

# EWI-2 Is a Major CD9 and CD81 Partner and Member of a Novel Ig Protein Subfamily\*

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**A novel Ig superfamily protein, EWI-2, was co-purified with tetraspanin protein CD81 under relatively stringent Brij 96 detergent conditions and identified by mass spectrometric protein sequencing. EWI-2 associated specifically with CD9 and CD81 but not with other tetraspanins or with integrins. Immunodepletion experiments indicated that EWI-2-CD9/CD81 interactions are highly stoichiometric, with ~70% of CD9 and CD81 associated with EWI-2 in an embryonic kidney cell line. The EWI-2 molecule was covalently cross-linked (in separate complexes) to both CD81 and CD9, suggesting that association is direct. EWI-2 is part of a novel Ig subfamily that includes EWI-F (F2 $\alpha$  receptor regulatory protein (FPRP), CD9P-1), EWI-3 (IgSF3), and EWI-101 (CD101). All four members of this Ig subfamily contain a Glu-Trp-Ile (EWI) motif not seen in other Ig proteins. As shown previously, the EWI-F molecule likewise forms highly proximal, specific, and stoichiometric complexes with CD9 and CD81. Human and murine EWI-2 protein sequences are 91% identical, and transcripts in the two species are expressed in virtually every tissue tested. Thus, EWI-2 potentially contributes to a variety of CD9 and CD81 functions seen in different cell and tissue types.**

Tetraspanin proteins contain cytoplasmic N and C termini, four transmembrane domains, one small and one large extracellular loop, and at least 30 amino acids distributed throughout the molecule that are highly conserved among most family members (1–3). Conserved cysteines within the large extracellular loop stabilize the folding of five  $\alpha$ -helical domains (4). Tetraspanins may be molecular facilitators (2) that organize other tetraspanins and other cell surface proteins into a network of complexes described as the tetraspan web (5). Indeed, tetraspanins form extensive complexes with each other and with other proteins such as integrins (5–17), major histocompatibility complex molecules (5, 18–20), CD4 and CD8 (21), CD19 and CD21 (22, 23), and transforming growth factor- $\alpha$  and HB-EGF<sup>1</sup> (24, 25). The large number of reported tetraspanin-

associated proteins raises critical questions about complex size, specificity, and functional relevance.

These concerns may be addressed by evaluating tetraspanin complexes with respect to four criteria: detergent stability, specificity, proximity, and stoichiometry. For example, CD151- $\alpha_3\beta_1$  integrin complexes are stable in conditions (Triton X-100 plus SDS detergent) that disrupt all other known tetraspanin associations, and at least 90% of  $\alpha_3\beta_1$  is associated with CD151 under these conditions (26). Covalent cross-linking of CD151 to  $\alpha_3$  integrin confirmed that CD151 and  $\alpha_3$  integrin are directly associated (27). Furthermore, CD151 participation in  $\alpha_3\beta_1$ -mediated neurite outgrowth and growth cone motility points to a specific functional relationship between CD151 and  $\alpha_3\beta_1$  integrin (28).

To observe many other tetraspanin complexes, detergents less disruptive than Triton X-100 are required. We often utilize the moderately stringent Brij 96/97 detergent because (i) tetraspanin complexes in Brij 96 have a discrete size (significantly less than 4-million Da (29, 30), whereas milder detergents such as CHAPS may yield tetraspanin complexes in excess of 10-million Da (31); (ii) tetraspanin complexes in Brij 96 are fully soluble and do not depend on poorly solubilized low density vesicular membrane microdomains for their stability (29, 30); (iii) tetraspanin complexes with distinct protein compositions can be readily solubilized in Brij 96 (30); and (iv) tetraspanin associations observed in Brij 96 have typically proven to be functionally relevant. For example, CD81 contributes to  $\alpha_3\beta_1$ -mediated neurite outgrowth (28) and to signaling through CD19 (23, 32).

The tetraspanin CD81 is expressed by virtually every cell type tested, whereas CD9 is more restricted. As seen from co-expression in at least one cell type, the majority of CD9 may be in a complex with CD81. Consistent with this, in Brij 96 lysates, CD81 and CD9 complexes (with other proteins) appear strikingly similar but distinct from other tetraspanin complexes (30). Whereas CD9 plays an essential role in sperm-egg fusion during fertilization (33, 34) and acts as a coreceptor up-regulating the mitogenic and diphtheria toxin binding activities of membrane-bound HB-EGF (35, 36), CD81 binds to the hepatitis C virus (HCV) E2 coat protein (37) and may serve as a co-receptor for HCV, a virus that affects 300 million people worldwide and is a leading cause of chronic liver failure and hepatocellular carcinoma (38). Other studies further implicate CD81 and CD9 in virus-induced cell-cell fusion and virus entry into and/or release from cells (21, 37, 39–41). Also, CD9 and CD81 are involved in myoblast fusion into myotubes (42).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF407274.

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<sup>1</sup> The abbreviations used are: HB-EGF, heparin binding-epidermal growth factor; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; FPRP, F2 $\alpha$  receptor regulatory protein; DSP, dithiobis(succinimidylpropionate); EWI, glutamine-tryptophan-isoleucine; HRP, horseradish peroxidase; PBST, phosphate-buffered saline with 0.1% Tween 20; HCV, hepatitis C virus; IgSF, human Ig superfamily; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; h-, human.

Aside from cell fusion, CD9 and CD81 also function in cell motility (28, 43–48). CD9 in particular may negatively regulate tumor cell metastasis in a variety of cancers (49–56). CD81, first identified as the target of an antiproliferative antibody (57), has also emerged as a regulator of astrocyte proliferation (58) and of proliferation and activation of various hematopoietic cell subsets (22, 32, 57, 59–62). Despite the large body of evidence linking CD9 and CD81 to a wide range of developmental and pathogenic processes, no central organizing hypothesis has emerged to explain CD9 and CD81 functions. By identifying major protein partners that are specific, highly proximal, and highly stoichiometric, we hope to gain insight into the functions of CD9 and CD81 in disease and development.

To this end, we have used a mass spectrometry protein sequencing approach to identify proteins that co-purify with CD81 from Brij 96 lysates. Here we describe a novel Ig superfamily protein, EWI-2, that associates with CD81 and CD9 with high specificity, stoichiometry, and proximity. Among the many reported partners for CD9 and CD81, only one other molecule (FPRP, CD9P-1) appears to have a level of specificity, stoichiometry, and proximity comparable with that seen here for EWI-2 (30, 63). Remarkably, that FPRP/CD9P-1 molecule (here renamed EWI-F) belongs to the same novel subfamily of IgSF proteins as our newly discovered EWI-2 molecule.

#### EXPERIMENTAL PROCEDURES

**Antibodies and Cell Culture**—Anti-integrin mAbs used in this study were anti- $\alpha_2$ , A2-IIE10 (64); anti- $\beta_1$ , TS2/16 (65); and anti- $\alpha_6$ , A6-ELE (66). Anti-TM4SF mAbs used were anti-CD9, ALB6 (Chemicon); anti-CD81, M38 (67) and JS64 (68); and anti-CD151, 5C11 (26). The M2 anti-FLAG epitope mAb, either biotinylated or conjugated to agarose, was purchased from Sigma. Horseradish peroxidase (HRP)-conjugated goat-anti-mouse polyclonal antibody (Fc-specific) and non-immune mouse IgG were purchased from Sigma. Anti- $\beta_1$  integrin TS2/16 and non-immune mouse IgG were conjugated to agarose Affi-Gel-10 (Pierce) according to manufacturer's instructions. A431 epithelial carcinoma cells, NT2 teratocarcinoma cells, and 293 embryonic kidney cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Life Technologies, Inc.), penicillin-streptomycin, and 2 mM glutamine. To obtain NT2RA cells, NT2 cells were treated with 10  $\mu$ M retinoic acid for 4–5 weeks, split 1:6 into fresh flasks, and then treated for 10–14 days with mitotic inhibitors (69).

**Purification of CD81-associated Proteins and Mass Spectrometric Sequencing**—CD81-associated proteins were prepared as previously described (30). Briefly, CD81 complexes were affinity-purified from a Brij 96 lysate of NT2RA cells using anti-CD81 mAb JS64 and protein G-Sepharose. CD81-associated proteins were eluted in 1% Triton, 0.1% SDS, concentrated by Microcon (COSTAR), and resolved by SDS-PAGE. Silver-stained bands migrating at ~70 kDa were excised, rinsed with 50% HPLC-grade acetonitrile, and stored at -20 °C until analysis. Silver-stained bands were next subjected to in gel reduction, carboxyamidomethylation, and tryptic digestion (Promega). As previously described (30), peptide sequences were determined by microcapillary reverse-phase chromatography directly coupled to a Finnigan LCQ quadrupole ion trap mass spectrometer equipped with a custom nano-electrospray source. Interpretation of the resulting tandem mass spectroscopy spectra of the peptides was facilitated by programs developed in the Harvard Microchemistry Facility and by data base correlation with the algorithm Sequest (70, 71).

