected with both CD9 and KIAA1436 cDNAs, in which similar amounts of the two molecules were expressed, the anti-CD9P-1 mAb co-immunoprecipitated as much CD9 as the anti-CD9 mAb and reciprocally, showing the high stoichiometry of this association. Finally, the CD9/CD9P-1 association exists in living cells, as determined by cross-linking experiments.

The size of the smallest CD9/CD9P-1 complex revealed after chemical cross-linking is 150 kDa. This is the expected size for a CD9/CD9P-1 complex, which shows that this complex probably does not require additional components. Similarly, we have previously shown that the size of the smallest CD151-integrin complex is $\sim$250 kDa (24), which is close to the expected size for a complex containing CD151 and the $\alpha$ and $\beta$ integrin subunits. These two complexes were clearly and specifically detected by immunoprecipitations from digitonin lysates, showing that this approach is the best one to identify direct interactions inside the tetraspanin web. Although we did not directly address this question by cross-linking, the detection of CD81/CD9P-1 complexes in digitonin suggests that CD81 also interacts directly with CD9P-1. Moreover, CD81/CD9P-1 association could be observed after lysis with Triton X-100 supplemented with SDS. Thus, although more CD9P-1 associated with CD9, the CD81/
CD9P-1 interaction was more stable. The association of CD81 with CD9P-1 under conditions in which no CD9/CD81 interactions were detectable (a very low CD9/CD81 interaction in digitonin could be observed in rare experiments or cell lines (Fig. 4)) indicates that CD9 and CD9P-1 are separate complexes.

A high molecular mass band (>400 kDa) stained by both the anti-CD9P-1 and anti-CD9 mAbs also appeared with higher concentrations of DSP in both the CD9 and CD9P-1 immunoprecipitates. This indicates that the CD9 complexes can engage in higher order complexes. This band may correspond to a complex comprising CD9, CD9P-1, and the 225-kDa band observed in both the CD9 and CD9P-1 immunoprecipitates collected from digitonin lysates (Fig. 3A). Alternatively, this band may correspond to the association of the CD9P-1 complex with other tetraspanin partner complexes. Indeed, like the integrins α3β1, α4β1, and α6β1 (2, 3), CD9P-1 was found to associate with all tetraspanins studied after lysing the cells with mild detergents such as Brij 97. The fact that we did not detect this large complex in the CD151 immunoprecipitate after cross-linking does not exclude this hypothesis because these experiments might lack sensitivity. In a recent study, no association of CD9P-1 with tetraspanins other than CD9 and CD81 could be detected (32). Because the same detergent was used, this difference is likely to be due to a higher sensitivity of our experiments, which relied upon the use of a mAb. The interactions of CD9P-1 with the other tetraspanins were observed in Brij 97, but not in digitonin, suggesting that these interactions are indirect, probably through CD9 or CD81. In cross-linking experiments, no CD151-CD9P-1 complex could be detected, further indicating that these two molecules interact only indirectly.

It has been suggested that CD151 might be an obligatory molecular partner of the integrin α3β1 (22). Our data show that although all cell types studied expressing CD9P-1 also expressed CD9 or CD81, CD9 and CD81 are not obligatory partners of CD9P-1. Indeed, in HeLa cells, the anti-CD9 mAb precipitated only 70% of CD9P-1. Moreover, CD9P-1 was found to form a complex with at least one other molecule in the absence of CD9 or CD81. Indeed an 300-kDa complex present in the CD9P-1 immunoprecipitate after cross-linking was not readily immunoprecipitated or labeled by the anti-CD9 mAbs (Fig. 5), showing that, for the most part, CD9 is not present in this complex. Considering its molecular mass, the second component of this complex is probably the 175-kDa molecule that co-immunoprecipitated in digitonin with CD9P-1, but not with CD9 or CD81 (Fig. 3).

Reciprocally, CD9P-1 is not an obligatory partner molecule for CD9. This is implied by the higher expression of CD9 compared with CD9P-1 in most cell lines tested and also by the

