EWI2/PGRL Associates with the Metastasis Suppressor KAI1/CD82 and Inhibits the Migration of Prostate Cancer Cells

Xin A. Zhang, William S. Lane, Stephanie Charrin, Eric Rubinstein, and Lei Liu

Vascular Biology Center and Department of Medicine, University of Tennessee Health Science Center, Memphis, Tennessee 38163 [X. A. Z.]; Harvard Microchemistry and Proteomics Analysis Facility, Harvard University, Cambridge, Massachusetts 02114 [W. S. L.]; INSERM U268, Hopital Paul Brousse, 94807 Villejuif Cedex, France [S. C., E. R.]; and W. M. Keck Center for Comparative and Functional Genomics, University of Illinois at Urbana-Champaign, Urbana-Champaign, Illinois 61801 [L. L.]

ABSTRACT

Cancer metastasis suppressor KAI1/CD82 belongs to the tetraspanin superfamily and inversely correlates with the metastatic potential of a variety of cancers. The mechanism of KAI1/CD82-mediated metastasis suppression remains unclear. In this study, we found a M, 68,000 cell-surface protein physically associated with KAI1/CD82 and named it KASP: a KAI1/CD82-associated surface protein. Distinctive from known KAI1/CD82 associations that usually occur in the context of “tetraspanin web,” the KAI1/CD82-KASP association is likely to be direct because it is: (a) highly stoichiometric; (b) stabilized by chemical cross-linking; and (c) independent of cholesterol-enriched lipid rafts. Therefore, KASP is one of the major transmembrane proteins that associates with KAI1/CD82. Consistent with the wide distribution of KAI1/CD82, KASP is expressed ubiquitously in human tissues. Through peptide sequencing, KASP was identified as an immunoglobulin superfamily member called EWI2 or PGRL. Although EWI2/PGRL has been found to associate with tetraspanins CD9 and CD81, it forms distinct complexes with different tetraspanins, and its association with KAI1/CD82 could be independent of CD81 and CD9. Overexpression of EWI2/PGRL in DU145 metastatic prostate cancer cells inhibits cell migration on both fibronectin- and laminin-coated substratum, indicating that EWI2/PGRL directly regulates cell migration. Furthermore, EWI2/PGRL synergizes KAI1/CD82 in inhibiting cell migration, indicating that EWI2/PGRL is likely required for the function of KAI1/CD82. In summary, we identified a major KAI1/CD82-associated protein, EWI2/PGRL, that is important for KAI1/CD82-mediated suppression of cancer cell migration.

INTRODUCTION

KAI1/CD82 is closely related to cancer metastasis and has been defined as a metastasis suppressor (1–10). The role of KAI1/CD82 in metastasis suppression was originally identified in metastatic prostate cancer (1). The expression of KAI1/CD82 inversely correlates with metastasis potential of prostate cancer (2–4). Reintroducing KAI1/CD82 into prostate cancer cells resulted in a significant suppression of distal metastasis in nude mice (1). Then it was found that KAI1/CD82 expression was actually down-regulated in the advanced stages of various epithelial malignancies (5–7). Although the mechanism underlying these pathological phenomena remains unknown, studies show that ectopic expression of KAI1/CD82 can inhibit cancer metastasis in animal models, as well as cancerous cell invasiveness in vitro, a process imitating the initial steps of the dissemination of tumor cells in vivo (8–10).

KAI1/CD82 is a type III transmembrane protein and belongs to the tetraspanin superfamily, in which all of the members possess four transmembrane domains, NH2- and COOH-terminal cytoplasmic domains, and two extracellular loops (11–13). The cytoplasmic domains and an intracellular loop between the second and third transmembrane domains form the intracellular face of the KAI1/CD82 molecule (14–16), and these regions have not been tested for a role in KAI1/CD82-mediated functions. The small and large extracellular loops of KAI1/CD82 have 23 and 114 amino acids, respectively, and the large loop between the third and fourth transmembrane domains contains six cysteines that form disulfide bonds (11–16). The extracellular loops, especially the large one, may contribute to interactions of KAI1/CD82 with other transmembrane molecules, because a role for the large extracellular loop in the lateral interaction has been demonstrated in other tetraspanins such as CD81 and CD151 (17, 18).

KAI1/CD82 associates with other tetraspanins such as CD9, CD63, and CD81 in the plasma membrane, and forms a transmembrane multimolecular complex called the “tetraspanin web” (19). Meanwhile, KAI1/CD82 binds to a list of other transmembrane proteins such as αβ2 integrin, CD4, CD8, CD19, and MHC molecules (13, 19–25). Because the latter occurs at a relatively low stoichiometry and within the context of the tetraspanin web, whether these transmembrane proteins interact with KAI1/CD82 directly or through other tetraspanins remains to be investigated. KAI1/CD82 localizes subcellularly in cell peripheral “dot-like structures” and endosome/lysosome compartments, as well as in exosomes (24, 26, 27), suggesting that KAI1/CD82 may participate in the turnover of its associated transmembrane partners. Indeed, KAI1/CD82 plays a role in attenuating EGF3 signaling by accelerating EGF receptor endocytosis via its interaction with the EGF receptor (28). The tetraspanin complexes that contain KAI1/CD82 interact not only with transmembrane molecules but also with the intracellular signaling molecules such as protein kinase C and phosphatidylinositol 4-kinase (29, 30). Therefore, KAI1/CD82 may mediate cross-membrane signal transduction. Consistent with its role in signaling, KAI1/CD82 functions as a costimulator and regulates cytoskeleton rearrangement during T-cell activation (31, 32).

We hypothesize that the mechanism of metastasis suppression mediated by KAI1/CD82 is closely related to its participation in the tetraspanin complexes where it interacts with other molecules including transmembrane and intracellular signaling molecules. Therefore, understanding the molecular anatomy of the tetraspanin web becomes fundamentally important. In this study, we identified a KAI1/CD82 associated protein, EWI2/PGRL, which belongs to a novel Ig subfamily, forms a highly stoichiometric complex with KAI1/CD82, regulates prostate cancer cell migration, and synergizes the function of KAI1/CD82.