**Construction and Expression of FLAG-tagged EWI-2**—EWI-2 with a C-terminal FLAG-tag was obtained by polymerase chain reaction from EWI-2 cDNA. The cDNA clone used for the polymerase chain reaction (GenBank™ accession number AI700165) contained an intron that was removed by recombinant polymerase chain reaction. Primers for amplifying the N-terminal exon were: sense, 5'-GATATCGTCGACCCACGCG-3'; and antisense, 5'-CCAGCACCACACCTTCCTCCCGCACATGTAGAGG-3'. Primers for amplifying the C-terminal exon were: sense, 5'-GTGCGGGAGGAGGTGTGGTGTGCTGGAGGCTGTG-3'; and antisense, 5'-GGAATTCCTACTTGTCATCGTCGTCCTTGAATCCCGTTTTCGAAGCCTCTTCATG-3'. The recombinant polymerase chain reaction product was digested with *SalI* and *EcoRI* and cloned into *SalI/EcoRI* I sites of the pLXIZ retroviral vector. The resulting retroviral expression construct was transfected by the calcium phosphate method into  $\Phi$ NX-packaging cells. 48 h

after transfection, the  $\Phi$ NX cell supernatant was passed through a 0.45- $\mu$ m filter, supplemented with 4  $\mu$ g/ml Polybrene (Sigma), and used to infect 293 cells. Stable EWI-2-expressing cells were obtained by selection for 2 weeks in 100  $\mu$ g/ml zeocin (Invitrogen). The resulting cells, 293-EWI-2 cells, were maintained in 100  $\mu$ g/ml zeocin as polyclonal population.

**Northern Blot Analysis**—A multiple tissue Northern blot (CLONTECH) was hybridized with EWI-2 or  $\beta$ -actin probes using ExpressHyb hybridization solution (CLONTECH) according to the manufacturer's instructions. EWI-2 probe was prepared by digestion of pLXIZ-EWI-2 construct with *BstXI* and gel purification of a 0.9-kilobase 3'-fragment of EWI-2 open reading frame. Radioactive probes were generated by random priming with Prime It II kit (Stratagene) with [ $\alpha$ - $^{32}$ P]dCTP (PerkinElmer Life Sciences) according to the manufacturer's instructions.

**Immunoprecipitation and Immunoblotting**—Cells were biotinylated with 0.2 mg/ml sulfo-NHS-LC biotin (Pierce) in 20 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM MgCl<sub>2</sub> (HBSM) for 1 h at room temperature. After 3 rinses with HBSM, cells were lysed by scraping into 1% Brij 96 (Fluka) or 1% Triton X-100 (Sigma) in HBSM with 2 mM phenylmethylsulfonylfluoride (Sigma), 20  $\mu$ g/ml aprotinin, and 10  $\mu$ g/ml leupeptin (Roche Molecular Biochemicals). After a 1-h extraction at 4 °C with rocking, insoluble material was removed by centrifugation, and lysates were precleared for 1 h at 4 °C with protein G-Sepharose (Amersham Pharmacia Biotech). Specific antibodies were added along with protein G-Sepharose, and immune complexes were collected overnight at 4 °C. Alternatively, lysates were precleared with non-immune mouse IgG coupled to agarose before immunoprecipitation with M2 anti-FLAG-agarose beads. After rinsing four times with lysis buffer, immune complexes were eluted by boiling in sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose. Blots were blocked with 3% nonfat milk in phosphate-buffered saline with 0.1% Tween 20 (PBST). After rinsing with PBST, blots were developed and visualized using HRP-extravidin (Sigma) followed by chemiluminescence detection (Renaissance reagent, PerkinElmer Life Sciences).

For CD81 immunoblotting, immunoprecipitates prepared as above were separated by non-reducing SDS-PAGE and transferred to nitrocellulose. After blocking with 5% milk in PBST, the blots were developed with the M38 anti-CD81 mAb (2  $\mu$ g/ml) followed by HRP-goat-anti-mouse-IgG and chemiluminescence. FLAG epitope immunoblots were prepared and blocked as above, rinsed with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and blotted for 30 min with biotinylated M2 anti-FLAG mAb. After 10 rinses, blots were developed with a 30-min exposure to HRP-extravidin followed by 10 more rinses and chemiluminescence detection. For immunodepletion, 293-EWI-2 cells were cell surface-biotinylated and lysed in 1% Brij 96 as described above. The lysate was divided into 3 parts and passed 7 times over 0.4-ml columns of mouse Ig-agarose, M2 anti-FLAG agarose, or TS2/16 agarose poured in 1-ml syringe barrels. The depleted samples were divided into four parts each, immunoprecipitated, and analyzed by SDS-PAGE as above.

**Covalent Cross-linking**—1% Brij 96 extracts of 293-EWI-2 cells were clarified by two rounds of centrifugation at 15,000  $\times g$  for 15 min and then treated with the indicated concentrations of the cross-linkers dithiobismaleimidoethane or dithiobis(succinimidylpropionate) (DSP) (Pierce) for 1 h at room temperature. DSP reactions were quenched by reacting for 15 min at room temperature with 10 mM glycine, pH 7.5. Triton X-100 was added to 1% to cross-linked lysates and a portion of the non-cross-linked control lysates, and samples were immunoprecipitated with the indicated antibodies and analyzed by SDS-PAGE followed by immunoblotting as described above.

**Size Exclusion Chromatography and Sucrose Density Gradient Centrifugation**—Cell surface biotinylated 293 cells were lysed in 1% Brij 96 as above. Lysate-containing ~1  $\times 10^7$  cell equivalents was loaded in 5% glycerol with blue dextran and phenol red to a 25.5  $\times$  1.0-cm Sepharose 6B column that had been pre-equilibrated in 1% Brij 96 in HBSM at room temperature (Brij 96 solutions cloud upon prolonged storage at 4 °C). 22 fractions of ~540  $\mu$ l were collected spanning from the leading edge of the blue dextran elution to the phenol red elution point. 400  $\mu$ l of fraction 11 from this column was mixed with 400  $\mu$ l of 90% sucrose, 1% Brij 96 in HBSM loaded over a 0.5-ml cushion of 50% sucrose and overlaid with layers of 40% sucrose (1.5 ml), 20% sucrose (1.5 ml), and 5% sucrose (0.7 ml) prepared in HBSM without detergent. After centrifugation for 21 h at 45,000 rpm in a Beckman SWTi55 rotor at 4 °C, 14 fractions of 360  $\mu$ l were collected from the top of the gradient. The pellet was included in the final fraction. 1 ml of 1.36% Brij 96 in HBSM was added to each fraction, and CD81 complexes were immunoprecipitated and analyzed by blotting with extravidin-HRP, as described above.

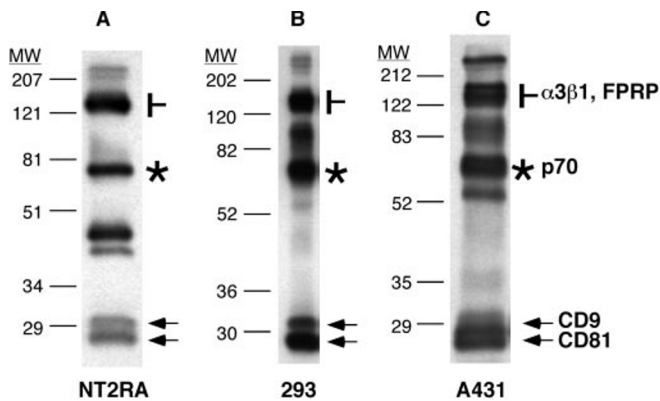


FIG. 1. **CD81 complexes contain a major 70-kDa species.** Retinoic acid-differentiated NT2 teratocarcinoma cells (NT2RA) (lane A), 293 embryonic kidney epithelial cells (lane B), and A431 epithelial carcinoma cells (lane C) were cell surface-labeled with biotin and lysed in 1% Brij 96 detergent. CD81 complexes were immunoprecipitated using mAb M38, resolved by non-reducing SDS-PAGE, and visualized by blotting with horseradish peroxidase-conjugated extravidin followed by chemiluminescence. Bands corresponding to CD81, CD9,  $\alpha_3\beta_1$  integrin, and prostaglandin F $2\alpha$  receptor regulatory protein (FPRP) are indicated. An asterisk indicates a major unidentified 70-kDa species (p70) present in all three immunoprecipitates. MW, molecular mass.

