The Tetraspan Protein Epithelial Membrane Protein-2 Interacts with \( \beta_1 \) Integrins and Regulates Adhesion*

The growth arrest-specific-3 (GAS3)/PMP22 proteins are members of the four-transmembrane (tetraspan) superfamily. Although the function of these proteins is poorly understood, GAS3/PMP22 proteins have been implicated in the control of growth and progression of certain cancers. Epithelial membrane protein-2 (EMP2), a GAS3/PMP22 family member, was recently identified as a putative tumor suppressor gene. Here, we addressed the normal function of EMP2 by testing the prediction that it influences integrin-related cell functions. We observed that EMP2 associates with the \( \beta_1 \) integrin subunit. Co-immunoprecipitation and immunodepletion experiments indicated that \( \sim 60\% \) of \( \beta_1 \) integrins and EMP2 can be isolated in common protein complexes. Whereas this association between EMP2 and \( \beta_1 \) integrin may be direct or indirect, it has features of integrin heterodimer selectivity. Thus, by laser confocal microscopy, EMP2 colocalized with \( \alpha_6 \beta_1 \) but not \( \alpha_5 \beta_1 \) integrin. Increased expression of EMP2 also influenced the integrin heterodimer repertoire present on the plasma membrane. EMP2 specifically increased the surface expression of the \( \alpha_6 \beta_1 \) integrin while decreasing that of the \( \alpha_5 \beta_1 \) protein. Reciprocally, reduction in EMP2 expression using a specific ribozyme decreased surface expression of \( \alpha_5 \beta_1 \) integrin. Accordingly, these EMP2-mediated changes resulted in a dramatic alteration in cellular adhesion to extracellular matrix proteins. This study demonstrates for the first time the interaction of a GAS3/PMP22 family member with an integrin protein and suggests that such interactions and their functional consequences are a physiologic role of GAS3/PMP22 proteins.

Connexins, tetraspanins, and GAS3/PMP22\(^1\) comprise the major families of tetraspan proteins. Functionally, the best understood tetraspan proteins are connexins, which form the major structural elements of gap junctions. On the other hand, tetraspanins have recently gained prominence for their role as “molecular facilitators” in the assembly of mixed protein signaling complexes, including those involving integrins (1, 2). Functionally, tetraspanins have been shown to influence integrin-mediated events such as cell proliferation, migration, and tumor cell invasion in a variety of cell types (3–8).

Currently, there are at least six known members of the GAS3/PMP22 family: PMP22, EMP1 (or TMP), EMP2 (or XMP), EMP3 (or YMP), PERP, and brain cell membrane protein 1 (9–12). These genes share \( 30–40\% \) amino acid identity (11, 13). All GAS3/PMP22 members are predicted to contain two extracellular loops and a small cytoplasmic tail, but beyond this structural information, little is known about the endogenous function(s) associated with most of the family members. What is known is that many of these proteins appear to be involved with cell proliferation, cell-cell, and cell-matrix interactions and/or myelin formation. Dysregulation of certain family members has been linked with disease (11, 14). For example, PMP22 has gained prominence due to the fact that genetic alterations in this gene (e.g. mutations, deletions, or duplications) lead to peripheral neuropathies such as Charcot-Marie-Tooth type 1A and Dejermie Sottas syndrome (14–17).

Epithelial membrane protein-2 (EMP2) was first identified based on its homology to PMP22 (11). Our laboratory initially encountered EMP2 as part of a search for genes involved in the malignant progression of B cell lymphomas using suppression subtractive hybridization (13). Disruption of EMP2 yielded dramatic phenotypes. Specifically, in a model of B cell lymphoma progression, EMP2 appears to act as a tumor suppressor (13). Moreover, ectopic overexpression of in cultured cells promoted stress-induced apoptosis (13).

In this study, we begin to address the biochemical function of EMP2/PMP22 family members. We test the idea that they may share with tetraspanins the capacity to interact with integrins (1, 18, 19). Integrins are \( \alpha \beta \) heterodimeric receptors that bind and organize cellular responses to ECM proteins and cellular receptors (21, 22). There is exceptional combinatorial diversity in this receptor system, with 16 \( \alpha \) and 9 \( \beta \) subunits and alternate splicing of individual subunits (11, 23). Individual integrin \( \alpha \beta \) heterodimers display distinct specificities for various ECM proteins and cell surface receptors, so changes in the expression of these various integrins have profound effects on the repertoire of cell proliferation, survival, adhesion, and migration (12, 14, 24).

