EWI-2 is a new component of the tetraspanin web in hepatocytes and lymphoid cells

Stéphanie CHARRIN*, François LE NAOUR*, Valérie LABAS†, Martine BILLARD*, Jean-Pierre LE CAER†, Jean-François EMILE*, Marie-Anne PETIT‡, Claude BOUCHEIX* and Eric RUBINSTEIN*¹

*INSERM U268, Institut André Lwoff, Hôpital Paul Brousse, 14 Av Paul Vaillant Couturier, Villejuif 94807 Cedex, France, †ESPCI, 10 rue Vauquelin, 75005 Paris, France, and ‡INSERM U271, 69424 Lyon Cedex 03, France

Several tetraspanins bind directly to a few molecular partners to form primary complexes, which might assemble through tetraspanin–tetraspanin interactions to form a network of molecular interactions, the tetraspanin web. We have produced a monoclonal antibody directed to a 63 kDa molecule (determined under non-reducing conditions) associated with CD9. This molecule was first identified by MS as a molecule with four Ig domains, EWI-2. Like the related molecule CD9P-1, EWI-2 was found to be a partner not only for CD9, but also for CD81, a tetraspanin required for hepatic infection by the parasite responsible for malaria, and also a putative hepatitis C virus receptor. Using chimaeric CD9/CD82 molecules, two separate regions of CD9 of 40 and 47 amino acids were demonstrated to confer

the ability to interact with EWI-2. Both EWI-2 and CD9P-1 were detected in the human liver at the surface of hepatocytes and were found to associate with CD81 on freshly isolated hepatocytes. EWI-2 also co-localized with CD81 in the liver. CD9P-1 was not detected on most peripheral blood cells, whereas EWI-2 was expressed on the majority of B-, T- and natural killer cells and was not detected on monocytes, polynuclear cells or platelets. This distribution is identical to that of CD81. Finally, EWI-2 associated with all tetraspanins studied after lysis under conditions preserving tetraspanin–tetraspanin interactions, showing that EWI-2 is a new component of the tetraspanin web.

Key words: CD9, CD81, tetraspanin, tetraspanin web.

INTRODUCTION

Tetraspanins are a family of widely expressed cell-surface proteins, having sequence similarities, especially in the four transmembrane (TM) domains, and a ‘tetraspanin fold’ in the largest of the two extracellular loops. They have been implicated in many cellular functions such as adhesion, migration, co-stimulation, signal transduction and differentiation [1–3]. The importance of these molecules is supported by genetic evidence. For example, mutations in human Talla-1/TM4SF2 are associated with mental retardation [4] and CD9-null mouse oocytes have a fusion defect [5,6]. Targeted deletion of CD81 in mice has shown its role in the immune response to antigens that preferentially stimulate a T-helper 2 response [7]. Several tetraspanins seem to play a role in other pathological processes. Thus CD9 and CD82 can act as metastasis suppressors (for review, see [1]). Additionally, it was demonstrated recently that the tetraspanin CD81 is required for the hepatic development of the malaria parasite *Plasmodium* [8]. Also, it is now well established that the hepatitis C virus (HCV) E2 envelope glycoprotein binds human CD81 [9,10]. HCV infection develops into liver disease and is also accompanied frequently by various autoimmune extrahepatic manifestations, of which mixed cryoglobulinaemia, a B-lymphocyte proliferative disorder, is the most common [11]. Interaction of E2 with CD81 may act directly on immune cells. Thus ligation of CD81 by a truncated soluble form of E2 induced aggregation of B-lymphoid cells and altered their proliferation [10]. Other studies have shown that ligation of CD81

by monoclonal antibody (mAb) or by E2 co-stimulates T-cells [12] and blocks natural killer (NK) cell activation [13,14], which could be an efficient HCV evasion strategy to limit the antiviral activities of NK cells and contribute to the development of a persistent infection.

Even if tetraspanins are associated with a host of cellular activities, their specific molecular functions are not yet understood. There is no identity with well-defined structural modules or motifs in their cytoplasmic domains (with the exception of a tyrosine-based internalization motif found at the C-termini of several tetraspanins) and no soluble or membrane protein has yet been shown to be a physiological ligand for these molecules except murine CD9, which was identified recently as a pregnancy-specific glycoprotein 17 receptor [15]. Rather, the various effects of tetraspanins might be explained by the organization by these molecules of a network of molecular interactions, the tetraspanin web [1–3]. In a current model, it is proposed that each tetraspanin binds directly to a few molecular partners to form primary complexes, and that they might assemble through tetraspanin–tetraspanin interactions [1,2] by a mechanism involving, in part, palmitoylation of these proteins [16–18]. The primary complexes can be visualized by co-immunoprecipitation after lysis in detergents that do not allow the observation of tetraspanin–tetraspanin interactions, such as digitonin (or in a few cases Triton X-100) [19,20], whereas higher-order complexes are observed using detergents like CHAPS, Brij98 or Brij97 that maintain tetraspanin–tetraspanin interactions [21–24]. The primary complexes (tetraspanin–partner), CD151–integrin $\alpha 3 \beta 1$,

Abbreviations used: mAb, monoclonal antibody; IgSF, immunoglobulin superfamily; TM, transmembrane; LEL, large extracellular loop; PGF2 α , prostaglandin F2 α ; HCV, hepatitis C virus; MALDI-TOF, matrix-assisted laser-desorption ionization-time-of-flight; DSP, dithiobis(succinimidyl propionate); NK cells, natural killer cells; CHO cells, Chinese hamster ovary cells; PE, phycoerythrin.

¹ To whom correspondence should be addressed (e-mail erubin@vjf.inserm.fr).

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CD151–integrin $\alpha 6\beta 1$, CD81–integrin $\alpha 4\beta 1$ and CD81–CD19, were the first ones to be identified [19,25–27].

In several examples, the tetraspanins and their associated molecules have been shown to have overlapping functions. For instance CD81, like CD19, lowers the threshold for B-cell activation through the B-cell receptor [25], and ligation of CD81 and other tetraspanins at the surface of lymphoid B-cell lines induces the phosphorylation of CD19 on tyrosine residues [26]. Additionally, it has been established clearly that tetraspanins modulate cell migration, a process in which integrins play a crucial role (for reviews, see [1,3]). Recently, it was demonstrated that the tetraspanin CD151 can modulate the role played by integrins in the formation of cord-like structures by fibroblastic and endothelial cells [28–30]. Thus the identification of specific partners appears to be a key step to better understanding of the role of tetraspanins in physiological and pathological conditions.

Recently, MS analysis of CD81-containing complexes led to the identification of two related immunoglobulin superfamily (IgSF) proteins [31–33]. The first of these proteins, CD9P-1 (also called FPRP or EWI-F), had formerly been identified in rat as a molecule reducing the number of prostaglandin F 2α (PGF 2α)-binding sites on PGF 2α -receptor-transfected COS cells [34]. The production of a mAb to CD9P-1 allowed the demonstration that it is linked to the tetraspanin web, being a molecular partner for both CD9 and CD81 [35]. Because of the lack of specific mAb, only a partial characterization of the interaction of the second molecule, EWI-2 (also called PGRL in the mouse), with tetraspanins could be performed [32,33]. We now report the production of a mAb directed against EWI-2, which was used to demonstrate that this molecule is linked to the tetraspanin web and is like CD9P-1, i.e. a partner for both CD81 and CD9. This molecule is expressed on *Plasmodium* and/or HCV target cells such as hepatocytes and lymphoid cells.