MATERIALS AND METHODS

Antibodies. Antitetraspanin antibodies were anti-CD9, DuALL (Sigma, St. Louis, MO); anti-CD63 and 6H1 (33); anti-CD81 and M38 (34); anti-CD82, M104 (34), 50F11 (BD PharMingen, San Diego, CA), and 4F9 (16); and the abbreviations used are: EGF, epidermal growth factor; DSP, dithiothenylsuccinimidylpropanate; FPRP, prostaglandin F2α receptor regulatory protein; Ig, immunoglobulin; IgSF, immunoglobulin superfamily; KASP, KAI1/CD82-associated surface protein; mAb, monoclonal antibody; mIgCD, methyl-β-cyclodextran; HRP, horseradish peroxidase; PBST, PBS with 0.1% Tween 20; MS, mass spectrometry; MS/MS, tandem mass spectrometry.

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1 Supported by the Elsa U. Pardee Foundation.
2 To whom requests for reprints should be addressed, at Vascular Biology, University of Tennessee Health Science Center, Coleman Building, Room H300, 956 Court Avenue, Memphis, TN 38163. Phone: (901) 448-3448; Fax: (901) 448-7181; E-mail: xazhang@utmem.edu.

3 The abbreviations used are: EGF, epidermal growth factor; DSP, dithiobis(succinimidylpropanate); FPRP, prostaglandin F2α receptor regulatory protein; Ig, immunoglobulin; IgSF, immunoglobulin superfamily; KASP, KAI1/CD82-associated surface protein; mAb, monoclonal antibody; mIgCD, methyl-β-cyclodextran; HRP, horseradish peroxidase; PBST, PBS with 0.1% Tween 20; MS, mass spectrometry; MS/MS, tandem mass spectrometry.
anti-CD151 and 5C11 (35). Integrin antibodies were intergins αm mAb B5G10 (36), αm mAb PUJ2 (37), αm mAb ELE (38), and β3, mAb TS2/16 (39). The mAb SA12 against human EWI2/PGRIL was generated using the tetraspanin-containing immune complexes. Other antibodies used in this study were CD98 mAb GB12 (40), MHC-I mAb W6/32 (41), and Myc tag mAb (Invitrogen, San Diego, CA). HRP-conjugated goat antimouse and goat antirabbit antibodies were purchased from Sigma.

**Cell Culture and Transfectants.** Jurkat, a T-cell leukemia cell line, and Molt4, an acute lymphoblastic leukemia cell line, were obtained from American Type Culture Collection and maintained in RPMI 1640 with 10% fetal bovine serum (Invitrogen), penicillin, and streptomycin. HT1080 fibroblastsoma cells or Du145 metastatic prostate carcinoma cells were transfected with pCDNA3.1-KAII1/CD82 plasmid DNA using Superfect reagent (Qiagen, Valencia, CA) and selected with G418 (Invitrogen) at a concentration of 1 mg/ml. The G418-resistant clones were pooled, and the KAII1/CD82-positive clones were collected by flow cytometry.

The full-length DNA of KASP was obtained by PCR using IMAGE clone 3613821 plasmid DNA as a template and pCDNA3.1-myc/HisA vector (Invitrogen) in which a sequence encoding the myc tag was fused in-frame to the COOH terminus of KASP. The construct DNA was transiently transfected into the HT1080/KAII1/CD82 cells. Du145-Mock cells, Du145-KAII1/CD82 cells, Molt4 cells, or the Jurkat-T cells, a Jurkat cell line that was stably transfected with SV40 large T antigen and originally obtained from the laboratory of Dr. Jun Liu (Massachusetts Institute of Technology, Cambridge, MA).

**Immunoprecipitation, Reimmunoprecipitation, Chemical Cross-linking, and Immunoblotting.** All of the experiments were basically performed as described previously (29, 30). Briefly, cells were lysed with 1% Brij 97 (Sigma) or 1% NP40 (Sigma) lysis buffer containing 10 mM HEPES, 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 2 mM sodium orthovanadate, and 2 mM sodium fluoride. For the experiments using surface-labeled cells, cells were biotinylated with 100 mg/ml EZlink sulfo-NHS-LC biotin (Pierce, Rockford, IL) in PBS for 1 h at room temperature followed by three rinses with PBS. After a 1-h extraction at 4°C with rocking, insoluble material was removed by centrifugation at 10,000 × g, and lysates were preincubated for 3 h at 4°C twice with a mixture of protein A- and G-Sepharose (Amersham Pharmacia, Uppsala, Sweden). Specific antibodies were added along with protein A- and G-Sepharose, and immune complexes were collected from 3 h to overnight at 4°C. 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 1% BSA in PBS. After rinsing with PBS, blots were developed with HRP-Extravidin (Sigma) and detected with Renaissance chemiluminescence kit (NEN Biotechnology, Boston, MA).

For reimmunoprecipitation, the immunoprecipitates from the 1% Brij 97 cell lysate were incubated with 1% NP40 at 4°C for 60 min to dissociate KASP from the immunocomplex. The 1% NP40 elutes were reprecipitated with the EW12/PGRIL mAb SA12 at 4°C for 6 h.

For immunoblotting, KAII1/CD82- or myc-tagged KASP-associated proteins were eluted from rinsed immune complexes with Laemmli sample buffer. Eluted proteins were separated by SDS-PAGE, transferred to nitrocellulose, and detected with antibody-specific HRP-conjugated antibodies. The blots were washed twice in 2% SSC/0.1% SDS for 30 min at room temperature and twice in 0.1× SSC/0.1% SDS for 30 min at 60°C. The hybridized signals were detected with Spotlight Chemiluminescence Detection kit (Clontech) and visualized on Kodak X-OMAT film (Eastman Kodak, Rochester, NY) by fluorography.