## RESULTS

**Identification of Novel CD81-associated Proteins**—Elsewhere we have established methodology for isolation of specific CD81-associated proteins from 1% Brij 96 detergent lysates. Size and density estimations confirmed that our detergent conditions yielded complete solubilization of discrete CD81 complexes (30). CD81 complexes isolated from cell surface-labeled NT2RA cells (Fig. 1, lane A), 293 embryonic kidney cells (Fig. 1, lane B), and A431 epithelial carcinoma cells (Fig. 1, lane C) contained CD9 (~30 kDa) as well as major species migrating above 120 kDa. These were previously identified as a mixture of  $\alpha_3\beta_1$  integrin and a novel Ig superfamily protein (FPRP, CD9P-1 (30, 63)). All three cell types also contained a major unidentified species of ~70 kDa. To identify the CD81-associated p70 protein, CD81 complexes were affinity-purified from a Brij 96 lysate of NT2RA cells and fractionated by SDS-PAGE. The 70-kDa band was excised after silver-staining and digested with trypsin. Tryptic peptides were separated by reverse phase liquid chromatography and sequenced by ion trap tandem mass spectroscopy. A few of the peptides obtained (from actin, keratin, and heat shock proteins) are commonly observed in sensitive mass spectroscopy-sequencing protocols. A few other peptides corresponded to proteins for which CD81 association could not be confirmed.<sup>2</sup> By far the most well represented species was a protein for which 13 peptides were obtained. These peptides were initially sorted into six groups based on overlap with distinct ESTs (Table I). The first five peptides in Table I clustered to an EST (GenBank<sup>TM</sup> accession AI336860) described as “similar to a leukocyte surface antigen.” The UniGene tool (National Center for Biotechnology) revealed several large, known cDNA clones in this cluster. Sequencing of one clone (GenBank<sup>TM</sup> accession AI700165) revealed an insert of ~2.7 kilobases with an ATG start codon conforming to the Kozak consensus (72), 1312 base pairs of coding sequence, a 228-base pair intron, and an additional 527 base pairs of coding region beyond the intron. Peptide 9 in Table I contained sequences on both sides of this intron. A second cDNA (GenBank<sup>TM</sup> AI356969) with an insert of ~2.3 kilobases, had an identical coding region but contained no introns. An open reading frame of 1839 base pairs encoded a predicted protein of 613

TABLE I  
Peptides recovered from the p70 CD81-associated protein

| Peptides                     | EST cluster; GenBank <sup>TM</sup> accession No. |
|------------------------------|--|
| 1. (R) MTVHEGQELALGCLAR      |  |
| 2. (R) STLQEVVGIR            |  |
| 3. (R) SDLAVEAGAPYAER        | AI336860   |
| 4. (K) HTHLAVSFGR            |  |
| 5. (K) LAAGELR               |  |
| 6. (R) VVAGEVQVQR            |  |
| 7. (K) DTQFSYAVFK            | R90815   |
| 8. (R) LQGDAVVLK             |  |
| 9. (R) EEGVVLEAVAWLAGGTVYR   | W64732 <sup>a</sup>                              |
| 10. (R) SRPLPVHVR            |  |
| 11. (R) LEARPGDAGTYR         | AA322589   |
| 12. (R) LVAQLDTEGVGSLGPGYEGR | H41877   |
| 13. (R) PGGGPVSVELVGPR       | AI910243   |

<sup>a</sup> This EST corresponds to the murine homologue of the human p70 protein.

amino acids. All 13 of the peptides in Table I were found within the coding region (see Fig. 2).

**A Novel Ig Subfamily**—The p70 protein sequence features a 27-amino acid leader peptide characteristic of secreted or transmembrane proteins, a 556-amino acid extracellular domain, a putative transmembrane domain of 21 amino acids, and a very short, highly charged cytoplasmic tail. The mature p70 polypeptide has a predicted molecular mass of 62 kDa. The additional 8 kDa are likely the result of *N*-linked glycosylation at three potential NX(S/T) glycosylation sites in the extracellular domain. The p70 extracellular domain contains 8 cysteine residues within four sequence motifs characteristic of the B and F strands of four V-type Ig domains (73, 74).

A BLAST search of the GenBank<sup>TM</sup> data base revealed three additional Ig proteins with significant similarity to p70. These proteins include FPRP/CD9P-1, which like p70 is also a major CD9 and CD81 partner (30, 63), as well as IgSF3 and CD101. Collectively, these four proteins are more similar to each other than to any other Ig protein currently identified. Using the ClustalW program, the individual Ig domains of each protein were compared pairwise to create the alignment shown in Fig. 2A and schematically in Fig. 2B. The approximate locations of the  $\beta$ -strands in each Ig domain were assigned based on established criteria (73, 74). The most striking homology is among the distal two Ig domains of each protein. Although alignment of other Ig domains is less certain, the membrane proximal Ig domains align somewhat better to each other than to the more N-terminal Ig domains. Since a function-based nomenclature is premature, we have adopted a nomenclature based on a shared EWI protein sequence motif present within the F-G loop in the second Ig domain of each protein (Fig. 2A). Hence, FPRP/CD9P-1 becomes EWI-F, the p70 protein (being the second CD81-associated Ig protein discovered) becomes EWI-2, CD101 becomes EWI-101, and IgSF3 becomes EWI-3. Fig. 2C summarizes the strong similarity of the EWI family members to each other overall (23–35%) and especially within the first two Ig domains (34–57%). EWI-3 and EWI-101 are clearly more closely related to each other than to the other two members of the EWI subfamily.

Peptides 9 and 10 in Table I, although contained in the human EWI-2 protein, were first clustered to a mouse EST. ESTs containing peptides 9 and 10 belonged to mouse EST 34,

<sup>2</sup> C. Stipp and M. Hemler, unpublished data.



A

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hEWI-3 M--K---CFFPVLSCVAVGVSACQVTVQGGFLYRTGSHITWCVSGYQGPSECFWNSYILSSPEREVOIVTMDSSFFPVAITVTVRVRGGKI
hEWI-101 MAG-----ISYVASFPFLLLTKLSIGCREVTVQKGLFRAEGYFVSGICVTHGQGPSECFWNSYILSSPEREVOIVTMDSSFFPVAITVTVRVRGGKI
hEWI-F MGRLL-----ASRPLLLALLSLALCRGRVVRVPTATTVRVGTGLVIPCVSQYDGPSECFWNSYILSSPEREVOIVTMDSSFFPVAITVTVRVRGGKI
hEWI-2 MGALRPTLLPPLSLPLLLMLMGMCWAEVLPVGGFLYRTGSHITWCVSGYQGPSECFWNSYILSSPEREVOIVTMDSSFFPVAITVTVRVRGGKI
mEWI-2 MGVPSPPTLSS--LLLLLLILGTRCYARVHVHVPGLYRTGSHITWCVSGYQGPSECFWNSYILSSPEREVOIVTMDSSFFPVAITVTVRVRGGKI

hEWI-3 FIERVQ--STLLHITDLQARDAGEYECHTPTSDTKQVPGSYAKMNEVIPDSL-----OTTAMP-----QTLHRVQDPLELTCVASETIQHSHLSV
hEWI-101 YVERVQNSVLLHISKLQMKDAGEYECHTPTSDTKQVPGSYAKMNEVIPDSL-----SATMSS-----QTLKKEEPEPLATCEASAKATAGHTLSV
hEWI-F LLRRTANDAVELHIXNQVPSDQGHYKSTPTSDATVQGVNEDTVQVVLADSLHW-----GPSAR--P--PFLSLRKEEPEPLATCEASAKATAGHTLSV
hEWI-2 QVQRLQDQAVLKIALRQADAGIYECHTPTSDTKQVPGSYAKMNEVIPDSL-----GPSAR--P--PFLSLRKEEPEPLATCEASAKATAGHTLSV
mEWI-2 QVQRLKQDSVVLKIALRQADAGIYECHTPTSDTKQVPGSYAKMNEVIPDSL-----GPSAR--P--PFLSLRKEEPEPLATCEASAKATAGHTLSV

hEWI-3 ANLRQKVG-----EKPVEVLSRDFMLHSSSEYACROSLGEVRLDKLGRITFRLTIFHLQPSDQGEFYCEAAEWIQDQDGSNMYAMTRKRSEGVVNVQV
hEWI-101 TWYLTQDGG-----GSQATEIISLQKDFILVPLVTERFPAASDVQNLKGLPTTFRLLSIERLQSSDQQLFCEATEWIQDQDGSNMYAMTRKRSEGVVNVQV
hEWI-F LWEVHRGPAP-----RSVLALTHEGRFHPGLQYEQRYHSGDVRDLTVGSDAYRLSVSRALSADQGSYRCIVSENIAR--QGNWQRIQKAEVAVETVQV
hEWI-2 SFQSPVPEAPVGRSTLQEVVGIKSDLAEGAPYAEIRLAGEELRQKSGTDTRYRMVVGQAQAGDAGTYHCTAAEWIQDQDGSNMYAMTRKRSEGVVNVQV
mEWI-2 SFQRAIPAPVGRATLQEVVGIKSDLAEGAPYAEIRLAGEELRQKSGTDTRYRMVVGQAQAGDAGTYHCTAAEWIQDQDGSNMYAMTRKRSEGVVNVQV