EXPERIMENTAL

mAbs

Anti-tetraspanin mAbs used in this study were SYB-1, ALB-6, 10B1 (CD9), Z81 [36] and TS81 (CD81), TS53 (CD53), TS82 (CD82) and TS151 (CD151) [35]. Other mAbs were 1F11 (CD9P-1) [35], v5-vj6 (integrin $\alpha 5$), 12A12 (CD55) and ALB-2 (CD10) [37]. For generation of 8A12 mAb, BALB/c mice were injected intraperitoneally twice with 10^7 HeLa cells and a final boost was performed 3 weeks later with CD9-containing complexes collected from a Brij97 lysate of $\approx 10^9$ HeLa cells. Spleen cells were fused with P3x63AG8 mouse myeloma cells (5×10^7 and 3×10^7 cells respectively) according to standard techniques and distributed into 96-well tissue-culture plates. After 2 weeks, hybridoma culture supernatants were harvested and tested for staining of HeLa cells by indirect immunofluorescence, using a microplate fluorescence reader (Cytofluor II; PerSeptive Biosystems, Framingham, MA, U.S.A.) and a FACScalibur flow cytometer (Becton-Dickinson, San Jose, CA, U.S.A.). Positive supernatants were then further characterized by immunoprecipitation. The anti-EWI-2 mAb 8A12 is of the IgG2a subclass.

Cells, cell culture and transfection

The lymphoid B-cell lines Raji, Daudi and NALM-6, as well as CHO (Chinese hamster ovary) cells, were cultured as described in [22,35]. The hepatoma cell line Mahlavu was cultured in Dulbecco's modified Eagle's medium supplemented with 10 %

fetal calf serum, 2 mM glutamine and antibiotics (all from Invitrogen, Cergy-Pontoise, France). CHO cells were electroporated as described previously [35]. CD9-transfected Daudi cells have been described previously [26].

Human hepatocytes were prepared from surgical biopsy specimens obtained during hepatic surgery. The study was approved by the Institutional Ethical Committee. Cells were isolated by a two-step collagenase (Boehringer Mannheim) perfusion procedure [38]. Freshly isolated hepatocytes were seeded at a density of 10^6 cells/ml and cultivated as described previously [39]. The William's E medium was supplemented with 10 % fetal calf serum, 5 g/l bovine insulin (Sigma, St Louis, MO, U.S.A.), 100 units/ml penicillin and 100 μ g/ml streptomycin (Invitrogen); 24 h later this 'seeding' medium was replaced with a fetal calf serum-free 'maintenance' medium supplemented with antibiotics and insulin as described above, plus 3.5×10^{-7} M hydrocortisone hemisuccinate. Hepatocytes do not grow in this medium, and therefore cultures were not passaged during the culture period (4–6 days after seeding).

Plasmids and construction of chimaeric tetraspanins

The Bx19c11 cDNA in pCMVSPORT6 was obtained from Dr G. Wistow (National Institutes of Health, Bethesda, MD, U.S.A.). To increase the level of expression of B $\times$ 19c11 product after transfection, most of the 3'-untranslated region was removed by digestion with *SalI* and *XhoI* and subsequent ligation. The chimaeric molecules CD82 $\times$ 9 and CD9 $\times$ 82 have been described previously [35].

The molecules CD82LEL9 and CD82CCG9 were constructed according to the PCR-based method described by Chen and Przybyla [40]. For constructing CD82LEL9, a first PCR was performed on the CD82/pSVSPORT1 plasmid (antisense direction) between a pSVSPORT1-specific oligonucleotide located immediately ahead of the multiple cloning site (5'sport, 5'-CGC-CTGCAGGTACCGGTCCGG-3') and oligonucleotide OX9D [5'-(CD9)-CGACAATAAATTTTCAC-ATCATC-CTCGGCGTG-GGCGT-(CD82)-3']; where the transition between CD9 and CD82 is indicated by •, the sequence between the two • being common to both CD9 and CD82] which allow the amplification of the sequence coding the last TM region of CD82 with flanked CD9 sequences. The resulting PCR product was purified and used as an oligonucleotide, together with a T7 primer (5'-AATACGACTCACTATAGGG-3'), in a second PCR on CD82 $\times$ 9/pCDNA3 (sense direction), creating a second swap at the beginning of the fourth TM domain [(CD9)-DNKFF-II-LGV-(CD82)]. There was a Val²³⁴-to-Met mutation in the fourth TM region that was not changed because it was in a CD82 region. To construct the CD82CCG9 chimaeric molecule, the sequence encoding CD9 downstream of the CCG conserved motif was first amplified using primers T7 and OX8D [5'-(CD82)-CAGG-CTCAGGTGAAG-TGCTGT-GGTTTGGCTGGGG-(CD9)-3'] on CD9/pSVSPORT1 (sense direction). The latter primer is flanked by CD82 sequences. The resulting PCR product was used as an oligonucleotide, together with the 5'sport primer, in a second PCR on CD82/pSVSPORT1 (sense direction), creating a swap at the level of the CCG conserved motif present in the large extracellular loop (LEL) of all tetraspanins [(CD82)-AQVK-CCG-LAGG-(CD9)].

CD82x(67)9 and CD82x(34)9 chimaeras were made using a PCR-ligation technique [41]. Briefly, the 5' part of CD82 was amplified from a CD82 cDNA in pSVSPORT using 5'sport and specific CD82 reverse primers. The remaining 3' part of CD9 was amplified from a CD9 cDNA in pSVSPORT1, using

T7 and specific CD9 direct primers. The PCR fragments were purified and phosphorylated for 30 min at 37 °C with 10 units of polynucleotide kinase (New England Biolabs, Beverly, MA, U.S.A.), in the buffer provided by the supplier supplemented with 1 mM ATP. The fragments were then ligated in the same buffer with 2.5 units of T4 DNA ligase for 30 min at room temperature. The fusion gene was finally amplified using T7 and sport1 primers. The primers used for CD82x(67)9 were 5'-GGCGCCCTCATGATGCTGGT-3' (CD9, direct) and 5'-CACGCCGATGAAGACATAGG-3' (CD82, reverse). For CD82x(34)9, 5'-TGA CTCCGATTCGACTCTCA-3' (CD9) and 5'-CACCCGAAGCCAGGATCA-3' were used.