**Sequence Analysis.** The sequence analyses were conducted using the databases and searching tools provided by the National Center for Biotechnology and Information. Homology searches of the sequence databases were performed with the BLASTn program for nucleic acid sequence and the BLASTp for protein sequence. The full-length sequence or open reading frame of KASP was subjected to in-frame analysis using human genome database by searching against human genome database using a tBLASTn program. The pairwise multiple alignments of amino acid sequences of IgSF3, CD101, KASP, and CD9-P.1 were obtained with the CLUSTALW program. The KASP gene was localized on human chromosome 1 using the Human Map Viewer program. The genome organization of EW12/PGRIL was determined using the Genescan program. The Ig domains in EW12/PGRIL were analyzed with the RPS-BLAST program by searching the conserved domain database.

**Cell Migration Assays.** The cell migration assays were performed in modified Boyden chambers as described previously (44). Briefly, the transwell membrane filter inserts that were placed in a 24-well tissue culture plate (BD Bioscience, Bedford, MA) were 6.5 mm in diameter, 8-μm pore size, 10-μm thick polycarbonate membrane. The lower surface of the porous membrane was coated with either human plasma fibronectin (10 μg/ml) or mouse laminin 1 (10 μg/ml) at 4°C overnight and then blocked with 0.1% heat-inactivated BSA (Calbiochem, San Diego, CA) at 37°C for 45 min. The Du145 cells transfected with vector or myc-tagged KASP were resuspended in serum-free DMEM containing 0.1% heat-inactivated BSA, and 3 × 10⁵ cells were added to each insert (or the top wells). The serum-free DMEM containing 0.1% heat-inactivated BSA was added to the bottom wells. After overnight incubation at 37°C, the cells that had not migrated through the filter were removed from the upper face of the filter using cotton swabs, and cells that had migrated to the lower surface of the filters were fixed and stained with Diff-Quick (Baxter Healthcare Corp., McGraw Park, IL). The number of cells per field was counted under a light microscope at magnification ×40.

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S. Charrin and E. Rubinstein, unpublished observations.
RESULTS

KASP, a Cell Surface Protein, Specifically Associates with KAI1/CD82. In Jurkat cells, KAI1/CD82 coprecipitates a protein with a molecular weight of $M_r \sim 68,000$ (Fig. 1A). The protein is apparently a cell surface protein because it can be biotinylated in intact cells. This $M_r \sim 68,000$ cell surface protein specifically binds to KAI1, not to other cell surface molecules such as integrin $\alpha_\beta_1$ and CD98, which are expressed abundantly in Jurkat cells. We named this protein KASP, a KAI1/CD82-associated surface protein. The biotinylated KAI1 in Jurkat cells has three forms with apparent molecular weights of $M_r 45,000, 36,000$, and 32,000, and, as reported elsewhere, they represent the molecule with different degrees of glycosylation (34). We confirmed these bands were KAI1 by peptide sequencing (data not shown). The association of KASP with KAI1 was observed in the detergent 1% Brij 97 but disrupted when the Jurkat cells were lysed with 1% NP40, a stringent detergent (Fig. 1B), indicating that the KASP-KAI1 association is stable only in a relatively mild lysis condition. Also, the KASP-KAI1 interaction was observed in other cells that express KAI1, such as Moll4, Du145, and HT1080 cells (see “identification of KASP”) (see Fig. 4, 5, and 7).

Identification of KASP. To identify KASP, we prepared KAI1/CD82 complexes from 1% Brij 97 lysates of Jurkat cells, and KASP was eluted with 1% NP40, because this detergent dissociates KASP from KAI1/CD82. After SDS-PAGE separation and silver staining, a silver-stained band corresponding to where KASP migrates was excised and digested in situ with trypsin. The eluted tryptic peptides were separated by reverse-phase liquid chromatography and sequenced by ion trap MS/MS. We obtained four peptide sequences: STLQEVVGIR, SDLAVEAGAPYAER, VLPDVLQVSAAPPGPR, and SVPEAPVGR, which are derived from a novel protein with GenBank accession no. BC004108. The DNA sequence under this GenBank accession number is only a partial open reading frame that lacks the NH2-terminal sequence. To obtain the full-length KASP sequence, we performed a BLASTp search against the expressed sequence tag database using this partial sequence. Several NH2-terminal extended clones were found, and after DNA sequencing, one IMAGE clone with GenBank accession no. BE384230 encoded an open reading frame of 1839 bp that starts with the ATG start codon (45). To confirm that this sequence represented the full-length KASP, we searched the KASP partial sequence against human genome draft sequence using the tBLASTn program and obtained identical results, suggesting that this ATG is indeed the start codon. The amino acid sequence of KASP is shown in Fig. 2A. KASP contains 613 amino acids that include an extracellular domain, a putative transmembrane domain, and a short but highly charged cytoplasmic domain. The NH2-terminal signal sequence, which is typically present in secreted and transmembrane proteins, is potentially cleaved at amino acid position 27 (46). The predicted molecular weight of KASP is $M_r 62,000$. Three NH2-linked glycosylation motifs (N-X-S/T) were found in the extracellular domain (Fig. 2A), and glycosylation at these positions may be responsible for the difference between the predicted molecular weight and the actual molecular size of KASP (Fig. 1).

Through searching the conserved domain database by using RPS-BLAST program, KASP was found to be a member of IgSF, as depicted schematically in Fig. 2B, and all of the Ig-like domains in KASP were revealed as V-type Ig domains. An IgSF protein named EWI2 or PGRL was identified recently as a CD81- and CD99-associated protein (47, 48). KASP is identical to EWI2 or PGRL in amino acid sequence, indicating that they are the same protein.

Characteristics of EWI Ig Subfamily and EWI2/PGRL. In the protein sequence database, IgSF proteins V7/CD101 (49), FPRP/CD9P-1 (50–52), and IGSF3 (53) share significant sequence and structural similarities with EWI2/PGRL (Fig. 2C). They had been grouped into a novel Ig subfamily called EWI subfamily because a CXXXEWIXD/E (X can be any amino acid) sequence motif that is fully conserved among all four of the members lies in the F-G loop region of the second IgV domain (Fig. 2C; Ref. 47). Besides the EWI motif, we found two more sequence motifs that are fully conserved among all four of the proteins and localized in the NH2-terminal Ig-like domains. One is the CXXPXXTD sequence located in the F-G loop region of the first IgV domain (Fig. 2C); and another one is the HXHLXV/L sequence in the B-C loop region of the second IgV domain (Fig. 2C). Furthermore, all of the proteins contained short but highly charged cytoplasmic domains.