hEWI-3 TDKEFTVLETEKRLHTVGEPEFRQILE-----AQNVDPDYFAVSWAF-----SSLATMGPNAPVPINSEFAHREARQKLVAKSSDSVFLKIYH
hEWI-101 AVKDFQWITADS--LFAEGKPLELVCLVW-----SSGR--DPQLQGIWFE-----GTEIAHIDAGGVGLKNDYKERASQGLQLSKLQPKAFSLKIFS
hEWI-F SVLRAAVPK-----SVSVAEGKELDLTCTIT-----TDRAVDVPEVTVSESR-----MPDSTLPGSRVLARLDRLSLVHSSP--HVALSHVDARSYHLVRD
hEWI-2 LSSQLAVTVGPERRIGPGEPLLELCVSGALFPAGR--HAAYSQWEMAPAGAPGRGLVAQLDTEGIGSLGPGYEDRH-----IAMEKVASRTYRLRLEA
mEWI-2 LSSQLAVTVGPERRIGPGEPLLELCVSGALFPAGR--HAAYSQWEMAPAGAPGRGLVAQLDTEGIGSLGPGYEDRH-----IAMEKVASRTYRLRLEA

hEWI-3 LRQEDSGKYNCRVTEREKTVTGEFIDKESKPKNIPIIIVLPLTDNWNVQVQHHQLSQGHLESISSEVASASVILEGDELRFSCSVRT--AGRPQGR
hEWI-101 LGPEDEGAYRCVVAEVMKTRTGSQVQLQKQSPDHSVHLKPKAAR-----SVVSTYKQVWVWEGSTLAELCKAGG--AESP--
hEWI-F VSKNSGYCHVSLWAPGHRSWHKVAEAVSSPAGVGVTLLEP-----DYQVYLASKVPGFADDPTELAACRVVDTKSGEANVR
hEWI-2 AREGAGTYRCLAKAYVRSGTRLREASARSRLPLVHVREEGVVL-----
mEWI-2 AREGAGTYRCLAKAYVRSGTRLREASARSRLPLVHVREEGVVL-----

hEWI-3 FSVIQL--VDRQNR--SNIMW--LDRDGTVPQSSYWNERSFGQVQMBQVQNSFSLQIFNSRKEDEGQVCHVTEWVRVADGEMQIVGERRASTPISI
hEWI-101 LSVSWH--IPRQDT--QPEFVAGQDGIQVGLALLNQTSYHNTREKMDWATFOLEITFTAITDSQTEYCRVSEKSNQARDLSWT--QKISVTV
hEWI-F FTVSYMYNRNSDNVVTSELLAVMDGNTLKYGERSKQRAQDGFIFSKHEITDTNFRQRTTEEDRGNYCVVSANTKQRNSWKSQD--VFSFVNTI
hEWI-2 SLQDSVVKLEWTDASRVVG--VILEK--VQEDFERYMYQTVSDAGLYRCMTANSFVRGSLWREATSLSNPIEIDPQTSQPIF
mEWI-2 SLQDSVVKLEWTDASRVVG--VILEK--VQEDFERYMYQTVSDAGLYRCMTANSFVRGSLWREATSLSNPIEIDPQTSQPIF

hEWI-3 TALEMGFAVTAISRTPGVTYSDFDLQCIIP--HYPAWVSVTVWRQVPGVTVEFDELVTFTDQGVQNGDRSSSPRTRTAIEKAESSNNVRLSISRASD
hEWI-101 KSLSSQVLSMRQVQVMTNTEDLSCVVRAGYSLKVLTVWQFQASSHIFHOLIRITHGTEINGNLSRFPQKTKVQSLSLFRS--QLLVHDATE
hEWI-F FWALEDV-----
mEWI-2 FWALEDV-----

hEWI-3 TEAGYKCVASLWRKN--Y--NTWTRLAERTSNLIRVLPQVTKLVQSKSKRTLTIVENKPIQLCSVKSTSONSHFAVLMYVHKE--SDADG--KLIL
hEWI-101 QETGVYQCEVEYDRLNLYNPPPRASIAISHPLRIATVLPESKLVNSRQGLSINSNTDIECLSRNGNLQAIHMYFSPVST--ASN--LKIL
hEWI-F LTVKARQKPPFAAGNTFEMTKVYSKNIKSPRYSLVMAEKPVGLDSSPETYII
hEWI-2 LTVKARQKPPFAAGNTFEMTKVYSKNIKSPRYSLVMAEKPVGLDSSPETYII
mEWI-2 LTVKARQKPPFAAGNTFEMTKVYSKNIKSPRYSLVMAEKPVGLDSSPETYII

hEWI-3 KTHNSAFETYAYEEGLRRLQFERHVSGLFSLTVQRAEVSQSGSYCHVEEWLLSPNYAWYKLAEEVSGRTEVTVKQPSRLRLSQAQGLSVSGD
hEWI-101 EMDQNVITKQDEFHTPQRKQKPHTEK--VSQDLFQLHILAVEQSDRGYHCAVEWLLSTGTWHLKGLKKSGLTELKLPQTSKV--
hEWI-F SLQDSVVKLEWTDASRVVG--VILEK--VQEDFERYMYQTVSDAGLYRCMTANSFVRGSLWREATSLSNPIEIDPQTSQPIF
hEWI-2 SLQDSVVKLEWTDASRVVG--VILEK--VQEDFERYMYQTVSDAGLYRCMTANSFVRGSLWREATSLSNPIEIDPQTSQPIF
mEWI-2 SLQDSVVKLEWTDASRVVG--VILEK--VQEDFERYMYQTVSDAGLYRCMTANSFVRGSLWREATSLSNPIEIDPQTSQPIF

hEWI-3 PAGTAGVCGSQPHQHNLPAHGMVCMCAEQPPERETVARLSRDATPHYGEQAANNKGLRLHLESPPSGVYRLFQINQVAVQDSGTYSCHVEWLPSPSGMW
hEWI-101 PAGTAGVCGSQPHQHNLPAHGMVCMCAEQPPERETVARLSRDATPHYGEQAANNKGLRLHLESPPSGVYRLFQINQVAVQDSGTYSCHVEWLPSPSGMW
hEWI-F PAGTAGVCGSQPHQHNLPAHGMVCMCAEQPPERETVARLSRDATPHYGEQAANNKGLRLHLESPPSGVYRLFQINQVAVQDSGTYSCHVEWLPSPSGMW
hEWI-2 PAGTAGVCGSQPHQHNLPAHGMVCMCAEQPPERETVARLSRDATPHYGEQAANNKGLRLHLESPPSGVYRLFQINQVAVQDSGTYSCHVEWLPSPSGMW
mEWI-2 PAGTAGVCGSQPHQHNLPAHGMVCMCAEQPPERETVARLSRDATPHYGEQAANNKGLRLHLESPPSGVYRLFQINQVAVQDSGTYSCHVEWLPSPSGMW

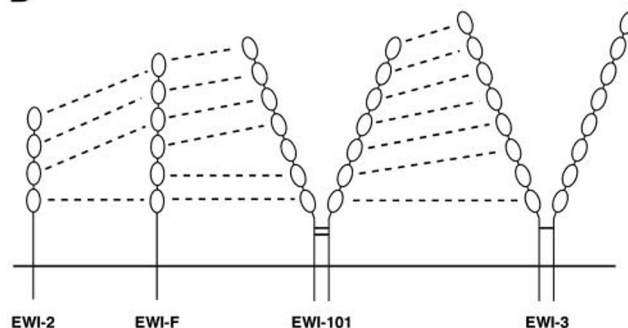
hEWI-3 SKRAEDTAGQALTVMRPDASLQVDTVVPAITVSEKAAPQDCSTVSRSSQDSR--FAVANYSLRTKAGKRSPPGLEEQEEEEEEEEEEEEEDDDDD
hEWI-101 -----RVSKYVWTEVTEHREVAIRCLESVSSATL-----YSVMYWNRENSGS
hEWI-F -----ASVHSQDTPSIRGLDLKLFCTITVGAALDPDMAFVSVFAVHSPG-----L
hEWI-2 -----EAVANLAGQTVYRGETASLLCISVRGPPGLR-----LAASWVVERPEDGEL--SSVPA
mEWI-2 -----EAVANLAGQTVYRGETASLLCISVRGPPGLR-----LAASWVVERPEDGEL--STOPA