Immuno-isolation of CD9-containing complexes and MS analysis

For identification of CD9-associated molecules, 8×10^8 cells were lysed in 12 ml of lysis buffer containing 10 mM Tris/HCl, 150 mM NaCl and 1 % Brij98, in the presence of protease inhibitors. Insoluble material was removed by centrifugation at 12 000 g for 30 min, and the lysates were precleared five times successively with Sepharose 4B beads (Amersham Biosciences, Saclay, France) coupled to BSA and twice with beads coupled to an isotype-matched mAb. Isolation of CD9-containing complexes was performed using beads coupled to mAb ALB-6. The beads were washed five times with lysis buffer and the proteins were eluted with 0.1 M glycine, pH 2.5, concentrated on Microcon YM3 (Millipore, Bedford, MA, U.S.A.) and then separated by SDS/PAGE (5–15 % gels) under non-reducing conditions. The gels were silver-stained as described previously [42]. Briefly, after fixing in 50 % methanol with 5 % acetic acid, the gels were incubated in 0.02 % sodium thiosulphate for 2 min and in 0.1 % silver nitrate for 40 min. The coloration was apparent following incubation of the gels in 0.014 % formaldehyde and 2 % sodium carbonate and then stopped by addition of 5 % acetic acid. The protein of interest was excised and destained for 5 min in 15 mM potassium ferricyanide and 50 mM sodium thiosulphate as described [43]. After three washes with water, the gel pieces were incubated in 100 % acetonitrile for 10 min and dried. The gel pieces were incubated in 100 mM ammonium bicarbonate containing 10 mM dithiothreitol for 30 min at 56 °C. After cooling to room temperature, the dithiothreitol solution was replaced with 55 mM iodoacetamide in 100 mM ammonium bicarbonate for 20 min at room temperature in the dark. The gel pieces were washed in 100 mM ammonium bicarbonate for 10 min, dehydrated in 100 % acetonitrile and dried in a vacuum centrifuge. The gel pieces were swollen in a digestion buffer containing 50 mM ammonium bicarbonate and 100 ng of trypsin (Promega, Madison, WI, U.S.A.). Following enzymic digestion overnight at 37 °C, the resultant peptides were extracted with 50 μ l of 5 % formic acid for 15 min at 37 °C followed by addition of 100 μ l of 100 % acetonitrile for another 15 min at 37 °C. After removal of acetonitrile by centrifugation in a vacuum centrifuge followed by rehydration in 1 % formic acid, the peptides were concentrated by using C₁₈ pipette tips (Millipore).

MS analyses were performed using a Voyager-DE-STR matrix-assisted laser-desorption ionization-time-of-flight (MALDI-TOF) mass spectrometer (PerSeptive Biosystems), operated in the delayed extraction mode. Peptide mixtures were analysed using a saturated solution of 2,5-dihydroxybenzoic acid (Aldrich, St Quentin, France) in 10 % acetonitrile containing 1 % trifluoroacetic acid (Sigma). Peptides were selected in the mass range 600–4000 Da. The search program MS-Fit, developed by the University of California at San Francisco (<http://prospector.ucsf.edu>), was used for searches in the NCBI database. Typical

search parameters were as follows: maximum allowed peptide mass error of 150 p.p.m., consideration of two incomplete cleavages per peptide, molecular mass $\pm$ 50 % and full pH range.

Cell labelling and immunoprecipitation

Surface labelling of cells with EZ-link-Sulpho-NHS-LC-biotin (Pierce, Rockford, IL, U.S.A.) was performed as described previously [35]. 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-Sulpho-NHS-LC-biotin. After 30 min of incubation at 4 °C, the cells were washed three times in 20 mM Tris, pH 7.4, 137 mM NaCl, 0.2 mM CaCl₂ and 0.2 mM MgCl₂ to remove free biotin and to inhibit the reactive group. Cells, labelled or not, were lysed in lysis buffer (1 % Brij97; Sigma) or digitonin (high purity; Calbiochem, San Diego, CA, U.S.A.) with 10 mM Tris, pH 7.4, 150 mM NaCl, 0.02 % NaN₃ and protease inhibitors. When Brij97 was used, the buffer was supplemented with either 1 mM CaCl₂ plus 1 mM MgCl₂ or 1 mM EDTA as indicated. Digitonin was first dissolved in methanol at 10 % (w/v) and then diluted 10 times in lysis buffer. Haematopoietic cells were lysed at the concentration of 2×10^7 /ml while adherent cells were lysed directly in the flask. After 30 min at 4 °C the insoluble material was removed by centrifugation at 12 000 g and the cell lysate was precleared for 2 h by addition of 1:1000 vol. heat-inactivated goat serum and 20 μ l of Protein G-Sepharose beads (Amersham Biosciences, Rainham, Essex, U.K.). Proteins were then immunoprecipitated by adding 1 μ l of ascitic fluid and 10 μ l of Protein G-Sepharose beads to 200–400 μ l of lysate. After 2 h incubation at 4 °C under constant agitation, the beads were washed five times in lysis buffer. For reprecipitation, the molecules co-precipitated with tetraspanins were eluted in lysis buffer supplemented with 1 % Triton X-100 and 0.2 % SDS and identified by a second immunoprecipitation using specific antibodies. The immunoprecipitates were separated by SDS/PAGE (5–15 % gel) under non-reducing conditions and transferred to a PVDF membrane (Amersham Biosciences). Western blotting on immunoprecipitates was performed using biotinylated mAbs and a streptavidin-biotinylated horseradish peroxidase complex (Amersham Biosciences), which was revealed by enhanced chemiluminescence (NEN, Boston, MA, U.S.A.). For identification of biotin-labelled surface proteins, only the latter step was performed.

Chemical cross-linking

Cells were lysed in the presence of 1 % Brij97 in a buffer containing 10 mM Hepes, pH 7.3, 150 mM NaCl, 1 mM EDTA and protease inhibitors. After removal of insoluble material, half was treated with 0.2 mM dithiobis(succinimidyl propionate) (DSP) for 30 min at 4 °C, and half with DMSO as a control. The labelling was then quenched with 10 mM Tris, pH 7.4, for 15 min at 4 °C and Triton X-100 was then added to both DSP-treated and untreated samples to reach a final concentration of 1 % (v/v). After 30 min at 4 °C, the samples were precleared and immunoprecipitated as above.

Immunofluorescence staining and confocal microscopy of frozen sections

Serial sections (4 μ m thick) of frozen human liver were fixed for 20 min in acetone at -20 °C. After drying at room temperature,

they were incubated for 10 min in PBS containing 10 % heat-inactivated goat serum and then with mAb (10 μ g/ml) in the same buffer for 20 min at room temperature in a moist chamber, washed in PBS containing 0.2 % BSA and further incubated for 20 min with FITC-labelled goat anti-mouse antibody diluted 1:50 in PBS/BSA. After three washes the sections were mounted in Mowiol and examined with a Leica DMR fluorescence microscope. For confocal microscopy, the sections were incubated with 10 μ g/ml Alexa 594-labelled anti-CD81 mAb Z81 and mAb 8A12 to EWI-2 or mAb ALB-2 to CD10, which were revealed with a FITC-labelled goat anti-IgG2a antibody diluted 1:50 (Caltag, Burlingame, CA, U.S.A.). Analysis was performed with a TCS SP2 confocal microscope (Leica, Wetzlar, Germany).

Flow-cytometric analysis

Blood samples were incubated with 4 vol. of erythrocyte lysis solution (155 mM NH_4Cl , 10 mM sodium bicarbonate and 0.1 mM EDTA) until a clear suspension resulted. Then the cells were pelleted, washed in PBS containing 0.2 % BSA and 1 mM EDTA and resuspended at the initial concentration in the same buffer supplemented with 10 % heat-inactivated goat serum to reduce non-specific binding. Cells (50 μ l of cell suspension) were stained at 4 °C for 30 min with biotin-labelled mAb 8A12, 1F11 or appropriate isotype-matched control mAbs, in combination with CD19-FITC or CD3-FITC + CD56-PE (all fluorochrome-conjugated mAbs, where PE is phycoerythrin; Beckman-Coulter, Marseille, France). Biotinylated mAbs were revealed by streptavidin conjugated to allophycocyanin (BD Biosciences, San Jose, CA, U.S.A.). Multiparameter analysis of cell-surface staining was performed using a four-color FACScalibur (BD Biosciences). For analysis of transfected CHO cells or hepatocytes of primary cultures, cells were detached using a non-enzymic solution, washed and stained with saturating concentrations of primary mAb. After washing three times with PBS containing 0.2 % BSA, they were incubated with 10 μ g/ml FITC-labelled goat anti-mouse antibody. After washing, cells were fixed with 1 % formaldehyde in PBS. All incubations were performed for 30 min at 4 °C.