The EWI2/PGRL gene consists of six exons (54) and spans ~7 kb of chromosomal DNA. The first exon codes only a 21-amino acid sequence as a portion of the signal peptide. The second, third, fourth, and fifth exons code, respectively, the IgV-1, -2, -3, and -4 domains, indicating that each Ig domain is conserved as a functional unit during evolution. The last exon encodes a 22-amino acid sequence of membrane-proximal extracellular domain, the transmembrane domain, and the cytoplasmic domain (data not shown).

Using the MapViewer program (National Center for Biotechnology Information), the EWI2/PGRL gene was mapped on human chromosome 1 between the Ig22 and Ig23.2 regions. Interestingly, we found that other members of this novel Ig subfamily are also located at chromosome 1. But instead of the q arm, FPRP/CD9P-1, CD101, and IGSF3 cluster together in the 1p11 to 1p12 region (Fig. 2D). The members of the CD2 subfamily, another subfamily of IgSF, play important roles in costimulation during T-cell activation. The genes of some members of the CD2 subfamily (e.g., CD2 and CD58) are also located in the 1p11 region, and are localized concomitantly with the
Fig. 2. Protein structure and chromosomal localization of KASP. A, amino acid sequence of KASP. The predicted KASP protein contains 613 amino acids shown in the single-letter code. The transmembrane domain is underlined. The eight cysteines that frame four Ig-like domains are shaded. The putative N-glycosylation sites are marked with H. The predicted signal peptide sequence is boxed. B, schematic representation of KASP. The peptide backbone is depicted by a heavy black line. Ig domains are represented by numbered loops linked by disulfide bonds. Potential N-linked glycosylation sites are shown by the letter N. The transmembrane domain is embedded in the lipid bilayer followed by the cytoplasmic domain in the inner side of the plasma membrane. C, sequence alignment of EWI2/PGRL, CD101, FPRP/CD9P-1, and IGSF3. The significant homology of amino acid sequence by a BLASTp search was found among EWI2/PGRL and three other IgSF proteins: FPRP/CD9P-1, CD101, and IGSF3. By the Clustal W program, the overall sequences were compared pairwise, and Ig domain pairs yielding the highest scores were aligned together. The complete conserved residues among all four sequences are shaded in green. The sequences are shaded in yellow when at least 50% of aligned residues are identical and in blue when the aligned residues are similar. The numbers on the top of the alignments indicate the start and end positions of each Ig-like domain in the EWI2/PGRL sequence. The putative transmembrane regions are outlined. D, chromosome localization of the KASP gene. The genes of EWI2/PGRL, other members of the EWI Ig subfamily, and members of the CD2 subfamily were localized on human chromosome 1 by using the Human MapViewer program. The p and q arms of chromosome 1 are represented schematically. The locations of the genes on chromosome 1 are indicated with lines.
EWI2/PGRL, a KAI1/CD82-associated IGSF Protein

To characterize the tissue distribution of EW12/PGRL, the mRNAs prepared from 16 human tissues were probed with EW12/PGRL full-length cDNA. The EW12/PGRL mRNA, a major band migrated to 2.4–2.5 kb, was detectable in all of the tissues tested (Fig. 3). A minor band around 3.1–3.2 kb was found in brain tissue. The expression of EW12/PGRL was high in brain, testis, and kidney, and low in peripheral blood cells, lung, and skeletal muscle. The ubiquitous expression pattern of EW12/PGRL was consistent with the wide distribution of KAI1 (1, 16).

EW12/PGRL Forms Distinct Complexes with KAI1/CD82 and Other Tetraspanins. To verify that the biotinylated M₆ 68,000 protein KASP is indeed EW12/PGRL, we analyzed KAI1/CD82 immunoprecipitates from the Jurkat-T cells transiently transfected with myc-tagged EW12/PGRL plasmid DNA by immunoblotting with an anti-myc tag mAb. We found that KAI1 associated with the myc-tagged EW1-2PGRL that migrated to M₆ ∼68,000 (Fig. 4A). Reciprocally, KAI1 was also readily detected in the immunoprecipitate of the myc mAb (Fig. 4B). Therefore, EW12/PGRL is indeed the M₆ 68,000 KASP protein coprecipitated with KAI1.

EW12/PGRL has been reported to interact with tetraspanins CD81 and CD9 (47, 48). We confirmed that CD81 and CD9, but not CD63, coprecipitated with a KASP-like protein in Jurkat cells under the 1% Brij 97 lysis conditions (Fig. 5A). Peptide sequence analysis indicated the CD81-associated KASP-like protein was indeed EW12/PGRL (data not shown). Also, in Du145 metastatic prostate cancer cells, which lack KAI1/CD82 expression, a M₆, 68,000 KASP-like protein coprecipitated with CD9 or CD81 but not CD63 or CD151, and reimmunoprecipitation using EW12/PGRL mAb M8A12 confirmed this M₆, 68,000 protein was indeed EW12/PGRL (Fig. 5A). Because certain proportions of CD81 and KAI1 associate with each other (19, 21) and both of them bind to EW12/PGRL, whether or not CD81 mediates the KAI1-EW12/PGRL needs to be addressed. In Fig. 5B, the CD81-EW12/PGRL complex does not contain biotinylated KAI1; therefore, it appears that the CD81-EW12/PGRL interaction occurs apart from the CD81-KAI1 complex. But interestingly, the KAI1/CD82 and EW12/PGRL precipitates do contain CD81. After preclearing CD81 from the cell lysate, a substantial amount of EW12/PGRL was lost, assuming that it was codepleted with CD81. But EW12/PGRL was still evident in KAI1 immunoprecipitates in the absence of CD81 (Fig. 5B). Also, upon depleting CD81 from the cell lysate, the M₆ 32,000 KAI1 was removed from the cell lysate, possibly indicating that this form of KAI1 associates with CD81. In the CD81-KAI1-EW12/PGRL complex, whether CD81 is required for the association of KAI1 to EW12/PGRL is still unknown. At least this result demonstrates that the formation of some KAI1-EW12/PGRL complexes, if not all, is independent of CD81. In other words, KAI1-EW12/PGRL complexes could exist in the absence of CD81. Thus, CD81-KAI1, CD81-EW12/PGRL, KAI1-EW12/PGRL, and KAI1-CD81-EW12/PGRL complexes are likely the discrete complexes in the plasma membrane.