The present study tests whether EMP2 phenotypic effects are mediated through its interaction with integrins. We show that EMP2 associates with the \( \beta_1 \) integrin subunit and appears to have a selective effect on the surface expression of the \( \alpha_5 \beta_1 \) integrin heterodimer. This change in integrin expression mediated by EMP2 confers unique binding properties onto the cell, namely adhesion to the ECM component laminin. These obser-
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vations reflect an important role for EMP2 in the regulation of an integrin-mediated cellular phenotype.

MATERIALS AND METHODS

Ribozyme Construction—An EMP2-specific hammerhead ribozyme was constructed and used to decrease endogenous expression of EMP2 in NIH3T3 cells (25). Briefly, two complementary 42-bp oligonucleotides, EMP2R1a and EMP2R1s, were synthesized by Invitrogen. The sequences were as follows: EMP2R1a, 5′-TCATCATCTGGTCCGCTTTCGAGCTCAGTTCACCCATGC-3′; EMP2R1s, 5′-CGGATGTTAGAATCCTTAGGCTGGAAACTCAGGAGTCCGAAAGGACGAAACAATGATGA-3′. Incorporation of these oligonucleotides was the 22 bases of the hammerhead ribozyme conserved catalytic core (underlined) and two 10 nucleotide EMP2-specific recognition domains (GenBank accession number AF346627; nucleotides 204–224 of the murine EMP2). To construct the ribozyme, the two oligonucleotides were phosphorylated, hybridized to one another, and ligated into the unique HpaI site located in the 3′-untranslated region of the green fluorescent protein gene in plasmid pEGFP-N3 (Clontech, Palo Alto, CA).

Cell Lines—NIH3T3 cells were cultured in Dulbecco’s modified Eagle medium (Invitrogen) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 2 mM l-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 100 units/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen). NIH3T3 cells stably expressing a FLAG-tagged murine EMP2 (referred to as “3T3/EMP2”) or an empty vector control (referred to as “3T3/V”) were established as previously described (13). NIH3T3 cells were also stably transfected with the EMP2-specific ribozyme vector (described above; pEGFP-N3-EMP2) using FuGENE 6 (Roche Molecular Biochemicals) per the manufacturer’s instructions. Stable clones were selected using Geneticin (800 μg/ml; Invitrogen). NIH3T3 cells transfected with the ribozyme construct were referred to as “3T3/RIBO.” Both 3T3/EMP2 and 3T3/RIBO represent pooled, heterogeneous populations of stably transfected cells. All cells were grown at 37 °C in a humidified, 5% CO2 atmosphere.

Northern Analysis—In vivo ribozyme cleavage of EMP2 transcripts was confirmed by Northern analysis. Total RNA was isolated from NIH3T3 or 3T3/RIBO cells using an RNA purification kit (Qiagen, Valencia, CA). RNA (5 μg) was subjected to agarose electrophoresis, transferred to a nylon membrane (Amersham Biosciences) by capillary action, and cross-linked by UV irradiation (Stratalinker; Stratagene, Valencia, CA). RNA (5 μg) was hybridized to a 5′-32P-labeled hammerhead ribozyme conserved catalytic core (underlined) and two 10 nucleotides, EMP2Rz1a and EMP2Rz1s, were synthesized by Invitrogen. The sequences were as follows: EMP2Rz1a, 5′-GTGGAACTCAGGAGTCCGAAAGGACGAAACAATGATGA-3′; EMP2Rz1s, 5′-TCATCATCTGGTCCGCTTTCGAGCTCAGTTCACCCATGC-3′. Blots were washed with a high-stringency buffer (60 °C, 0.1 SSC, 0.1% SDS) and exposed to x-ray film.