hEWI-3 PTERALLSVGPDAVFPQENWEGRLRFQRLSPVLYRLTVLQASQDTCVSCHEWMLPS-PQEWYRLTEESAIGIRVLDTSFTLQSIICNDAL
hEWI-101 ---K--LVLVQLHGDALVYEEELRGHLCYRSTDFVLYKHVYEMDAGMYKCAVAKLGHDPKWKINKHPSHSGWSPCCLOSRLPGLGAPRPP
hEWI-F DKAPVLSGLDRKGLVTSRDEWKSGLERVSDFLFLVQHSQEDQFNYYCSVTPWVKSPTOS--WKEAEIHSKP-----VPTVYMDVLNAFKYPL
hEWI-2 ---QLVGVQDQGVAEGLVREGQGVSVLVLQPSHRLRLHGLQPEDEGIYHCAPSANYQHADYS--WYQAGSARSGB-----VTYFYPHALDTLFLVL
mEWI-2 ---QLVGVQDQGVAEGLVREGQGVSVLVLQPSHRLRLHGLQPEDEGIYHCAPSANYQHADYS--WYQAGSARSGB-----VTYFYPHALDTLFLVL

hEWI-3 FYFVFPFPFPIFGLIITILLVRFKSNSSKNSDGKNGVPLLIKEPHLNSPTCLEPVLISHPGAID
hEWI-101 EYFLEICPEVLLLLLSLLGLYKARKLSTLRNTRKEKALWDLKEAGGVTRNRREDEEEDGN
hEWI-F LIGVGLSLVIGLLSCLIGYCSHCKCKEVOETRRRRRLMSMEMD
hEWI-2 LVGTGVALVTGATVLTITTCFMKRLRRR
mEWI-2 LVGTGVALVTGATVLTITTCFMKRLRRR

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B



C

|                                     | 2   | F   | 101 | 3   |                                  |
|-------------------------------------|-----|-----|-----|-----|----------------------------------|
| 2                                   | —   | 27% | 30% | 30% | % IDENTITY OVER<br>ENTIRE LENGTH |
| F                                   | 37% | —   | 24% | 23% |                                  |
| 101                                 | 43% | 34% | —   | 35% |                                  |
| 3                                   | 44% | 36% | 57% | —   |                                  |
| % IDENTITY OVER<br>1st 2 lg DOMAINS |     |     |     |     |                                  |

FIG. 2. EWI-2/p70 is a member of a novel Ig subfamily. A, a BLASTP search indicated that the human CD81-associated p70 protein, denoted here as *hEWI-2*, is significantly identical to three other human Ig superfamily proteins, *hEWI-3* (IgSF3), *hEWI-101* (CD101/V7), and *hEWI-F*

UniGene cluster Mm.29860. From this cluster of 94 EST sequences, we assembled the entire open reading frame of the murine p70 protein. Alignment of the human and mouse EWI-2 proteins revealed 91% identity and 96% similarity (including conservative substitutions) throughout their mature polypeptides (Fig. 2A). Among the non-identical residues, 75% fall within the first two Ig domains. The human EWI-2 protein localizes to chromosome 1 at position 584.71 cR3000 in the GeneMap99 GB4 map. In the mouse genome, EST 34 maps to chromosome 1, position 94.20 centimorgan, within a region that contains the *loop-tail* gene (see "Discussion"). The cDNA sources of the human and mouse EWI-2 ESTs include heart, brain, adrenal gland, lung, muscle, ovary, pancreas, testis, uterus, stomach, thymus, prostate, colon, and mammary gland. Probing of a multiple tissue Northern blot with human EWI-2 cDNA (Fig. 3) revealed especially high expression in human brain, kidney, liver, and placenta with moderate expression in all other tissues except for minimal expression in peripheral blood leukocytes. Thus EWI-2 is a broadly expressed member of a novel Ig subfamily and may carry out conserved functions in multiple species.

**Highly Specific and Proximal Association of EWI-2 with CD9 and CD81**—To confirm the association of EWI-2 with CD9 and CD81, we constructed an EWI-2 cDNA with a C-terminal FLAG epitope tag. This EWI-2-FLAG cDNA was cloned into a retroviral expression vector, which was used to infect 293 cells. Stable EWI-2-FLAG infectants (293 EWI-2 cells) and parental 293 cells were cell surface-labeled with biotin and lysed in Triton X-100 or Brij 96. Anti-FLAG immunoprecipitates from Triton X-100 lysates of 293 EWI-2 cells but not the parental 293 cells contained a major band of 70 kDa, as expected (Fig. 4A, lanes 1 and 2). Also present were additional bands of ~52, 47, and 43 kDa (*asterisks* in Fig. 4A), which may be cleavage products of the intact EWI-2 protein (see Fig. 4D). The higher  $M_r$  bands in EWI-2 immunoprecipitates may contain EWI-F (see Fig. 6) but may also contain aggregates of EWI-2 itself since they are diminished under reducing conditions.<sup>3</sup> In Brij 96 lysates, a major band corresponding to EWI-2-FLAG co-precipitated with both CD81 (Fig. 4A, lane 4) and CD9 (Fig. 4A, lane 6). Reciprocally, bands corresponding to CD9 and CD81 co-precipitated with EWI-2-FLAG (Fig. 4A, lane 8). Neither EWI-2 nor CD9 nor CD81 were detected in  $\alpha_2\beta_1$  or  $\alpha_6\beta_1$  integrin immunoprecipitates (Fig. 4A, lanes 9–12). The EWI-2-FLAG band co-precipitating with CD9 and CD81 in Fig. 4A, lanes 4 and 6, migrated slightly above the endogenous p70 band seen in CD9 and CD81 immunoprecipitates from parental 293 cells (Fig. 4A, lanes 3 and 5). This may be the result of the strong overexpression of the EWI-2-FLAG protein affecting post-translational modification (*e. g.* incomplete "trimming" of N-linked glycans).

To confirm the identities of the cell surface-biotinylated bands in Fig. 4A, CD9 and CD81 immunoprecipitates from Brij 96 lysates of 293 EWI-2 cells were immunoblotted with an

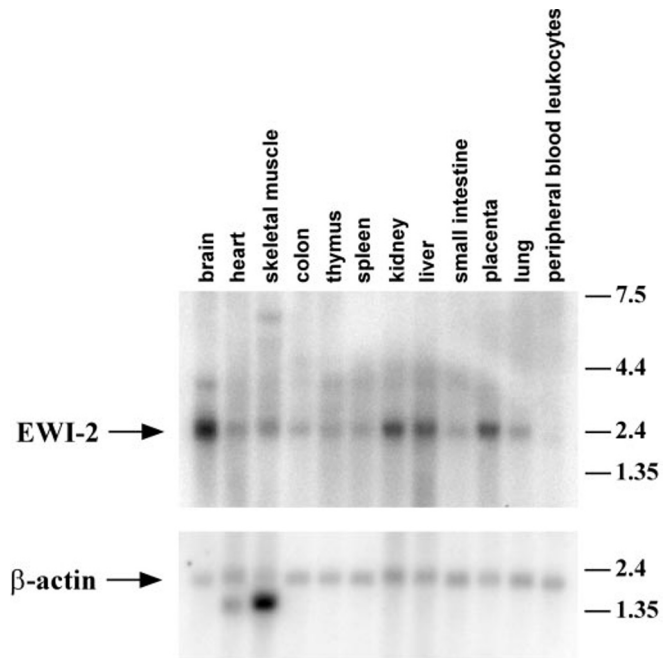


FIG. 3. Northern blot analysis of EWI-2 expression. Multiple tissue Northern blot containing 1  $\mu$ g of poly(A)<sup>+</sup> RNA/lane (CLONTECH) was hybridized with EWI-2 or  $\beta$ -actin probes. RNA size markers (in kilobases) are indicated on the right.

anti-FLAG monoclonal antibody. EWI-2 was detected specifically in CD9 and CD81 immunoprecipitates (Fig. 4B, lanes 1 and 2) but not in  $\alpha_2$  integrin,  $\alpha_6$  integrin, or CD151 immunoprecipitates (Fig. 4A, lanes 4–6). In the converse experiment, anti-CD81 immunoblotting revealed CD81 co-precipitating with CD9 and EWI-2 but not with  $\alpha_6$  integrin or CD151 (Fig. 4C). These results confirm the highly specific association of EWI-2 with CD9 and CD81.