To confirm this conclusion, we tested the EW12/PGRL-KAI1/CD82 association in HT1080 fibroblasts cells that are stably transfected with KAI1. The wild-type HT1080 cells do not express KAI1. Interestingly, in the HT1080-KAI1 cells that were labeled with biotin on the surface, the KAI1 immunoprecipitates contained a M₆, 60,000–70,000 KASP-like protein but little CD81 (Fig. 5C). Because of heavy glycosylation, KAI1 was revealed as a M₆, 45,000–60,000 smear from the HT1080-KAI1 lysates. However, CD81 coprecipitated with the KASP-like protein and KAI1. The M₆, 68,000–70,000-associated protein was readily reimmunoprecipitated by EW12/PGRL mAb, confirming that the protein is EW12/PGRL. Another tetraspanin able to bind to EW12/PGRL is CD9 (Fig. 7; Ref. 47). Apparently, CD9 does not bridge the KAI1-KASP interaction in this case because HT1080 cells barely express any CD9. Therefore, the KASP-KAI1 association is independent of other tetraspanins such as CD81 and CD9 in the HT1080-KAI1 cells.

Characterization of the EW12/PGRL-KAI1/CD82 Complexes. Most known KAI1/CD82 associations are low stoichiometric interactions (13, 19–25, 28). This is due to the fact that the stoichiometry of EW12/PGRL-KAI1 complex, a Brij 97 lysate of the HT1080-KAI1 cells transiently expressing myc-tagged KASP was depleted using a KAI1 mAb M104, but the CD81-KAI1 complex is not depleted.

Fig. 3. Tissue distribution of EW12/PGRL. Northern blots containing polyadenylated RNA (1 μg/lane) from 16 human tissues were hybridized with biotinylated EW12/PGRL or β-actin cDNA probe, and the hybridized signals were then detected in a fluorography. RNA size markers (Kb) are indicated on the right. P.B.L., peripheral blood leukocyte.

Fig. 4. The KAI1/CD82-associated M₆ 68,000 surface protein is the EW12/PGRL protein. A, the Jurkat cells transiently transfected by the Myc-tagged EW12/PGRL were lysed with 1% Brij 97 or 1% NP40. The lysates were immunoprecipitated with indicated mAbs followed by a Western blot using anti-Myc mAb. B, the experimental procedures were performed as described in the legend for Fig. 6A, except that the immunoprecipitates from Mol14 cell lysates were resolved by nonreducing SDS-PAGE and blotted with the KAI1/CD82 mAb M104.

Fig. 5. Characterization of the KAI1-EW12/PGRL complexes. (A) Western blot analysis of the KAI1/CD82 complexes in the cell lysates. (B) Western blot analysis of the KAI1-EW12/PGRL complexes in the cell lysates. (C) Western blot analysis of the KAI1/CD82 complexes in the cell lysates.
a myc mAb, or a β₁ integrin mAb TS2/16. Anti-KAI1, anti-myc, and anti-β₁ integrin immunoblotting were performed on each depletion (Fig. 6A). The β₁ integrin depletion served as a negative control in this experiment, because β₁ integrin did not associate with EW1/PGRL (see above, 33). Also, the β₁ integrin did not bind KAI1 in HT1080-KAI1 cells under Brij 97 lysis condition (data not shown). Therefore, in the β₁ depletion lysate, KAI1 or myc-tagged EW1/PGRL should not be removed on the depletion of β₁ integrin. After densitometry analyses, we estimated that anti-myc immunodepletion removed 100% of myc-tagged EW1/PGRL from the lysate and 40–45% of KAI1 compared with the β₁ depletion. On the other hand, around 45–50% of myc-tagged EW1/PGRL was removed on completely depleting KAI1. These results imply stoichiometries of 45% for the

Fig. 5. The EW1/PGRL forms distinctive complexes with KAI1/CD82 and other tetraspanins. A, KASP selectively associates with tetraspanins. The biotinylated Jurkat cells or Du145 cells were lysed in 1% Brij 97 and then immunoprecipitated with mAbs against the indicated tetraspanins. The precipitated proteins were resolved by SDS-PAGE. After electrical transfer, the membranes were incubated with peroxidase-conjugated extravidin followed with a chemiluminescence. In Du145 cells, the co-precipitated proteins were eluted with 1% NP40 and reprecipitated with EW1/PGRL mAb 8A12. The reimmunoprecipitates were detected with a chemiluminescence after SDS-PAGE separation and electrical transfer. The expression levels of the immuno-precipitated proteins at cell surface were measured by flow cytometry. In Jurkat cells, the mean fluorescent intensity of CD9 is 11, CD63 24, CD81 336, and KAI1/CD82 190. In Du145 cells, the mean fluorescent intensity of CD9 is 83, CD63 129, CD81 143, and CD151 187. B, the EW1/PGRL-KAI1/CD82 association is independent of CD81. The Molt4 cells were labeled with biotin on the cell surface and lysed with 1% Brij 97 detergent. Some of the lysate was subjected to the immunoprecipitation as described in “Materials and Methods.” The rest of the lysate was precleared with CD81 mAb M38-conjugated Sepharose beads to deplete CD81. The CD81-depleted lysate was then immunoprecipitated with the indicated mAbs. All immunoprecipitates were resolved by SDS-PAGE and visualized with peroxidase-extravidin followed by chemiluminescence. C, the HT1080-KAI1/CD82 stable transfectant cells were labeled with biotin on the cell surface and then lysed with 1% Brij 97 detergent. The lysates were immunoprecipitated with an integrin α₅ mAb PUJ2, a CD81 mAb M38, a KAI1/CD82 mAb 50F11, or a CD151 mAb 5C11. Half of each individual immunoprecipitate was eluted with 1% NP40 and reimmunoprecipitated with EW1/PGRL mAb 8A12. All precipitates were resolved by SDS-PAGE and observed with peroxidase-extravidin followed by chemiluminescence. The middle panel shows a long exposure of the immunoprecipitation experiment to highlight the precipitated CD81 proteins.