Antibodies—Rabbit polyclonal anti-EMP2 antibodies were produced as previously described (13). The anti-integrin monoclonal antibodies used were from cell clones 346-11A (anti-β1), G0H3 (anti-αv), and 5H10-27 (anti-α5) (BD Biosciences, San Diego, CA). The anti-β1 integrin antibody clones 9EG7 and 18 were purchased from BD Biosciences. Rabbit preimmune sera or isotype-matched anti-light chain antibody (0.25 μg/ml, light chain antibody (0.25 μg/ml) was subjected to agarose electrophoresis, transferred to a nylon membrane (Amersham Biosciences) by capillary action, and cross-linked by UV irradiation (Stratalinker; Stratagene, Valencia, CA). A standard static adhesion assay (15–20 min) was performed as previously described (29). Briefly, 96-well plates were precoated overnight with the ECM substances laminin, fibronectin, polyn-l-lysine (Roche Molecular Biochemicals; 5–10 μg/ml), or 1% fatty acid-free bovine serum albumin (Sigma). For collagen I or collagen IV (Becton Dickinson Labware, Bedford, MA), plates were coated 2 h as per the manufacturer’s instructions. Cells (7 × 104) were plated onto the ECM in serum-free conditions and incubated at 37 °C. Unbound cells were washed away. Bound cells were stained with toluidine blue and then lysed using 2% SDS (BioWhittaker, Walkersville, MD). The result was detected by quantitating the absorbance at 595 nm. Binding to each ECM was performed in triplicate. Each experiment was repeated at least three times. An unpaired Student’s t test was used to confirm significance between 3T3/EMP2 and 3T3/V as well as between 3T3/V and 3T3/RIBO.

In antibody blocking experiments, cells were preincubated with various dilutions of anti-αv or anti-α5 integrin monoclonal antibody for 60 min at 4 °C. Cells (7 × 104) were aliquoted into a 96-well plate pre-coated with laminin or polyn-l-lysine and allowed to adhere for 30 min. Unbound cells were washed away, and bound cells were quantitated as described above. Each experiment was repeated five times. 

Total RNA from 3T3/RIBO or 3T3/EMP2 cells were plated overnight onto glass coverslips (Fisher). Cells were fixed and permeabilized in cold methanol for 30 min at –20 °C and then blocked with 1% normal goat serum for 45 min. Cells were incubated 2 h at room temperature in a humidified chamber with the primary antibody and then washed 3–4 times with PBS plus 0.01% Triton X-100 (PBST). Cells were incubated overnight with a fluorescein isothiocyanate (FITC)-conjugated donkey anti-rat IgG and Texas Red-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at 4 °C in a humidified chamber. Cells were washed with PBST, rinsed briefly with double-distilled H2O, and mounted onto microscope slides using a 3.5% n-propyl gallate-glycerol solution (Sigma).

Flow Cytometry—The membrane expression of αv, α5, and β1 integrin subunits was assessed by flow cytometry. Cells were fixed in 2% paraformaldehyde (w/v) in PBS for 20 min on ice. Cells were incubated with primary antibody (1:200) for 30 min on ice in PBS plus 2% fetal calf serum, washed two times, and then incubated with red-phycocerythrin-conjugated anti-rat Ig, κ light chain antibody (0.25 μg/ml) cells; BD
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A. Northern (EMP2)

B. Western (EMP2, CD9)

Fig. 1. Recombinant modification of EMP2 expression in NIH3T3 cells. A, Northern analysis probed for the EMP2 transcript. Wild type cells (NIH3T3) show moderate levels of a full-length EMP2 transcript. Transcript levels are dramatically reduced in NIH3T3 cells stably bearing an EMP2-specific hammerhead ribozyme (3T3/RIBO) and show an additional band, corresponding to the predicted size of the EMP2 transcript ribozyme cleavage product (arrow). B, Western analysis with anti-EMP2 or anti-CD9. In the upper panel (anti-EMP2), levels of the EMP2 protein are detectable in NIH3T3 cells stably transfected with vector control (3T3/V) and are increased or absent, respectively, in cells transfected with an EMP2 expression vector (3T3/EMP2) or an EMP2-specific hammerhead ribozyme (3T3/RIBO). In the lower panel (anti-CD9), levels of the tetraspanin protein CD9 were unaltered in the three recombinant cell lines.

Biociences) for 30 min on ice. Negative control cells were incubated with the secondary antibody alone. After two consecutive washes, cells were resuspended in PBS and analyzed with a FACScan flow cytometer (BD Biociences). Integrin expression levels were calculated as mean fluorescent intensity (MFI). Experiments were repeated three times.