RESULTS

Identification of EWI-2 in CD9-containing complexes

In a search for tetraspanin partners, we found that CD9 and CD81 regularly co-immunoprecipitated a 63 kDa molecule (determined under non-reducing conditions) under conditions disrupting tetraspanin–tetraspanin interactions [19,35]. To identify this protein, CD9 immunoprecipitates collected from Brij98 extracts of wild-type and CD9-transfected Daudi cells were compared after silver staining (Figure 1). This permitted the distinction of the proteins specifically co-immunoprecipitated with CD9 from non-specific bands, that cannot be eliminated with the use of 'mild' detergents required to preserve the tetraspanin web. This approach to identify tetraspanin-associated molecules was chosen because ectopically expressed CD9 gives a pattern of co-immunoprecipitation identical to that of endogenous CD81 and other tetraspanins, reflecting its incorporation into the tetraspanin web [22].

The molecular mass of a major band specifically detected in the CD9 immunoprecipitate collected from Daudi/CD9 cells was consistent with that of the 63 kDa molecule. The band was cut out from the gel and digested with trypsin. The resulting peptides were analysed by MALDI-TOF MS and the spectrum

Figure 1 A 63 kDa molecule is the major band detected by silver staining in CD9 immunoprecipitates of Daudi/CD9 cells

CD9-associated proteins collected from Daudi/CD9 cells were fractionated by SDS/PAGE and visualized by silver staining. To discriminate specifically immunoprecipitated proteins from non-specific bands, the same procedure was applied to parental CD9-negative Daudi cells. The proteins that are specifically immunoprecipitated are indicated on the right. The identity of HLA-DR and CD9 was determined by MALDI-TOF MS. CD9 co-migrated with a contaminant on this particular gel.

was used for protein identification in the NCBI database using the MS-FIT search program. Eleven peptides were consistent with those derived from an unknown partial protein encoded by the clone IMAGE 3687782, covering 25 % of this putative protein. Related sequences were retrieved from Unigene (cluster Hs.332012) or through a BLAST search of the human EST database, and a contig was generated. The clone Bx19c11 [44] was sequenced completely. This molecule is a TM molecule of 613 residues. The sequence starts with a 28-amino-acid signal peptide, followed by a 552-residue extracellular domain containing four Ig domains. The TM domain precedes a short 11-amino-acid highly charged cytoplasmic domain. A BLAST search revealed that it is most similar to three other proteins, CD101, IgSF3 and CD9P-1/FPRP, a molecule recently described as a major CD9 and CD81 molecular partner [31,35]. As noted by others, these four molecules may represent an immunoglobulin protein sub-family. The coding sequence of the Bx19c11 cDNA appeared to be identical to that of EWI-2 and the orthologue of the PGRL protein, identified in mice [32,33].

Production of a mAb directed to EWI-2

We have previously reported the production of a mAb to CD9P-1, the major 135 kDa molecular partner of CD9 and CD81, by the use of CD9-containing complexes for immunizing mice. A mAb (8A12) that immunoprecipitates a molecule co-migrating with the 63 kDa molecule (determined under non-reducing conditions) present in the CD9 immunoprecipitate was generated in the same way (results not shown). Several experiments were conducted to confirm that the 8A12 mAb is directed against the product of Bx19c11/EWI-2 cDNA. First, 8A12 stained CHO cells transfected with the Bx19c11/EWI-2 cDNA, but not mock-transfected cells, as determined by flow cytometry (Figure 2A). Secondly, by Western blotting, it recognized a 63 kDa protein in extracts of CHO cells transfected with Bx19c11/EWI-2 cDNA (Figure 2B, left-hand panel) but not with a control plasmid.

Figure 2 Characterization of mAb 8A12

(A) CHO cells were transiently transfected with the EWI-2/Bx19c11 cDNA or mock-transfected and analysed 24 h later for the binding of mAb 8A12 by indirect immunofluorescence and flow cytometry. (B) Transfected CHO cells were lysed in the presence of 1% Triton X-100 and 0.2% SDS and a fraction was subjected to immunoprecipitation (IP) with mAb 8A12 or a control mAb. Extracts (left-hand panel) and immunoprecipitates (right-hand panel) were analysed by Western blot using 8A12 mAb, conjugated or not with biotin. (C) CHO cells transiently transfected with an EWI-2 cDNA or a control plasmid, together with a CD81 or a CD9 cDNA, were surface labelled with biotin, lysed with digitonin and immunoprecipitations with antibodies against tetraspanins CD81 or CD9 and EWI-2 were performed. The additional band appearing in the CD9 and CD81 immunoprecipitates when EWI-2 is expressed is indicated by *. These experiments were carried out twice with identical results.

Thirdly, following lysis of transfected cells in stringent conditions, using Triton X-100 (Figure 2B, right-hand panel), the same 63 kDa protein was immunoprecipitated by mAb 8A12 and recognized by this mAb by immunoblotting. Several additional controls were run to check for the specificity of mAb 8A12. First, we determined that this mAb did not stain CHO cells expressing CD9P-1 and did not bind to Jurkat cells expressing CD101 at a higher level than wild-type cells, excluding cross-reactivity with these two closely related molecules. Moreover, EWI-2 was the only biotin-labelled molecule immunoprecipitated by mAb 8A12 from Triton X-100/SDS lysates of HeLa and A431 cells (results not shown). From these data, we conclude that the mAb 8A12 specifically recognizes EWI-2. This is the first mAb directed to this molecule.

EWI-2 is a molecular partner for both CD9 and CD81 in transfected CHO cells

Immunoprecipitations from digitonin lysates provide a way to identify tetraspanin molecular partners [19,35]. CD9 or CD81 co-immunoprecipitated EWI-2 from digitonin lysates of transfected CHO cells, and reciprocally (Figure 2C). Surprisingly, two bands appeared in the CD9 and CD81 immunoprecipitates after transfection of EWI-2. The molecular mass of the higher band is $\approx 63/78$ kDa (determined under non-reducing and reducing conditions, respectively). The lower band (≈ 47 kDa; determined under non-reducing conditions) may be an unrelated molecule whose expression or association with CD9/CD81 is induced by EWI-2 expression, or may correspond to a cleavage product of EWI-2, not recognized by mAb 8A12, that remains associated with CD9 or CD81. In favour of this latter hypothesis, cleavage products of FLAG-tagged EWI-2 have been described [32].