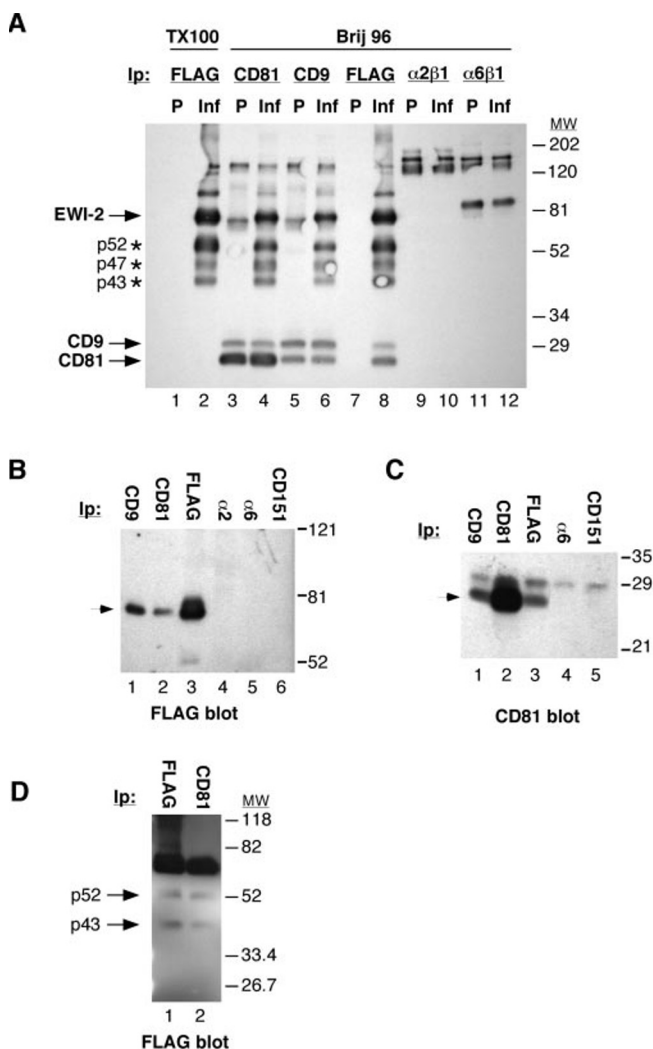
The apparent proteolytic cleavage of EWI-2 observed in Fig. 4A allowed a preliminary assessment of the region of EWI-2 involved in the CD9/CD81 interaction. An anti-FLAG immunoprecipitate from a Triton X-100 lysate of 293 EWI-2 cells was compared with an anti-CD81 immunoprecipitate from a Brij 96 lysate. Anti-FLAG blotting revealed that the 52- and 43-kDa bands observed as biotinylated species in Fig. 4A, lane 2, still contain the C-terminal FLAG epitope (Fig. 4D) and, thus, are authentic cleavage products of the EWI-2 protein. These same cleavage products also co-precipitated with CD81. This suggests that the CD9/CD81 interaction domain resides within the C-terminal 43 kDa of the EWI-2 molecule and, thus, may not involve the distal 2 Ig domains of the molecule. The variability in the apparent ratios of cleavage products to intact EWI-2 seen in Fig. 4, A *versus* D, is likely accentuated by the cutoff in detection limits seen with the M2 anti-FLAG antibody in immunoblots.

To assess the proximity of CD9 and CD81 to EWI-2, a portion of a Brij 96 lysate of 293 EWI-2 cells was treated with the

<sup>3</sup> C. Stipp, T. Kolesnikova, and M. Hemler, unpublished data.

(FPRP/CD9-P1). Using the ClustalW program, individual Ig domains of each protein were compared pairwise, and Ig domain pairs yielding the highest scores were aligned together. The Ig domain strands A–G are indicated above the alignment and numbered 1–8 according to the largest member of the family, EWI-3. The conserved EWI protein motif that helps to define the EWI protein family lies within the F2–G2 loop. The murine EWI-2 protein (*mEWI-2*), deduced from overlapping ESTs, is also included. The conserved sequence is indicated by shading when at least 60% of aligned residues are identical. Conserved cysteines in the B and F strands are shown in **bold font**. Potential N-linked glycosylation sites are outlined (*N*). Non-Ig extracellular cysteines in the membrane proximal regions of hEWI-3 and hEWI-101 are underlined, and the putative transmembrane regions are indicated by a dotted underline. Nine of the peptides sequences obtained by mass spectroscopy (listed in Table I) are indicated by a wavy underline. The four other peptides listed in Table I (not underlined) are overlapping with the nine that are underlined. The complete cDNA sequence of human EWI-2 has been deposited at GenBank<sup>TM</sup> (accession number AF407274). B, a schematic representation of the alignment in A is shown, with dotted lines connecting most closely related Ig domains (*open ovals*). EWI-101 and EWI-3 are depicted as dimers stabilized by disulfide bonds (*solid horizontal lines*) in their membrane proximal extracellular regions. C, the relatedness of the human EWI family proteins was measured by calculating percent identity over the entire alignment (*gray numbers*) or over the first two Ig domains only (*black letters*).





**FIG. 4. EWI-2 associates specifically with CD9 and CD81.** A, parental 293 cells (P) and 293 cells infected with a FLAG-tagged EWI-2 cDNA (Inf) were cell surface-labeled with biotin and lysed in 1% Triton X-100 (lanes 1–2) or 1% Brij 96 (lanes 3–12). Lysates were immunoprecipitated with anti-FLAG, anti-CD81 (M38), anti-CD9, anti- $\alpha_2\beta_1$  integrin, or anti- $\alpha_6\beta_1$  integrin monoclonal antibodies. Immunoprecipitates were resolved by SDS-PAGE and visualized with HRP-extravidin followed by chemiluminescence. Bands corresponding to CD9, CD81, and EWI-2 are indicated with arrows. Asterisks mark bands that are most likely EWI-2 cleavage products. B, 293 cells infected with the FLAG-tagged EWI-2 cDNA (293 EWI-2 cells) were lysed in 1% Brij 96 and immunoprecipitated (Ip) with anti-CD9, anti-CD81 (M38), anti-FLAG, anti- $\alpha_2$ , and anti- $\alpha_6$  antibodies. Immunoprecipitates were blotted with an anti-FLAG antibody after separation by SDS-PAGE. C, 293 EWI-2 cells were lysed in 1% Brij 96 and immunoprecipitated as in B followed by blotting for CD81. D, 293 EWI-2 cells were lysed in 1% Triton X-100 (lane 1) or in 1% Brij 96 (lane 2) and immunoprecipitated with anti-FLAG or anti-CD81 antibodies respectively. Immunoprecipitates were analyzed by blotting with an anti-FLAG antibody. CD151,  $\alpha_2$ , and  $\alpha_6$  integrin are all abundant on 293 cells, as judged by immunoprecipitation of cell surface-labeled proteins and by flow cytometry (not shown). MW, molecular mass.

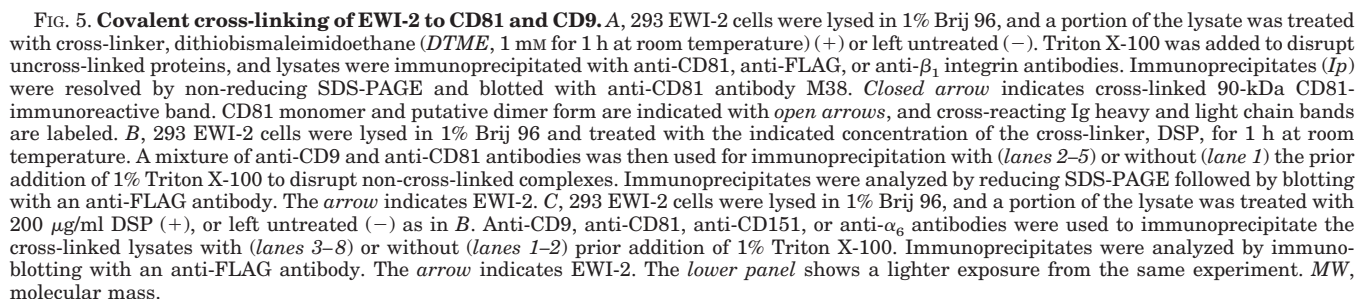
cross-linker dithiobismaleimidoethane before the addition of Triton X-100 and immunoprecipitation. The immunoprecipitates were resolved by SDS-PAGE and blotted for CD81 (Fig. 5A). In response to cross-linking, an ~90-kDa band appeared in both the CD81 and the EWI-2-FLAG immunoprecipitates (Fig. 5A, lanes 2 and 4) that was not present in the absence of cross-linker (lanes 1 and 3) or in the  $\beta_1$  immunoprecipitates (lanes 5 and 6). This CD81 immunoreactive band is of a size consistent with one molecule of CD81 cross-linked to one molecule of EWI-2. Cross-linking also resulted in the significant

stabilization of CD81 immunoreactive material of a size consistent with a dimer of CD81. Indeed, crystallography of the CD81 large loop has revealed that CD81 is likely to form a dimer (4).