RESULTS

Production of Cell Lines with Varying Levels of EMP2—In order to elucidate the cellular function of EMP2, we first created a number of cell lines engineered to express different levels of the gene. We capitalized on the fact that wild type NIH3T3 cells express moderate levels of EMP2, so we could create variants that overexpressed (3) or underexpressed (described below) this gene compared with the wild type cells. The latter was accomplished through the stable introduction of an EMP2-specific hammerhead ribozyme vector (30), which down-regulates EMP2 expression by specific cleavage of its transcript. Northern blot analysis was used to directly validate that the EMP2-specific ribozyme cleaved the EMP2 message (Fig. 1A). Wild type NIH3T3 cells yielded a single EMP2 transcript at 4.8 kb (Fig. 1A). However, NIH3T3 cells stably transfected with an EMP2 ribozyme (called 3T3/RIBO cells) retained only a minimal signal for the normal transcript and displayed an additional lower band representing the cleaved EMP2 transcript (arrow). The residual intact EMP2 transcript probably reflects variable ribozyme expression in this nonclonal transfecnt population.

We used Western analysis to characterize the protein levels of EMP2 in empty vector control NIH3T3 cells (3T3/V), cells expressing a FLAG-tagged recombinant EMP2 (3T3/EMP2), and cells expressing the EMP2-specific ribozyme (3T3/RIBO) (Fig. 1B, upper panel). Compared with 3T3/V cells, EMP2 protein levels were higher in 3T3/EMP2 cells and undetectable in 3T3/RIBO. Western analysis was also performed to measure the levels of an independent tetraspan protein (the tetraspanin, CD9). Protein levels of CD9 were unaffected among the three recombinant 3T3 cell lines (Fig. 1B, lower panel). These observations indicated that our recombinant 3T3 cell lines were modified as intended for EMP2 expression and suggested that the modification was specific for EMP2.

EMP2 Associates with the β₁ Integrin Subunit—Using these engineered NIH3T3 variants, we examined the normal cellular function of EMP2. Initially, we chose a candidate approach drawing on the observation that numerous members of the tetraspanins associated with integrin dimers containing the β₁ subunit (12, 13, 22). Specifically, we sought to establish whether EMP2 associated with integrins and/or modified integrin-dependent function. In order to determine whether EMP2 and the β₁ integrin subunit could associate, a coimmunoprecipitation approach was employed. Cellular lysates were produced from NIH3T3 (or 3T3/V) and 3T3/EMP2 cells, incubated with an anti-β₁ integrin antibody to immunoprecipitate this molecule, and subjected to Western analysis using EMP2 antisera. As shown in Fig. 2A, Western analysis demonstrated that EMP2 immunoprecipitated with the β₁ integrin subunit. The reciprocal experiment showed similar results: lysates treated with an anti-EMP2 antibody immunoprecipitated EMP2 plus β₁ integrin protein (Fig. 2B). Positive controls, in which EMP2 and β₁ integrin immunoprecipitates were probed for the cognate protein (Fig. 2, C and D), suggested that a substantial amount of these proteins were in a common coimmunoprecipitable complex.

To estimate the fraction of the β₁ integrin subunit that associates with EMP2, immunodepletion experiments were performed according to the procedure of Stipp et al. (28) (see “Materials and Methods”). Lysates of 3T3/V or 3T3/EMP2 cell lysates were incubated overnight with anti-β₁ integrin antibody or EMP2 antisera, and the antibody with bound proteins was then immunodepleted using protein A/G beads. The amounts of EMP2 and β₁ integrin in the lysates before and after immunodepletion were quantitated by Western analysis and densitometry, and these values were used to calculate the percentage of protein in the coimmunoprecipitation (Fig. 2, E and F). As expected, anti-EMP2 and anti-β₁ integrin each depleted >90% of their cognate antigen (EMP2 and β₁ integrin, respectively). Anti-EMP2 immunoprecipitation coimmunoprecipitated 56% of the β₁ integrin subunit from 3T3/V cells and 70% from 3T3/EMP2 cells. Anti-β₁ integrin antibody coimmunoprecipitated ~65% of EMP2 in both cell types. Taken together, these data indicate that ~60% of β₁ integrin and EMP2 pools (the averages of the depletion numbers above) are present in a common co-immunoprecipitable complex after Nonidet P-40 solubilization. This association between EMP2 and β₁ integrin may be direct or indirect. However, it should be noted that the interaction between EMP2 and β₁ integrin was preserved in the presence of 1% Nonidet P-40, which unlike certain other nonionic detergents (CHAPS, Brij-97) disrupts most tetraspan-tetraspan interactions observed after such detergent solubilization (7, 8, 32). It thus appears that the association was not due to this type of nonspecific hydrophobic interactions.