The interaction of EWI-2 with CD9 or CD81 is direct

Cross-linking experiments were performed to determine whether CD9 and CD81 interact directly with EWI-2. EWI-2 was expressed in CHO cells in combination with CD9, CD81 or CD82 as a control, and cells were lysed with Brij97. A fraction of each sample was treated with the cross-linking reagent DSP before disruption of non-stabilized interactions with Triton X-100. Immunoprecipitations with anti-CD9, -CD81, -CD82 and -EWI-2 mAbs were carried out and immunoprecipitates were run under non-reducing conditions. The complexes containing CD9, CD81 or CD82 were visualized by Western blot using appropriate biotin-labelled mAb (Figure 3). After cross-linking, an ≈ 87 kDa band recognized by the CD9 mAb appeared in the EWI-2 immunoprecipitate collected from cells transfected with both CD9 and EWI-2. This is the expected size for a CD9-EWI-2 complex, which shows that these molecules interact directly. Reciprocally, the same band appeared in the CD9 immunoprecipitate, indicating that CD9 associates with other molecules in CHO cells. Similarly, a band present in the EWI-2 immunoprecipitate collected from cells transfected with both CD81 and EWI-2 was stained with the CD81 mAb and had the size expected for a complex containing only EWI-2 and CD81. As a control, no band recognized by the CD82 mAb could be observed in the EWI-2 immunoprecipitate collected from cells transfected with both EWI-2 and CD82. Altogether, these data show that CD9 and CD81 associate directly with EWI-2.

Multiple regions of CD9 contribute to the interaction with EWI-2

Because CD82 did not associate with EWI-2 after lysis in digitonin (Figure 4), we tested the ability of chimaeric CD9/CD82 molecules to associate with EWI-2 in transfected CHO cells, after lysis with digitonin. All chimaeras were expressed on the cell surface as determined by flow-cytometric analysis of transfected cells (results not shown). Regions of CD9 were substituted progressively by the corresponding sequences of CD82. Replacing the N-terminal short intracellular domain of CD9 and the first TM region by that of CD82 [CD82x(34)9] did not affect the association with EWI-2. In addition, a chimaeric molecule in which the first extracellular loop and ≈ 10 residues of the second TM region of CD9 were further replaced by the corresponding part of CD82 [CD82x(67)9] retained the full ability to interact with EWI-2. In contrast, the chimaeric molecule consisting of the first half of CD82 joined to the LEL (from Val¹¹⁷),

Figure 3 Covalent cross-linking of EWI-2 to CD9 and CD81

CHO cells transiently expressing EWI-2 together with CD9 (A), CD81 (B) or CD82 (C) were lysed in the presence of Brij97 before treatment (+) or not (–) with the cross-linking reagent DSP and dissociation of interactions not stabilized by DSP with Triton X-100. The different molecules were immunoprecipitated (IP) as indicated and the immunoprecipitates were analysed by Western blot using biotinylated CD9 (SYB-1), CD81 (Z81) and CD82 (TS82b) mAbs as indicated. The ≈ 87 kDa band appearing after cross-linking in the CD9, CD81 and EWI-2 immunoprecipitates is indicated (arrow). These experiments were carried out twice with identical results.

fourth TM region and C-terminal cytoplasmic end of CD9 (CD82 $\times$ 9) still interacted with EWI-2, but this interaction was greatly decreased. The fourth TM region of CD9 did not play a role in this residual interaction since a chimaeric molecule in which the LEL of CD82 was replaced by that of CD9 (CD82LEL9) interacted with EWI-2 to the same extent as CD82 $\times$ 9. Several lines of evidence indicate that CD82 $\times$ 9 does not have an altered conformation. (i) It was expressed on the cell surface at the same level as CD9, as determined by flow cytometry using three different mAbs. In particular, this chimaera is recognized by mAb ALB-6 and SYB-1, which are conformation-dependent. (ii) CD82 $\times$ 9 is fully competent for interacting with CD9P-1, another CD9 partner [35], and with other tetraspanins (results not shown).

The first half of the LEL of tetraspanins (up to the CCG) appears to be conserved in size and secondary structure, while the second half is more variable [1,45]. We have engineered a chimaeric molecule (CD82CCG9) in which a CD82/CD9 sequence switch was made at the level of the CCG conserved

motif. This molecule was not recognized by mAbs TS82 and TS82b directed to CD82, or by a large panel of established (ALB-6, SYB-1, DULL-1, CLB-thromb, FMC56, FMC8, J2, J30, J9, ML13 and SJ-9A4) and new anti-CD9 mAbs (a total of 14 CD9 mAbs obtained in our laboratory). This indicates that the epitopes recognized by these mAb are either non-linear or span the CCG sequence. Only one mAb, 10B1, could recognize CD82CCG9 expressed in CHO cells (results not shown). This mAb was able to co-immunoprecipitate EWI-2 from cells transfected with either CD9 or any of the chimaeric CD82/CD9 molecules tested, including CD82CCG9. The level of EWI-2 co-immunoprecipitation with CD82CCG9 was similar to that with CD82 $\times$ 9, indicating that the first half of the LEL of CD9 does not contribute to the specificity of the interaction with EWI-2.

The above data indicated that the CD9 LEL (and in particular the second half of the LEL) was sufficient to allow the interaction with EWI-2, and that sequences before Val¹¹⁷ were necessary for achieving a maximal level of interaction. To determine whether this region could interact with EWI-2 independently from the LEL of CD9, we tested the ability of a chimaeric molecule consisting of the first half of CD9 (up to Glu¹¹⁶) joined to the LEL and fourth TM region of CD82 (CD9 $\times$ 82) to interact with EWI-2. As shown in Figure 4, CD9 $\times$ 82 was able to co-immunoprecipitate EWI-2, although with reduced efficiency compared with CD9.

Altogether, these data indicate that two different regions of CD9 contribute independently to the interaction with EWI-2, and that the combination of both is necessary for achieving a maximal level of interaction. The first region is located between Leu⁶⁹ and Glu¹¹⁶ in a portion comprising ≈ 14 amino acids of the second TM domain, a small cytoplasmic sequence, the third TM domain and approximately six residues of the CD9 LEL. The second site is located in the LEL, after the CCG conserved motif.

One of the regions of CD9 involved in the interaction with EWI-2, and delimited by the use of CD82x(67)9 and CD82 $\times$ 9, comprises three cysteines that attach palmitate moieties covalently. This led us to test whether a mutant CD9 that lacks all potential palmitoylation sites [16] could interact with EWI-2. The mutant interacted with EWI-2 with the same efficiency as CD9, excluding a role for CD9 palmitoylation in this interaction (results not shown).

Expression of EWI-2 and CD9P-1 on peripheral blood cells

The availability of a mAb recognizing EWI-2 provided a unique opportunity to study the interaction of endogenous EWI-2 with endogenous tetraspanins. We first examined whether EWI-2 was expressed in peripheral blood cells. Indeed, the HCV is thought to functionally affect lymphoid cells including T-, B- and NK cells [10,12–14]. Expression of EWI-2 and CD9P-1 on peripheral blood cells was determined by multi-colour flow cytometry. EWI-2 was detected on the majority of B-, T- and NK cells as determined by the labelling of CD19⁺, CD3⁺ and CD56⁺/CD3[–] cells, respectively (Figure 5). No staining of monocytes, granulocytes or platelets could be observed (results not shown). This distribution is identical to that of CD81 [46], but different from that of CD9 (expressed on basophils, eosinophils, platelets and possibly monocytes [37]). CD9P-1 was clearly not expressed by T-cells, NK cells or granulocytes. Surprisingly, whereas its expression was detected on most megakaryocytic cell lines [35], it was absent from platelets (results not shown). A weak staining for CD9P-1 was observed on monocytes (results not shown) and lymphoid B-cells (Figure 5).