To confirm that EWI-2 can be cross-linked to CD81 and/or CD9, a Brij 96 lysate of 293 EWI-2 cells was treated with various concentrations of the reducible cross-linker DSP. Triton X-100 was added to disrupt non-cross-linked complexes, and a combination of anti-CD9 and anti-CD81 antibodies was used for immunoprecipitation followed by immunoblotting with an anti-FLAG antibody. As shown in Fig. 5B (as well as Fig. 4A, lane 1), Triton X-100 completely eliminated co-precipitation of EWI-2-FLAG with CD9 and CD81 (compare lane 1 to lane 2). However, in response to increasing the DSP concentration, successively more EWI-2 could be cross-linked to CD81 and/or CD9 and recovered in Triton X-100 (Fig. 4A, lanes 3–5). In a separate experiment, a fraction of EWI-2 was cross-linked to CD81 and to a lesser extent to CD9 (Fig. 5C). A lighter exposure (Fig. 5C, lower panel) indicated that the ratio of EWI-2 cross-linked to CD9 versus CD81 (lanes 5 and 6) was lower than the ratio of EWI-2 that co-precipitated with CD9 versus CD81 in Brij 96 without cross-linking (lanes 1 and 2). No cross-linking to CD151 or  $\alpha_6$  integrin was detected (Fig. 5C, lanes 7 and 8). These data indicate that the CD81/EWI-2 interaction is highly proximal and likely to be direct. The CD9/EWI-2 interaction might also be direct but cross-linked at a lower efficiency.

**EWI-2 Interaction with CD9 and CD81 Is Highly Stoichiometric**—To estimate the stoichiometry of the EWI-2 interaction with CD9 and CD81, a Brij 96 lysate of cell surface-biotinylated 293 EWI-2 cells was depleted with non-immune mouse IgG-agarose, with anti-FLAG agarose, or with TS2/16 anti- $\beta_1$  integrin agarose, and then anti-FLAG, anti-CD81, anti-CD9, and anti- $\beta_1$  integrin immunoprecipitations were performed on each depletion. Based on results shown in Fig. 6A and semi-quantitative densitometry, we estimated that anti-FLAG immunodepletion removed ~90% of the EWI-2 from the lysate and ~60–65% of CD9 and CD81, implying a stoichiometry of ~70% for the EWI-2 interaction with CD9 and CD81. Little if any  $\beta_1$  integrin was removed upon depletion with the anti-FLAG antibody, and conversely,  $\beta_1$  integrin depletion removed little if any EWI-2, CD9, or CD81 from the lysate. A separate experiment yielded very similar results regarding EWI-2 stoichiometry (not shown).

**Comparison of EWI-2 and EWI-F Complexes with CD9 and CD81**—In 293 cells expressing only low levels of  $\alpha_3\beta_1$  integrin, CD9 and CD81-associated material migrating above 120 kDa is primarily EWI-F (30). To assess the relationship between CD9/CD81-EWI-2 and CD9/CD81-EWI-F complexes, we fractionated Brij 96 lysate from cell surface-biotinylated 293 cells. After size exclusion chromatography (which reduces overall heterogeneity), a peak fraction containing CD81 complexes was further fractionated by sucrose density gradient equilibrium centrifugation, and CD81 complexes from each fraction were immunoprecipitated. As shown in Fig. 6B and quantitated in 6C, EWI-2 and EWI-F associated with CD81 showed distinct but overlapping peaks, with EWI-2 shifted toward lower density regions of the gradient. For example the EWI-2/EWI-F ratio was 14:1 in fraction 5 but 1:3 in fraction 11 (Fig. 6C). Evidence for distinct EWI-2 and EWI-F complexes was also obtained from immunodepletion experiments. Whereas anti-FLAG-EWI-2 antibody removed ~65% of CD9 and CD81, it simultaneously removed only ~40% of the EWI-F associated with CD9 and CD81 (Fig. 6A). Thus although some CD9/CD81 complexes may contain both EWI-2 and EWI-F, a significant fraction of CD9/CD81-EWI-F complexes contains no EWI-2.



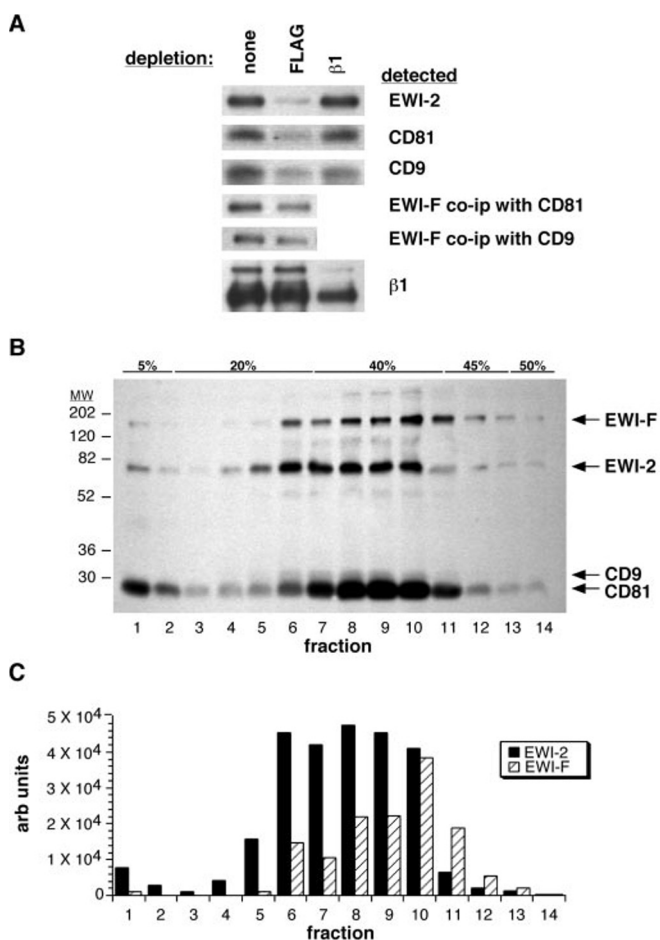
**Identification of EWI-2**—To gain insight into the functions of tetraspanins CD9 and CD81, we sought to identify major associated protein partners. Using Brij 96 detergent (see the Introduction), we purified a major CD81-associated protein of 70 kDa. Next we identified it using ion trap tandem mass spectroscopic peptide sequencing, characterized it as a novel Ig superfamily protein, and named it EWI-2 (because it is the second major CD81/CD9-associated protein discovered that contains a shared EWI motif). The human EWI-2 protein is 91% identical to its mouse counterpart, suggesting a highly conserved function. EWI-2 is widely expressed. Human and mouse cDNAs have been obtained from a wide range of tissue types, and human and mouse EWI-2 transcripts were found in almost every tissue tested including brain, liver, lung, kidney, heart, skeletal, and smooth muscle, pancreas, thyroid, thymus, spleen, testis, ovary, prostate, and uterus (Fig. 3 and Ref. 75).

**EWI-2 Is a Member of a Novel Ig Subfamily**—A BLASTP search of available protein data bases revealed a highly significant similarity between EWI-2 and three other Ig superfamily proteins, IgSF3, CD101, and FPRP/CD9-1P (*E* values = 3e-72, 1e-66, and 1e-55, respectively, where *E* represents the number of equally good sequence matches expected by chance alone). This high degree of similarity is comparable with the range of similarities seen within other subfamilies of Ig proteins (*e. g.* for ICAM-1 compared with ICAM-3, ICAM-5, and ICAM-2). The next most significant similarity to EWI-2 is the chicken Igα

Besides the EWI sequence motif, other features are shared by EWI family members. The ectodomains of all EWI proteins are composed exclusively of V-type Ig domains, an unusual feature for Ig superfamily proteins in general. All have short, highly charged cytoplasmic tails, and for all family members, the distal two Ig domains are the regions of highest homology to the other proteins in the family. This points to a potentially related function for these domains, perhaps involving binding to unknown ligands. Remarkably, EWI-F also has been identified as a major CD9 and CD81 partner (30, 63). It is not yet determined whether EWI-3 and EWI-101 are also CD9 and CD81 partners. In contrast to EWI-2 and EWI-F, the EWI-3 and EWI-101 proteins both contain extra cysteines C-terminal to their final Ig domain. Disulfide-linked dimerization of EWI-101 likely occurs via these non-Ig cysteines (78).