We further examined the association and colocalization of EMP2 and the β₁ integrin subunit using laser-scanning confocal microscopy. NIH3T3, 3T3/V, or 3T3/EMP2 cells were stained with anti-β₁ integrin and anti-EMP2, and serial laser confocal images were captured and analyzed using Fluoview image analysis software (Fig. 3). In NIH3T3 (not shown) or 3T3/V cells (Fig. 3), EMP2 expression is predominantly perinuclear, with an additional component of dispersed cytoplasmic granules and low levels of plasma membrane expression. The perinuclear staining is consistent with endoplasmic reticulum and Golgi apparatus localization. Overexpression of EMP2 in 3T3/EMP2 cells resulted in increased EMP2 on the plasma membrane. When 3T3/V cells were double-labeled with anti-EMP2 and anti-β₁ integrin, there was a significant colocalization of staining both in the perinuclear region and cytoplasmic

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2 M. Wadehra, L. Goodglick, and J. Braun, unpublished data.
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**Fig. 2. Stoichiometric analysis of the binding of EMP2 and β₁ integrin subunit.** A–D, cell lysates were prepared from 5 x 10⁶ 3T3/V cells or 3T3/EMP2 cells in 1% Nonidet P-40 and immunoprecipitated using rabbit anti-EMP2 antisera, anti-β₁ integrin antibody (clone TS2/16), or the appropriate isotype control IgG antibodies. Precipitates were fractionated by SDS-PAGE and examined by Western analysis using anti-EMP2 (FITC secondary) and anti-β₁ integrin, since no colocalization was observed between EMP2 and another tetraspan protein, CD9, in either 3T3/V or 3T3/EMP2 cells (data not shown).

**E.** Immunodepletion: None EMP2 β₁ integrin EMP2 β₁ integrin % Depletion

|          | 3T3/V | 3T3/EMP2 |
|----------|-------|----------|
| EMP2     | 56%   | 95%      |
| β₁ integrin | 70%   | 90%      |

In 3T3/EMP2 cells, β₁ integrin colocalized with EMP2 on the plasma membrane. These results were specific for β₁ integrin, since no colocalization was observed between EMP2 and another tetraspan protein, CD9, in either 3T3/V or 3T3/EMP2 cells (data not shown).

**EMP2 Expression Modulates Binding of Cells to the ECM**—We next examined whether the apparent association of EMP2 with the β₁ integrin subunit had functional consequences. Specifically, we tested whether modulation of EMP2 expression affected the binding of cells to the ECM. To evaluate this, 3T3/V, 3T3/EMP2, and 3T3/RIBO cells were incubated for 15–20 min in plates coated with various ECM proteins (laminin, fibronectin, collagen I, collagen IV). Poly-L-lysine and 1% fatty acid-free bovine serum albumin were used as positive and negative substrates, respectively. Unbound cells were rinsed away, and the attached cells were stained with toluidine blue and quantitated as described under “Materials and Methods.” Interestingly, the cells that overexpressed EMP2 (3T3/EMP2 cells) exhibited an approximate 2-fold increase in laminin binding compared with 3T3/V cells (p < 0.05; Fig. 4A), and a 4-fold increase in laminin binding compared with 3T3/RIBO (p < 0.005; Fig. 4A).

In contrast to the results with laminin, EMP2 expression levels inversely correlated with fibronectin binding. Specifically, the cells with lower levels of EMP2 (3T3/RIBO cells) demonstrated significantly greater binding to fibronectin compared with either 3T3/V or 3T3/EMP2 cells (Fig. 4B). Within the 15–20-min incubation period, none of the cell variants displayed significant binding to collagen I or IV even in the presence of Mn²⁺, which is known to enhance integrin-mediated adhesion (34) (data not shown).

Laminin binding is primarily, although not exclusively, mediated by the α₅β₁ integrin (35). To verify that laminin binding in 3T3/V and 3T3/EMP2 cells was mediated by α₅ integrins, we tested whether an anti-α₅ integrin would successfully block this interaction. Cells were incubated with anti-α₅ integrin or with anti-α₅ integrin as a control and then allowed to adhere to a laminin-coated plate for 30 min. As anticipated, anti-α₅ integrin but not anti-α₅ integrin antibodies specifically reduced binding of 3T3/V and 3T3/EMP2 cells to laminin-coated plates (Fig. 4C and D).