Figure 4 Association of CD9/CD82 chimaeric molecules with EWI-2

A schematic representation of different CD82/CD9 chimaeric molecules is shown on the left of each panel, with indication of the junctions (denoted by *). For CD82LEL9 only the second junction is indicated, the first one being the same as for CD82 × 9. In several cases the swap was performed at conserved residues; these residues are delimited by two dots, with common sequence between the dots. CHO cells were transiently transfected with EWI-2 and CD9, CD82, or various CD82/CD9 chimaeric molecules. At 24 h after transfection, the cells were lysed with digitonin before immunoprecipitation with mAbs against CD9 (SYB-1 and 10B1), CD82 (TS82) or EWI-2 (8A12) as indicated at the top of each lane, and Western-blot analysis using biotin-labelled mAbs (SYB-1, TS82b, 8A12) and peroxidase-labelled streptavidin. mAb 10B1 is the only anti-CD9 mAb so far tested that is able to recognize the CD82CCG9 chimaera. This chimaera was not recognized in Western-blotting experiments. All chimaeric molecules were tested at least twice for their association with EWI-2 with identical outcomes.

In addition, using indirect immunofluorescence, weak expression of EWI-2 was detected on lymphoid T- and B-cell lines such as Jurkat (T-cell line), NALM-6, KM3, Reh-6, RS4.11 (early B-cell lines) as well as Raji and Daudi (Burkitt lymphoid B-cell lines; results not shown).

In lymphoid B-cells, EWI-2 is a partner for CD9 and CD81, but not for other tetraspanins

Among the lymphoid cells expressing EWI-2, we focused on lymphoid B-cell lines because these cells express both CD9 and CD81 during early steps of differentiation while mature B-cells only express CD81 [26]. Moreover, these cells express a large panel of tetraspanins. As described previously, after lysis with digitonin the tetraspanins precipitated different sets of associated proteins [19]. In the CD9-negative lymphoid cell line Raji,

CD151 co-immunoprecipitated the integrin $\alpha 6 \beta 1$ while CD81 co-immunoprecipitated the integrin $\alpha 4 \beta 1$ and a molecule of molecular mass ≈ 50 kDa [19]. Both CD81 and CD53 co-immunoprecipitated proteins of just about the size of EWI-2. To assess the presence of EWI-2 in CD81 and/or CD53 immunoprecipitates, the proteins co-immunoprecipitated with the different tetraspanins were eluted and subjected to a second immunoprecipitation with mAb 8A12. This approach was used because it is more sensitive than Western blotting using biotin-labelled mAb. EWI-2 was only detected in the CD81 immunoprecipitate, showing that the molecule co-immunoprecipitated with CD53 is another unidentified molecule (Figure 6A).

In the CD9-positive early B-cell line NALM-6, EWI-2 was clearly present in both CD9 and CD81 immunoprecipitates collected from digitonin lysates, but absent from CD53 (Figure 6B). Thus, during lymphoid B-cell differentiation, EWI-2 is first associated with both CD9 and CD81, and afterwards only with

Figure 5 Expression of EWI-2 and CD9P-1 on peripheral blood lymphocytes

Peripheral blood cells were stained with biotin-labelled mAbs 8A12 and 1F11 directed to EWI-2 and CD9P-1 respectively, in combination with CD19-FITC or CD3-FITC + CD56-PE. The biotin-labelled mAbs were revealed using allophycocyanin-conjugated streptavidin. A region drawn to select lymphocytes was used for gating. The quads were set up on the isotype-matched controls dot plot. For CD56 labelling, CD3-positive cells were excluded. Results representative of four experiments are shown.

CD81. It will have to be determined whether this changes its activity.

In these experiments, EWI-2 co-immunoprecipitated molecules co-migrating with CD9 (in NALM-6 cells) and CD81 (in both Raji and NALM-6 cells), but at low levels, indicating that in these cells only a small fraction of CD9 and/or CD81 can be co-immunoprecipitated by mAb 8A12. This is most probably related to the much lower level of EWI-2 expression as compared with CD81 or CD9, so that only a fraction of these tetraspanins associates with EWI-2 directly.

EWI-2 associates with multiple tetraspanins under conditions allowing tetraspanin–tetraspanin interactions

In a previous study by Stipp et al. [32], using cells transfected with FLAG-tagged EWI-2, no association of EWI-2 with integrins or tetraspanins other than CD9 or CD81 could be detected, raising the hypothesis that CD9 and CD81 might form complexes with EWI-2 separated from the tetraspanin web. As shown in Figure 7(A), a fraction of EWI-2 was found to associate not only with CD81, but also with CD53, CD82 and CD151, when Raji cells were lysed in a lysis buffer (Brij97 and bivalent cations), preserving tetraspanin–tetraspanin interactions. Therefore EWI-2 is a component of the tetraspanin web.

Figure 6 EWI-2 is a CD9 and CD81 partner in lymphoid B-cells

Raji (A) or NALM-6 (B) cells were biotin-labelled, lysed in digitonin, and immunoprecipitations (IP1) were performed with anti-tetraspanin mAbs, the anti-EWI-2 mAb 8A12 or a CD55 mAb as a control, as indicated on the top of each lane. Proteins were separated by SDS/PAGE, transferred to a PVDF membrane and revealed using peroxidase-conjugated streptavidin. Tetraspanins and known associated molecules are indicated on the left. CD82 is not visible because of a poor labelling with biotin. In the lower panels (IP2), the co-immunoprecipitated proteins were eluted with 1% Triton X-100 and 0.2% SDS and subjected to a second immunoprecipitation with the anti-EWI-2 mAb. EWI-2 was present only in the CD9 and CD81 immunoprecipitates after lysis in the presence of digitonin. Bands co-migrating with CD81 (Raji) and both CD9 and CD81 (NALM-6) were detected in the EWI-2 immunoprecipitate but may not be visible on the figure. These experiments were carried out twice with identical results.

A new lysis buffer to detect primary complexes

The differences between the study of Stipp et al. [32] and ours could reflect the use of an epitope-tagged molecule in their study, the use of different cell lines or, alternatively, different immunoprecipitation conditions. This led us to look for other lysis conditions able to preserve the interaction of EWI-2 with CD9/CD81 while disrupting the interaction of EWI-2 with other tetraspanins. We found recently that lysis with Brij97 in the

Figure 7 Analysis of EWI-2-tetraspanin interactions after cell lysis with Brij97

Raji cells were biotin-labelled, and lysed in the presence of Brij97 and either bivalent cations (**A**) or EDTA (**B**). Immunoprecipitations (IP1) were performed with anti-tetraspanin mAbs, the EWI-2 mAb 8A12 or a CD55 mAb as a control, as indicated on the top of each lane. Proteins were separated by SDS/PAGE, transferred to a PVDF membrane and revealed using peroxidase-conjugated streptavidin. Tetraspanins and known associated molecules are indicated on the left. In the lower panels (IP2), the co-immunoprecipitated proteins were eluted with 1% Triton X-100 and 0.2% SDS and subjected to a second immunoprecipitation with the anti-EWI-2 mAb. EWI-2 was present in all tetraspanin immunoprecipitates when cells were lysed in a buffer containing Brij97 and bivalent cations, but only in the CD81 immunoprecipitate when cells were lysed with a buffer containing Brij97 and EDTA. In (**A**), the EWI-2 lane had a longer exposure. In (**B**) CD151 and CD82 are not visible because of a poor labelling with biotin. These experiments were carried out twice with identical results.