Fig. 6. Biochemical analyses of the EW1/PGRL-KAI1/CD82 association. A, KAI1/CD82 forms a high stoichiometric complex with EW1/PGRL. The HT1080-CD82 cells that transiently express the myc-tagged EW1/PGRL were lysed with 1% Brij 97 detergent. Portions of the lysate were immunodepleted with a KAI1/CD82 mAb M104, a myc tag mAb, or a β₁ integrin mAb TS2/16. Depleted lysates were resolved by nonreducing SDS-PAGEs and then immunoblotted with a KAI1/CD82 mAb M104, a myc tag mAb, or a β₁ integrin mAb TS2/16. B, effects of cholesterol depletion on KAI1/CD82-EW1/PGRL association. A Brij 97 lysate was prepared from cell surface-biotinylated Jurkat cells. The lysate was divided in half and treated with the solvent 1 × PBS buffer (Control) or with 40 mM MCD for 60 min at 4°C (MCD). Then the lysates were immunoprecipitated with the indicated mAbs. The immunoprecipitates were separated by a nonreducing SDS-PAGE and visualized with peroxidase-extravidin followed by chemiluminescence. C, covalent cross-linking of EW1/PGRL to KAI1/CD82. The Jurkat cells transiently transfected with EW1/PGRL were lysed with 1% Brij 97, and a portion of the lysate was treated with cross-linker, DSP (2 min for 1 h at room temperature). NP40 was added to both DSP-treated and untreated lysates to dissociate uncrosslinked proteins. The lysates were immunoprecipitated with the indicated mAbs. Then the immunoprecipitates were analyzed in a reducing SDS-PAGE followed by blotting with the myc mAb.
EWI2/PGRL, A KAI1/CD82-ASSOCIATED IGSF PROTEIN

EWI2/PGRL-KAI1 interaction in each direction. Because the endogenous EWI2/PGRL was not taken into account, the physiological stoichiometry is likely higher.

Although the EWI2/PGRL-KAI1/CD82 association is distinguishable from or independent of the classical tetraspanin web, the proximity of two transmembrane molecules is still an essential question that needs to be addressed. Lipid rafts are membrane microdomains, stabilized by a high content of cholesterol and sphingolipids (55, 56). Because tetraspanins can be found in detergent-insoluble cholesterol-enriched lipid raft-like domains (57, 58), the KAI1-EWI2/PGRL complex may occur in, or perhaps even depend on, raft-like domains. The importance of cholesterol in the lipid rafts formation is underlined by the raft destabilizing effect of the cholesterol-depleting agent MβCD (59). We analyzed KAI1-EWI2/PGRL complexes to determine whether they would be perturbed on cholesterol depletion with MβCD. The results shown in Fig. 6B indicate that in the presence of MβCD, both the KAI1-EWI2/PGRL and CD81-EWI2/PGRL associations were not altered, indicating that the tetraspanin-EWI2/PGRL associations, including the KAI1-EWI2/PGRL association, are independent of cholesterol-enriched rafts.

To further assess the proximity of KAI1/CD82 to EW12/PGRL, lysate made from the Jurkat cells transiently transfected with myc-tagged EW12/PGRL was treated with a membrane-permeable and reducible cross-linker DSP, which has an arm span of 12 Å and was used to define protein proximity in other studies (29, 52, 60). To disrupt noncross-linked complexes, 1% NP40 was added. A combination of KAI1 mAbs M104 and 4F9 was used for immunoprecipitation followed by immunoblotting with a mAb against the myc tag. As shown in Fig. 6C, NP40 completely eliminated coprecipitation of EW12/PGRL with KAI1. However, in the presence of cross-linker DSP, the myc-tagged EW12/PGRL was recovered from KAI1 immunoprecipitates. The result indicates that EW12/PGRL-KAI1 interaction is highly proximal and likely to be a direct protein-protein interaction.

EWI2/PGRL Inhibits Cancer Cell Migration. Because the KAI1/CD82 expression is frequently lost in invasive and metastatic cancer cells, we established the KAI1 stable transfectant in Du145 metastatic prostate cancer cells (Fig. 7A) to identify the functional relevance of KAI1-EWI2/PGRL association. The CD81 expression found no difference between the mock and KAI1/CD82 transfectant cells, and served as a control in this experiment (Fig. 7A). As expected, EW12/PGRL only forms complexes with KAI1 in Du145-KAI1/CD82 cells but not in Du145-mock cells (Fig. 7B), because KAI1 is absent in Du145-mock cells (Fig. 7A). To test whether EW12/PGRL can regulate cell migration, we overexpressed the myc-tagged EW12/PGRL in Du145-mock and Du145-KAI1 cells (Fig. 7C). The overexpression of EW12/PGRL markedly attenuated the haptotactic migration of Du145 cells on either fibronectin or laminin (Fig. 7D), indicating that EW12/PGRL directly inhibits cell migration, and this inhibition is independent of KAI1. As reported elsewhere (8–10, 28), the expression of KAI1 in Du145 cells resulted in a substantial reduction of cell motility compared with the Du145-mock cells (Fig. 7E). Moreover, EW12/PGRL-transfected Du145-KAI1 cells migrated much less than the Du145-KAI1 cells that did not express EW12/PGRL, indicating that EW12/PGRL synergizes with KAI1 in motility than that of its corresponding vector-transfected cells (P < 0.001).