### Table II

| Non-hematopoietic cells lines | CD9P-1 | CD9 | CD81 |
|--------------------------------|--------|-----|------|
| Cervical carcinoma, HeLa       | +++    | +++ | +++  |
| Lung carcinoma, A549           | +      | +++ | +    |
| Squamous carcinoma, A431       | +++    | +++ | +++  |
| Colon carcinoma                | LoVo   | +++ | +++  |
| COLO-205                       | +++    | +++ | +    |
| SW48                           | +++    | +++ | +    |
| Breast carcinoma, HBL-100      | ++     | +++ | +++  |
| Hepatocyte carcinoma           | ++     | +   | -    |
| Neuroblastoma, SK-N-SH         | +      | ++  | +    |
| Endothelial cell, ECV304       | +      | +++ | +++  |
| Fetal kidney, 293              | +      | +++ | +++  |
| Fibrosarcoma                   | HT1080 | +   | ++   |
| SHAC                           | +      | +++ | +++  |
| Fetal lung fibroblast          | ICIG-7 | -   | +++  |
| IMR-90                         | -      | +   | +++  |

| Hematopoietic cell lines       | CD9P-1 | CD9 | CD81 |
|--------------------------------|--------|-----|------|
| Myeloid cell                   | U937   | -   | ++   |
| KG1                            |        | +   | +    |
| K562                           |        | ++  | ++   |
| HEL                            |        | +   | +++  |
| DAMI                           |        | +   | +++  |
| Lymphoid cell                  |        | +   | +++  |
| Pre-B cell                     |        | +   | +++  |
| NALM-6                         |        | +++ | +++  |
| Hoon                           |        | ++  | +++  |
| KM3                            |        | ++  | +++  |
| Burkitt (lymphoid B)           | Daudi  | -   | +    |
| Raji                           | -      | +   | +++  |
| BJAB                            |        | +   | +++  |
| LGLC (lymphoid B),             |        | +++ | +++  |
| OCI-LY8                        |        | +++ | +++  |
| T cell                         | Jurkat | +   | +++  |
| CEM                            |        | +   | +++  |

CD9P-1 expression was studied by indirect immunofluorescence and flow cytometry using mAbs 1F11, ALB-6, and TS81, respectively. The staining by mAb TS81 is usually lower compared with other CD81 mAbs. The data indicate mean fluorescence intensity compared with the control staining based on the following scale: -, <6; +, >6, ≤25, ++, >25, ≤100, ++++, >100, ≤400, ++++, >400.
existence of cell lines expressing CD9 but not CD9P-1. We have also shown, by co-immunoprecipitations and cross-linking, that CD9 belongs to other complexes not containing CD9P-1. In particular, a 63-kDa molecule (under nonreducing conditions) was present in CD9 and CD81 immunoprecipitates (but not in CD9P-1 immunoprecipitates) collected from digitonin lysates, suggesting that this molecule could be another CD9 and CD81 partner. Other less intense bands were also detected in the CD9 immunoprecipitates in both HeLa (Fig. 3) and A431 (data not shown) cells. They may correspond to molecules forming complexes with CD9 at a low stoichiometry or, alternatively, to molecules that are poorly biotinylated. The detection by cross-linking of many CD9-containing complexes is in favor of this second hypothesis. Altogether, these data suggest that CD9 has several molecular partners, in contrast to CD151, which seems to associate directly only with the α2β1 and α6β1 integrins. CD9P-1 is the protein coded by the KIAA1436 gene (26). This protein is predicted to be a member of the Ig superfamily with six Ig domains. Molecules of this family play a role in cell/cell adhesion or communication, and some are also receptors for soluble proteins ligands such as Ig and cytokines (33). Therefore, the functional effects of CD9P-1 may be related in part to its association with CD9P-1. CD9P-1 is the human ortholog of FPRP, which was first identified as a bovine corpora luteal membrane glycoprotein that coeluted upon multiple chromatographic procedures with bound tritiated PGF-2α (34). The rat molecule was later found not to be a PGF-2α receptor, but to reduce the number of PGF-2α-binding sites on COS cells transfected with the PGF-2α receptor (35). The availability of a mAb directed to the human molecule will allow the determination of whether the same effect is observed in humans and to characterize the mechanism involved. It will be of interest to determine whether this effect occurs in the context of the tetraspanin web.

We and others have recently produced CD9-deficient mice (7–9). The major phenotype of these mice is a female infertility related to a defect in sperm-egg fusion. The profound effect on sperm-egg fusion contrasted with the absence of effect in other tissues strongly expressing this molecule. It has been proposed that in these tissues, the lack of CD9 could be functionally compensated by another tetraspanin. The association of both CD9 and CD81 with common molecular partners (CD9P-1 and possibly the unidentified 63-kDa (under nonreducing conditions) molecule) provides a molecular basis for such compensation. Also, it would be interesting to know whether CD9P-1 is expressed on oocytes.

In conclusion, we have identified a major molecular partner of CD9 and CD81. The identification of this molecule and the availability of a mAb will help to resolve the function of these molecules and also the role of CD9 in metastasis. Further work will also have to determine whether this molecule plays a role in hepatitis C virus infection.

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