**Stability, Stoichiometry, and Specificity**—Of the many proposed CD9 and CD81 partners, EWI-2 and EWI-F stand out as perhaps the most stable, specific, and highly stoichiometric identified to date. EWI-2 and EWI-F formed highly stable complexes with CD9 and CD81 in the relatively stringent detergent, Brij 96. Many other proposed CD9 and CD81-associated proteins such as  $\alpha_2\beta_1$ ,  $\alpha_{IIb}\beta_3$ ,  $\alpha_L\beta_2$ , and  $\alpha_5\beta_1$  integrin, HB-EGF, and transforming growth factor- $\alpha$  have only been observed in relatively mild detergents such as CHAPS or Brij 35 or in unspecified detergent conditions (10–13, 16, 24, 79). Aside from EWI-2 and EWI-F, other Brij 96-stable associations include CD9 and/or CD81 complexes with  $\alpha_3\beta_1$  and  $\alpha_6\beta_1$  integrin, CD4, CD8, CD19, CD21, and other tetraspanins such as CD63 and CD151. However, among these proteins, EWI-2 and EWI-F associate with CD9 and CD81 with higher stoichiometry





**FIG. 6. EWI-2 complexes with CD9 and CD81 are highly stoichiometric and separable from EWI-F complexes.** *A*, 293 EWI-2 cells were cell surface-labeled with biotin and lysed in 1% Brij 96. Portions of the lysate were immunodepleted with non-immune mouse IgG agarose (*none*), M2 anti-FLAG agarose (*FLAG*), or TS2/16 anti- $\beta_1$  agarose ( $\beta_1$ ). Depleted lysates were then immunoprecipitated with anti-FLAG (EWI-2), anti-CD81, anti-CD9, or anti- $\beta_1$  antibodies. Immunoprecipitates were resolved by SDS-PAGE and visualized with extravidin-HRP. The fourth and fifth panels show levels of 130-kDa EWI-F bands co-precipitating with CD81 and CD9 with or without EWI-2-FLAG immunodepletion. *B*, a 1% Brij 96 lysate of biotinylated 293 cells was fractionated by Sepharose 6B size exclusion chromatography. A single peak fraction was then further fractionated by isopycnic sucrose density gradient centrifugation (see "Experimental Procedures"). CD81 complexes from each sucrose gradient fraction were immunopurified and analyzed by SDS-PAGE followed by blotting with extravidin-HRP. *C*, intensities of bands corresponding to EWI-2 and EWI-F (as obtained in *B*) were quantitated by densitometry. *arb*, arbitrary.

(~70% for EWI-2 and 70–100% for EWI-F (30, 63) compared with ~10–20% at best for  $\alpha_3\beta_1$  integrin and other tetraspanins (6), less than 10% for CD19 (23), and only 1–2% for CD4 (80)). Furthermore, many of these lower stoichiometry associations have been observed in cellular environments (*e. g.* MDA-MB-231 or A431 cells) in which tetraspanin-tetraspanin interactions are more evident (6).<sup>2</sup> In contrast, the 293 cellular environment seems to lack an extensive "tetraspan web" such that CD151,  $\alpha_3\beta_1$ , and  $\alpha_6\beta_1$  associated with CD9 and CD81 only at a very low stoichiometry (30)), and no EWI-2 or EWI-F association with  $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_6$ ,  $\beta_1$ , or CD151 could be detected (Ref. 30 and Fig. 4). Nonetheless, EWI-2 and EWI-F associations with CD9 and CD81 remained robust in 293 cells, suggesting that these interactions are minimally dependent on a network of background tetraspanin-tetraspanin interactions.

Lacking an anti-EWI-2 antibody, we have utilized FLAG-EWI-2 for many of our studies. Results obtained using FLAG-EWI-2 are

pertinent to endogenous CD81-associated p70/EWI-2 for several reasons, which are as follows. 1) Anti-CD81 co-immunoprecipitated only a single major surface-labeled protein in the 70-kDa region, and mass spectrometry yielded the EWI-2 protein as by far the most prominent ~70-kDa protein obtained (13 different peptides corresponded to EWI-2). 2) Results in Fig. 1 predicted that p70/EWI-2 may be a major CD81-associated partner, and this has indeed been confirmed in reciprocal co-immunoprecipitation experiments and stoichiometry studies utilizing FLAG-EWI-2. 3) Similar conclusions regarding the partial overlap of CD81-EWI-2 with CD81-EWI-F complexes were obtained whether utilizing FLAG-EWI-2 (Fig. 6A) or endogenous EWI-2 (Fig. 6, B and C).

**How Many Complexes Are There?**—Several lines of evidence indicate that tetraspanin-EWI-2 and tetraspanin-EWI-F complexes are distinct (although they can be partly overlapping in Brij 96 detergent). First, covalent cross-linking studies are consistent with a 1:1 CD81-EWI-2 complex (this study) and a 1:1 CD9-EWI-F complex (63). Second, a prior study showed that an unidentified protein strongly resembling EWI-2 could be co-immunoprecipitated (under relatively stringent detergent conditions) using antibodies to CD9 or CD81 but not to EWI-F (63). Third, the wide distribution of EWI-2 and restricted distribution of EWI-F makes it likely that CD81-EWI-2 and CD9-EWI-2 complexes should often exist in the absence of EWI-F. Fourth, immunodepletion of EWI-2 from Brij 96 lysate removed a large fraction of CD9 and CD81 but a comparatively smaller fraction of the CD9-EWI-F and CD81-EWI-F complexes. Fifth, using a two-step fractionation protocol, CD81-EWI-2 and CD81-EWI-F complexes could again be at least partially resolved. It remains to be determined why CD81-EWI-2 complexes may be slightly less dense than CD81-EWI-F complexes. In a previous experiment involving gel filtration, CD81-EWI-2 complexes also appeared to be slightly smaller than CD81-EWI-F complexes (30), consistent with the relative sizes of the two EWI proteins. We suspect that CD81-CD81 and CD81-CD9 complexes may indirectly link a subset of EWI-2 to EWI-F, thus explaining the partial overlap between the two types of EWI protein complexes. Indeed, our own cross-linking experiments (Fig. 5A) as well as crystallographic studies (4) indicate that CD81 likely exists as a dimer, and we found previously that the majority of CD9 in 293 cells is complexed with CD81 (30).

Just as CD9-EWI-F and CD81-EWI-F complexes have already been shown to exist independently (30, 63), we suspect that CD9-EWI-2 and CD81-EWI-2 complexes may also exist independently. Indeed, immunoprecipitation of CD81 from HT1080 cells or purified NT2N neurons, both of which lack CD9, yielded a 70-kDa protein strongly resembling EWI-2 (28, 29). Furthermore, covalent cross-linking yielded a 1:1 CD81-EWI-2 complex, implying no obligatory role for CD9. Cross-linking results also suggest that CD9 may be directly associated with EWI-2. Because CD9-EWI-2 cross-linking was less efficient than CD81-EWI-2 cross-linking, it is possible that CD9 association occurs within a CD9-CD81-EWI-2 "double-cross-linked" complex. However, this seems very unlikely, because we have found CD9-CD81 cross-linking to be very inefficient.

In addition to EWI-2 and EWI-F, other proteins also may be cross-linked to CD9 and/or CD81. For example, cross-linking stabilized associations between CD9, CD81, CD63, and  $\beta_1$  integrins (6, 15), between CD81 and protein kinase C $\beta$ II (81), and between CD9 and HB-EGF (24), thus strongly suggesting close associations. However, in each case a much higher concentration of cross-linker was utilized (compared with the low levels needed for CD81-EWI-2 cross-linking), and discrete cross-linked complexes of defined size and defined protein composi-



tion were not obtained, thus making it difficult to ascertain precisely the nature of the molecular proximity. In another study,  $\alpha_3\beta_1$  integrin was suggested to covalently cross-link to CD9 (15), but in retrospect, EWI-F (which co-migrates with  $\alpha_3$ ) may have actually been the protein observed.

**Potential Functional Relevance of CD9-CD81-EWI-2 Complexes**—CD9 and/or CD81 have previously been implicated in sperm-egg fusion, myoblast fusion, cell migration, tumor cell metastasis, nervous system development, HB-EGF binding activities, and cell proliferation (see the Introduction). We propose that a widely expressed partner protein such as EWI-2 could conceivably play a key role in many of these functions. Also, the CD81 large extracellular loop is clearly involved in HCV binding (37), although it may be neither necessary nor sufficient for HCV entry (82–84). Thus again, a major partner protein such as the EWI-2 molecule could possibly make an important contribution during HCV pathogenesis. In this regard, EWI-2 transcripts are readily detectable in the liver (Ref. 75 and Fig. 3).

The murine EWI-2 gene lies within a region that contains the *loop-tail* (*Lp*) mutation (75), affecting neural tube closure at E8.5 (85). EWI-2, expressed in both the adult and developing nervous system, is a potential candidate for the *loop-tail* gene although perhaps not the strongest candidate (75, 86). Interestingly, genes within the *loop-tail* region in the mouse genome also cluster together in the human genome within a region that contains multiple break points associated with a variety of cancers (75).

In summary, we have identified a novel protein, EWI-2, as a major CD9 and CD81 partner. Remarkably, EWI-2 defines a subfamily of Ig proteins that includes EWI-F, another major partner for CD9 and CD81. Together, EWI-2 and EWI-F stand apart from other proposed CD9- and CD81-associated proteins because their associations are more stable, more highly stoichiometric, and more readily demonstrated by covalent cross-linking. Because of its wide distribution, EWI-2 is an especially strong candidate for involvement in the diverse functions ascribed to CD9 and/or CD81 such as oocyte fertilization, tumor cell metastasis, nervous system development, cell proliferation, myogenesis, and HCV and diphtheria pathogenesis. Future studies of EWI-2 and EWI-F, aimed at defining CD9 and CD81 interaction sites, as well as potential ligands or counter-receptors should provide further insight into CD9 and CD81 function.

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