Fig. 7. EW12/PGRL regulates cell migration. A, expression of KAI1 in Du145 cells. The same numbers of Du145-mock and Du145-KAI1 cells were biotinylated and lysed with 1% NP40, CD82 and CD81 were immunoprecipitated with their mAbs, respectively, resolved by SDS-PAGE, and detected by extravidin-conjugated peroxidase followed by chemiluminescence. B, KAI1/CD82 associates with EW12/PGRL in Du145 cells. The biotinylated Du145 cells were lysed in 1% Triton X-100 and immunoprecipitated with either KAI1/CD82 mAb M104 or an integrin α6 mAb ELE as a control. The precipitates were treated with 1% NP40 to elute KAI1/CD82- or integrin α6-associated proteins, respectively. The eluates were reimmunoprecipitated with EW12/PGRL mAb 8A12. C, overexpression of EW12/PGRL in Du145 cells. The myc-tagged EW12/PGRL or the plasmid vector was transfected into Du145-mock and Du145-KAI1/CD82 cells. The cell lysates were immunoblotted with the myc mAb. D, EW12/PGRL inhibits the haptotactic migration of Du145 cells on fibronectin and laminin. Du145 cells expressing the myc-tagged EW12/PGRL were added into trans-well inserts that were coated with fibronectin (10 μg/ml) or laminin (10 μg/ml) at the lower surface. The cells were allowed to migrate at 37°C overnight, and then the cells that migrated through the membrane were fixed, stained, and quantitated. The migration index represents the number of cells observed in a high-power field of the light microscope. Each bar represents the mean from three separate experiments, four random fields of view per experiment; error bars, ±SD. Cell migration of the EW12/PGRL-expressing Du145 cells was significantly lower than that of its corresponding vector-transfected cells (P < 0.001). E, EW12/PGRL synergizes with KAI1 in inhibition of prostate cancer cell migration. The cell motility of Du145 double transfectant cells were measured as described above. The Du145-KAI1/CD82 and vector cells migrated significantly less than the Du145-mock and vector cells (P < 0.001), and the Du145-KAI1/CD82 and EW12/PGRL cells migrated significantly less than the Du145-KAI1/CD82 and vector cells (P < 0.001).
hypothesis. KAI1 may generate signals that inhibit motility by using PGRL, is required for KAI1-mediated suppression of cancer cell motility. Among the KAI1-associated proteins, EWI2/PGRL is the major protein associating with KAI1, EWI2/PGRL likely serves as the most important functional partner in KAI1-mediated metastasis suppression. Because most IgSF proteins engage cell-cell interactions (63), EWI2/PGRL is likely to be a cell adhesion molecule, and it may regulate cell migration or cancer invasiveness directly. Indeed, overexpression of EWI2/PGRL in DU145 prostate cancer cells inhibits cell migration directly, which coincides with the general role of KAI1 (8–10, 28). Because DU145-mock cells do not express KAI1, this inhibitory effect of EWI2/PGRL is apparently independent of KAI1. However, in the DU145 cells expressing KAI1, KAI1 forms complexes with endogenous EWI2/PGRL; and these cells are much less migratory than DU145-mock cells in which no EWI2/PGRL-KAI1 complex was formed. More EWI2/PGRL-KAI1 complexes are formed on the overexpression of EWI2/PGRL in DU145-KAI1 cells and correlate with more reduced motility of DU145-KAI1 cells. Therefore, it is very likely that the EWI2/PGRL-KAI1 complex is required for KAI1-mediated motility inhibition. It will be interesting to test in future studies whether disruption of the EWI2/PGRL-KAI1 complex will overturn KAI1-mediated inhibition of cell migration. Also, the relationship of the EWI2/PGRL expression with the cancer metastatic potential becomes an important question.

On the other hand, KAI1/CD82 may regulate the intracellular signaling pathways that are linked to its associated transmembrane proteins to suppress cancer invasiveness and metastasis. For example, the KAI1/CD82-EGF receptor coupling down-regulates EGF receptor-mediated signaling by accelerating EGF receptor endocytosis and subsequently inhibits cell migration (28). EWI2/PGRL may be connected to the intracellular signaling machinery through its highly charged cytoplasmic domain. If such a connection is present, whether or not KAI1/CD82 affects the EWI2/PGRL-mediated signaling will be an interesting signal transduction paradigm to study. The EWI2/PGRL Protein and EWI Ig Subfamily. Saupe et al. (53) reported previously significant homology among FPRP/CD9P-1, CD101/V7, and IGSF3 proteins. Stupp et al. (47) found a common EWI sequence shared by all four of the proteins. In this study, we found that the EWI members share additional common features: (a) the CXTPTXD and HXHLXV/L motifs; and (b) the localization in human chromosome 1. These common structural features suggest that four proteins may carry some common functions. Because most IgSF molecules mediate cell-cell engagement, it is likely that the members of this Ig subfamily function as cell-cell adhesion molecules, and that their ligands or counter-receptors also share structural and functional similarities. Furthermore, high homology in protein structure and adjacency at genomic localization among members of this subfamily indicate that these molecules may have evolved from the same ancestor by gene duplication. The CD2 Ig subfamily, which plays important roles in costimulation during T-cell activation (64), also clusters in chromosome 1 and localizes next to EWI2/PGRL, CD101, FPRP/CD9P-1, and IGSF3 genes. These genes are tightly linked with the ATP1A genes on chromosome 1, which have evolved from gene duplication. By inference, it is likely that duplication of the primordial genes also gave rise to the structurally related the EWI subfamily or CD2 subfamily genes. But no significant amino acid sequence homology was found between EWI and CD2 subfamilies. Therefore, the functional meaning of colocalization of two Ig subfamilies needs to be studied additionally. Several biochemical features are shared only by some EWI members, implying that individual members carry unique functions. For example, there is an extra cysteine present in the extracellular domains of CD101/V7 and IGSF3, suggesting that these molecules may be