presence of EDTA dissociated tetraspanin-tetraspanin interactions from CD9-negative cells [16]. Figure 7(B) shows that, under this condition, EWI-2 could be co-immunoprecipitated with CD81, but not with other tetraspanins. Moreover, this condition

allows the visualization of other primary complexes also observed after lysis with digitonin. This is the case in particular for the CD151-integrin $\alpha 6 \beta 1$ complex or the complex consisting of CD53 linked to the unknown ≈ 68 kDa molecule. Interestingly, this buffer revealed two new primary complexes: the first one includes CD81 and a ≈ 200 kDa molecule while the other one consists of CD53 linked to a high-molecular-mass doublet protein (> 250 kDa). Finally, the only primary complex detected in digitonin but not in Brij97/EDTA was CD81- $\alpha 4 \beta 1$ integrin. This suggests that the CD81- $\alpha 4 \beta 1$ association might require bivalent cations, which is consistent with a previous report showing that mutants of integrin $\alpha 4$ within a putative bivalent cation binding site were deficient in their ability to associate with CD81 [47].

EWI-2 and CD9P-1 are molecular partners of CD81 in primary hepatocytes

To gain further information about the potential relevance of CD81-EWI-2 or -CD9P-1 complexes, we determined whether these molecules were expressed on hepatocytes. We first examined their distribution in human liver sections. Both EWI-2 and CD9P-1 stained hepatocytes with a pre-eminent staining on the surface bordering the sinusoids. A similar but brighter staining was observed for CD81, for which the staining of lateral membranes was more evident. In contrast, the expression of CD9 was not polarized (Figure 8A). Because the stainings with EWI-2 and CD9P-1 mAbs were relatively low and restricted mainly to the sinusoidal membranes, several controls were run to ensure the specificity of the signal. Thus no staining at the sinusoid surface could be observed with CD53 (IgG1, like the CD9P-1 mAb) and CD10 (IgG2a, like the EWI-2 mAb) mAbs. Only isolated cells were stained by the CD53 mAb. They are most probably Kupffer cells, since CD53 stains all leucocytes including macrophages in tissues [48]. As described previously, CD10 was expressed only by bile canaliculi [49].

To confirm further that EWI-2 and CD9P-1 are expressed by hepatocytes, we isolated hepatocytes and determined their phenotype by flow cytometry. As shown in Figure 8(B), these cells expressed low levels of EWI-2 and CD9P-1, and higher levels of CD81 and CD9. The tetraspanins CD63, CD82 and CD151 were also expressed in primary cultures of hepatocytes while CD53 was not detected (results not shown).

The distributions of CD81 and EWI-2 in the liver were compared by confocal microscopy (Figure 9). There was a substantial co-localization of these two molecules. Of note, EWI-2 was detected clearly on the lateral surfaces of hepatocytes, although at a lower level as compared with the sinusoidal surface. As a control, there was no co-localization of CD81 with CD10.

To determine whether EWI-2 and CD9P-1 are molecular partners of CD81 in hepatocytes, the cells were surface-labelled with biotin before lysis and immunoprecipitation. These experiments were performed after lysis in digitonin to detect the molecules associated directly with CD81. The major molecules co-immunoprecipitated with CD81 under these conditions co-migrated with EWI-2 and CD9P-1 (Figure 10). To confirm their identity, the proteins co-immunoprecipitated with CD81 were eluted and identified by a second immunoprecipitation. As shown in Figure 10, EWI-2 and CD9P-1, but not the integrin $\alpha 5 \beta 1$, were present in the CD81 immunoprecipitate. In other experiments, we demonstrated that CD81 co-immunoprecipitated many more proteins from Brij97 extracts of primary hepatocytes (results not shown). These additional interactions are likely to result from secondary interactions arising when milder detergent conditions are used.

Figure 8 EWI-2 and CD9P-1 are expressed in the liver on the hepatocyte cell surface

(A) Sections of human liver were stained by EWI-2, CD9P-1, CD9, CD81, CD53 and CD10 mAbs. Scale bar, 20 μ m. (B) The expression of EWI-2, CD9P-1, CD9 and CD81 was studied in primary cultures of hepatocytes by indirect immunofluorescence and flow cytometry using mAbs 8A12, 1F11, SYB-1 and Z81 respectively.

DISCUSSION

A major feature of the tetraspanins is their ability to associate with other surface molecules, many of which are so far unknown, and it is conceivable that their function is tightly linked to these associated proteins, especially to those they interact with directly, their molecular partners. In this study, we have produced a mAb to a molecule identified as a new IgSF protein with four Ig domains, called EWI-2. This molecule is a newly identified molecule of lymphocytes and hepatocytes, where it associates directly with CD81, a tetraspanin essential for the development of *Plasmodium* parasites in the liver and also a putative HCV receptor.

Figure 9 Co-localization of CD81 and EWI-2 in the liver

Cryostat sections of human liver were processed for double immunofluorescence confocal microscopy using mAbs Z81 against CD81 and 8A12 against EWI-2 or ALB-2 against CD10 as a control. Composite images were generated by superimposition of the green and red signals, with areas of overlap appearing as yellow. Scale bar, 20 μ m.

The production of a mAb directed to EWI-2 allowed the characterization of the association of endogenous EWI-2 with tetraspanins. EWI-2 was demonstrated to interact with all tetraspanins studied, provided that conditions maintaining tetraspanin–tetraspanin interactions are used in the co-immunoprecipitation experiments. This is a common property of all tetraspanin-associated molecules studied so far, such as the CD9 and CD81 partner CD9P-1, the CD81 partners CD19 and integrin $\alpha 4 \beta 1$, or the integrins $\alpha 3 \beta 1$ and $\alpha 6 \beta 1$, which are partners of CD151 [19,26,27,31,35]. We used two different sets of conditions to demonstrate that EWI-2 is a molecular partner of CD9 and a CD81 i.e. a molecule associated with these tetraspanins independently of other tetraspanins. The first of these conditions is lysis in the presence of digitonin. All complexes of tetraspanins with other molecules observed under these conditions could be confirmed (when tested) by covalent cross-linking showing a direct association ([20,31,32,35]; the present study). In addition to digitonin, we found a second

Figure 10 EWI-2 and CD9P-1 are associated with CD81 at the surface of human hepatocytes

Biotin-labelled hepatocytes were lysed in digitonin and immunoprecipitations with CD81, CD9P-1, EWI-2 or integrin $\alpha 5$ mAbs were performed (IP1). Proteins were separated by SDS/PAGE, transferred to a PVDF membrane and revealed using peroxidase-conjugated streptavidin. In the lower panel, the proteins co-immunoprecipitated with CD81 were eluted and reprecipitated with anti-EWI-2, anti-CD9P-1 or anti-integrin $\alpha 5$ mAb (IP2). Both EWI-2 and CD9P-1 were present in the CD81 immunoprecipitate, but integrin $\alpha 5\beta 1$ was not. This experiment was carried out twice with identical results.

condition allowing the observation of primary complexes, based on the presence or not of bivalent cations in a Brij97-based lysis buffer. In the presence of bivalent cations the different tetraspanins co-immunoprecipitate many other surface proteins, and the same molecules are present in each tetraspanin immunoprecipitate. The presence of EDTA induces a marked reduction of the number of proteins co-immunoprecipitated with each tetraspanin and, importantly, causes the different tetraspanins to co-immunoprecipitate different proteins, and in particular EWI-2 to be co-immunoprecipitated only with CD81 (in a CD9-negative cell line). This is related to the dissociation of tetraspanin–tetraspanin complexes as judged by the disappearance of CD81 from other tetraspanin immunoprecipitates (Figure 7B) and Western-blot analysis of tetraspanin–tetraspanin interactions [16]. This indicates further that second-order complexes form by assembly of primary complexes through tetraspanin–tetraspanin interactions. Therefore, on the basis of whether bivalent cations

are added or not in the lysis buffer, one would study primary or second-order complexes and this could explain why, in their recent studies, Stipp et al. [31,32] failed to observe the association of EWI-2 or CD9P-1 with tetraspanins other than CD9 or CD81. Further work will be necessary to determine whether bivalent cations decrease the ability of Brij97 to disrupt tetraspanin–tetraspanin complexes or whether this is related to a requirement for bivalent cations of tetraspanin–tetraspanin interactions.