**DISCUSSION**

**EWI2/PGRL Is One of the Major KAI1/CD82-associated Proteins.** Several KAI1/CD82-associated proteins have been identified; they include α5β1 integrin, CD4, CD8, CD19, MHC, and EGF receptor (13, 19–25, 28). The biochemical features of these interactions have not been well characterized. KAI1 binds loosely to these associated proteins, and the associations usually occur in low stoichiometry (<1%), suggesting that the interaction may be indirect. The localization of tetrascans in lipid rafts and the presence of tetrascans web make it especially difficult to assess these molecular interactions that occur among multiple transmembrane proteins. More importantly, except for KAI1-EGF receptor interaction, functional consequences of these associations are still unclear. The biochemical features of the EWI2/PGRL-KAI1 association underline the importance of this complex in KAI1-mediated functions. First, the association was found in the detergent Brij 97 (or Brij 96). Associations seen in Brij 96 have thus far proven to be functionally relevant. For example, CD81-CD19 complexes mediate B-cell signaling (61), and CD81-α5β1 complexes contribute to neurite outgrowth (62). As an intermediate stringent detergent, Brij 96 or 97 is less disruptive to transmembrane protein complexes than high-stringency detergents such as NP40 and Triton X-100 but sustains a better specificity than low-stringency detergents such as CHAPS. Brij 58, and Brij 99, in which too many associations are yielded. Secondly, at least some EWI2/PGRL-KAI1 complexes are distinct from EWI2/PGRL-CD81 complexes, KAI1-tetraspanin complexes, or tetrascan-integrin complexes. This distinction indicates that KAI1 may require different partners at different functional stages or in different subcellular environments. Furthermore, the interaction between KAI1 and EWI2/PGRL can be chemically cross-linked, is relatively highly stoichiometric, and is not mediated by lipid rafts, indicating that this association is highly proximal and likely to be a direct protein-protein interaction. Among the KAI1-associated proteins, EWI2/PGRL is the only one found thus far that is located in such proximity to KAI1. Finally, KAI1 is widely distributed in normal tissues (1), and so is EWI2/PGRL. Therefore, the EWI2/PGRL-KAI1 complex likely occurs in physiological scenarios and is functionally meaningful. In the case of the ubiquitously expressed CD81, EWI2/PGRL-CR81 complex may also be the major complex occurring in vivo compared with the recently identified CD81-FPRP/CD9P-1 complexes, because the expression of FPRP/CD9P-1 seems rather limited in normal tissues (50–52).

**The EWI2/PGRL-KAI1/CD82 Association Is Important for the Suppression of Cell Migration.** On the basis of the current understanding about KAI1/CD82, we hypothesize that the physical association of KAI1 with other transmembrane proteins, especially EWI2/PGRL, is required for KAI1-mediated suppression of cancer cell motility and metastasis. Results from the functional analyses of the KAI1-EWI2/PGRL association in this study are consistent with this hypothesis. KAI1 may generate signals that inhibit motility by using these lateral interactions through various putative mechanisms. On the one hand, KAI1 could directly use its associated transmembrane proteins to attenuate cell migration. Proteins that complex with KAI1/CD82, such as integrin α5β1, CD81, and EWI2/PGRL, play an important or direct role in cell migration. Because EWI2/PGRL is the major protein associating with KAI1, EWI2/PGRL likely serves as the most important functional partner in KAI1-mediated metastasis suppression. Because most IgSF proteins engage cell-cell interactions (63), EWI2/PGRL is likely to be a cell adhesion molecule, and it may regulate cell migration or cancer invasiveness directly. Indeed, overexpression of EWI2/PGRL in DU145 prostate cancer cells inhibits cell migration directly, which coincides with the general role of KAI1 (8–10, 28). Because DU145-mock cells do not express KAI1, this inhibitory effect of EWI2/PGRL is apparently independent of KAI1. However, in the DU145 cells expressing KAI1, KAI1 forms complexes with endogenous EWI2/PGRL; and these cells are much less migratory than DU145-mock cells in which no EWI2/PGRL-KAI1 complex was formed. More EWI2/PGRL-KAI1 complexes are formed on the overexpression of EWI2/PGRL in DU145-KAI1 cells and correlate with more reduced motility of DU145-KAI1 cells. Therefore, it is very likely that the EWI2/PGRL-KAI1 complex is required for KAI1-mediated motility inhibition. It will be interesting to test in future studies whether disruption of the EWI2/PGRL-KAI1 complex will overturn KAI1-mediated inhibition of cell migration. Also, the relationship of the EWI2/PGRL expression with the cancer metastatic potential becomes an important question.

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dimerized at the cell surface through this interchain disulfide bond. Also, in terms of the association with tetraspanins, at least EW2/PGRL and FPRP/CD9P-1 have been shown to bind to tetraspanins (47, 48, 51, 52). Whether other EWI members can associate with tetraspanins becomes an obvious question. Furthermore, because both EW2/PGRL and FPRP/CD9P-1 bind to tetraspanin web, we next need to address whether they associate with each other or if their tetraspanin-binding abilities rely on each other. Finally, an RGD sequence, the α and αi integrin-binding motif, is present in the membrane-proximal Ig-like domain of both mouse and human FPRP/CD9P-1. In the corresponding position, an RGD sequence that lacks integrin-binding affinity is found in the EW2/PGRL molecule. Whether these sites are cryptic or functionally meaningful remains to be tested.

The tissue expression patterns of the EWI subfamily members are quite diverse, indicating that individual members confine the function in a tissue-specific manner. EW2/PGRL expresses ubiquitously but is barely detectable in skeletal muscle, lung, and peripheral blood leukocytes. In contrast, CD101/V7 expresses in only lung and leukocytes (49). FPRP/CD9P-1 also has a limited expression pattern in mouse tissues (only positive in lung, uterus, and ovary; Ref. 50) but is expressed in multiple human cancer cell lines (52). The discrepancy in expression between normal tissue and cancer cell lines clearly points to the potential involvement of the EWI members in malignancy. The poorly studied IGSF3 shows an intermediate spectrum of tissue distribution and is highly expressed in lung, kidney, and placenta (53). Lung is the only organ that highly expresses CD101/V7, IGSF3, and CD9P-1 (only positive in lung, uterus, and ovary; Ref. 50) but is barely detectable in skeletal muscle, lung, and peripheral blood leukocytes.

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Xin A. Zhang, William S. Lane, Stephanie Charrin, et al.


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