The crystal structure of the CD81 LEL has been solved recently [50]. It has been described as a mushroom-shaped domain, with a stalk consisting of two α -helices in the continuity of the third and fourth TM domains. These two helices are predicted to be common features of all tetraspanins. The head consists of another well-conserved helix, located immediately before the CCG consensus sequence, and a region more variable in size, secondary structure and fold [1,45]. The LEL of CD9 is responsible for its ability to up-regulate the binding of diphtheria toxin to its receptor, and the use of monkey/mouse CD9 chimaeric molecules, as well as point mutations, have shown the critical involvement of the variable domain [51,52]. Also, two reports have studied the region of CD151 conferring the ability to stably interact with the integrin $\alpha 3\beta 1$ and the data showed that it encompasses the most variable region of the LEL [20,53]. In this paper we have used chimaeric CD9/CD82 molecules to define the regions of CD9 allowing interaction with EWI-2 and have shown the importance of two different regions. The first one is included in a 40-amino-acid region of the CD9 LEL, situated between the CCG consensus sequence and the last TM domain. A second region is located between the residues Leu⁶⁹ and Glu¹¹⁶, within a portion covering ≈ 14 residues of the second TM domain, a small cytoplasmic sequence, the third TM domain and approximately six residues of the CD9 LEL. Importantly, although both regions are required for achieving a maximal level of interaction, chimaeric proteins in which only one region was exchanged kept the ability to partially associate with EWI-2. This is the first demonstration that two independent regions in a tetraspanin act in concert to mediate an interaction with a partner molecule.

The results shown here and previous data from the literature [25,26,35] suggest that different CD9 and CD81 partners associate with CD9 or CD81 through different regions of these tetraspanins. EWI-2 and CD9P-1 interact with CD9 in different manners since the chimaeric molecule CD82 $\times$ 9, which lost a large part of its ability to interact with EWI-2, interacted strongly with CD9P-1 and no interaction of the reciprocal chimaeric molecule CD9 $\times$ 82 with CD9P-1 could be detected [35]. This indicates that the sequences of CD9 involved in EWI-2 binding and located between Leu⁶⁹ and Glu¹¹⁶ are not involved in the interaction with CD9P-1. Whether the LEL region of CD9 involved in the interaction with EWI-2 is also implicated in CD9P-1 interaction remains to be determined. A well-characterized molecular partner for CD81 is CD19, another IgSF molecule. There is little identity between CD19 and EWI-2. All anti-CD81 mAbs tested so far failed to co-immunoprecipitate CD19 [25,26], suggesting that they may dissociate the CD19–CD81 complex or not recognize CD81 in this complex. The fact that the same mAbs readily co-immunoprecipitated EWI-2 (and CD9P-1) suggests different binding sites on CD81 for interactions with CD19 and EWI-2. Among the two regions delineated in the CD9 sequences as being involved in the interaction with EWI-2, the Leu⁶⁹–Glu¹¹⁶ region has 62% identity with the corresponding region of CD81, while the second region, located in the LEL, has poor structural and sequence similarity with CD81. This suggests that the ways in which CD9 and CD81 associate with EWI-2 are different.

Recently, preliminary analyses of the region of EWI-2 interacting with CD81 were performed. In one study, it was found that a proteolytic fragment containing the cytoplasmic tag retained the ability to associate with CD81 and CD9 [32], strongly suggesting that the two membrane-proximal Ig domains mediate the association with these tetraspanins. A similar fragment of 47 kDa (determined under non-reducing conditions), not recognized by mAb 8A12, was observed in our experiments (Figure 2). On the other hand, removal of the two membrane-distal Ig domains of the mouse protein (PGRL) precluded the association with CD81 [33]. However, one cannot exclude the possibility that these two Ig domains mediate other properties of the molecule, e.g. compartmentalization and/or trafficking, rather than directly contributing to the association with CD81. Our data indicating that the Leu⁶⁹–Glu¹¹⁶ region of CD9 is implicated in the association with EWI-2 may favour the hypothesis that membrane-proximal sequences of EWI-2 interact with CD9.

The function of EWI-2 remains to be determined. Because tetraspanins have been shown in several studies to modulate cell migration [1,3], we tested whether the mAb 8A12 could modify haptotactic migration of Malhaviu cells (a hepatocellular cell line that was found to express the highest level of EWI-2) on various extracellular matrix proteins such as fibronectin, laminin-1 and laminin-10 (a ligand for the integrin $\alpha 3 \beta 1$), but we found no effect of this mAb. Also, the number of cells adhering to these substrates after 2 h of incubation was not changed in the presence of the mAb (results not shown). EWI-2 is most similar to three other molecules with Ig domains, CD101, CD9P-1/FPRP/EWI-F and IgSF-3, which form an IgSF subfamily [32,33]. The function of these molecules is poorly understood. CD9P-1 is the human orthologue of FPRP, which was first identified as a bovine corpora luteal membrane glycoprotein co-eluting from multiple chromatographic procedures with bound tritiated PGF2 α [54]. The rat molecule was later found not to be a PGF2 α receptor but to reduce the number of PGF2 α -binding sites on COS cells transfected with PGF2 α receptor. CD101 ligation on T-cells inhibits CD3-induced interleukin-2 production by blocking Ca²⁺ flux and NF-AT (nuclear factor of activated T-cells) nuclear translocation [55], while triggering CD101 on cutaneous dendritic cells inhibits T-cell proliferation via interleukin-10 production [56]. In contrast to CD9P-1 and EWI-2, we failed to observe an association of CD101 with CD9 or CD81 (results not shown). Moreover, the pattern of expression of CD101 on blood cells did not match that of CD81 since CD101 is expressed by a subset of T-cells, monocytes and granulocytes, but is absent from platelets and B-cells. In contrast, the patterns of expression of CD81 and EWI-2 are identical, both being expressed by T-, B- and NK cells and absent from granulocytes, monocytes and platelets ([46] and Figure 5). Moreover, EWI-2 and CD81 showed very similar patterns of staining in the liver where they were found by confocal microscopy to co-localize. These identical patterns of expression, together with the high stoichiometry of the interaction (Figures 2 and 3), further suggests that the functions of these two molecules are closely linked. It will be of special interest to determine whether EWI-2 can contribute to the pathogenesis of malaria and hepatitis C, as proposed for CD19, another CD81 partner [11].

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