Specific protein using protein A/G-agarose beads plus antibodies specific for EMP2 or β₁ integrin (clone TS2/16). Protein A/G-agarose beads alone without conjugated antibodies served as a negative control. The lysates were fractionated by SDS-PAGE and examined by Western analysis using anti-β₁ integrin (clone 18) or anti-EMP2 antisera. E and F show levels of EMP2 and β₁ integrin after immunodepletion, respectively.
**EMP2 Colocalizes Specifically with the αβ₅ Integrin Heterodimer**—We demonstrated above that EMP2 could coprecipitate with the β₅ integrin subunit and that EMP2 levels could modulate binding to laminin presumably through altering the expression and/or activity of the α₅ integrin subunit. Here we assessed whether EMP2 colocalized with the α₅ integrin subunit and whether altered protein levels of EMP2 would modulate the cellular distribution of the α₅ integrin protein. 3T3/V or 3T3/EMP2 cells were labeled with anti-EMP2 and anti-α₅ integrin antibodies, and analyzed for cellular distribution and colocalization using confocal microscopy. As shown in Fig. 5A, EMP2 colocalized with the α₅ integrin subunit in both 3T3/V and 3T3/EMP2 cells. Similar to the β₅ integrin chain, the distribution of the α₅ integrin subunit appeared more abundant on the cell surface with increased expression of EMP2 in 3T3/EMP2 cells (Fig. 5A). In contrast to the α₅ integrin subunit, EMP2 did not colocalize with the α₅ integrin chain (Fig. 5B). In 3T3/EMP2 cells, the α₅ integrin subunit was present on the plasma membrane; interestingly, overexpression of EMP2 (in 3T3/EMP2 cells) did not appear to increase surface expression of the α₅ integrin chain but rather increased α₅ integrin staining in the endoplasmic reticulum-Golgi and cytoplasmic vesicles (Fig. 5B).

**EMP2 Alters the Surface-expressed Repertoire of Integrins**—Flow cytometry was used to further assess the surface expression of the α₅, α₁, and β₅ integrin subunits in 3T3/V and 3T3/EMP2 cells. Cells were fixed, stained with antibodies detecting α₅, α₁, or β₅ integrins; and quantitated by MFI for levels of surface expression using a FACScan flow cytometer. As shown in Fig. 6, A–C, the level of the α₅ integrin subunit on the plasma membrane directly correlated with the level of EMP2. 3T3/EMP2 cells expressed ≈50% more α₅ integrin chain on their surface (MFI = 64.3) compared with 3T3/V cells (MFI = 43.2) or 3T3/RIBO cells (MFI = 39.7) (Fig. 6, A–C). In contrast, the expression of α₁ integrin protein on the cell surface was inversely correlated with the levels of EMP2, with 3T3/RIBO cells expressing ≈2-fold more α₁ integrin chain on their surface (MFI = 180.7) compared with 3T3/V cells (MFI = 87.7) and 3T3/EMP2 cells (MFI = 45.2) (Fig. 6). Whereas EMP2 levels directly or indirectly modulated the plasma membrane expression of α₅ and α₁ integrin subunits, the surface levels of the β₅ integrin subunit remained constant in 3T3/V, 3T3/EMP2, and 3T3/RIBO cells (Fig. 6, G–I). Moreover, the level of EMP2 expression had no effect on the surface expression of another membrane protein, CD9 (data not shown). These findings suggest that EMP2 expression can directly or indirectly regulate the surface repertoire of α₅ versus α₁ integrin subunits.

**DISCUSSION**

EMP2 belongs to the enigmatic GAS3/PMP22 family of tetraspan proteins, of which to date little is known regarding function or protein-protein interactions. Here we show for the first time a heterologous binding partner for a GAS3/PMP22 family member. We demonstrated by coimmunoprecipitation and immunodepletion that the majority of EMP2 and the β₅ integrin subunit exist in apparently common protein complexes. By laser confocal microscopy, EMP2 colocalized with...
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\( \alpha_6 \beta_1 \) but not \( \alpha_5 \beta_1 \) integrin, suggesting that EMP2 associates with a discrete subset of integrins. Moreover, modulating EMP2 expression levels reciprocally changed the surface expression of \( \alpha_6 \beta_1 \) and \( \alpha_5 \beta_1 \) integrin, with a corresponding alteration in cell adhesion to ECM proteins. These observations suggest that EMP2 selectively associates with certain integrin isoforms and that EMP2 levels in effect regulate integrin surface expression and function. The apparent association of EMP2 and \( \beta_1 \) integrins raises several issues. Our data indicate that the majority of both the EMP2 and \( \beta_1 \) integrin pools \((-60\% \) each\) can be isolated in reciprocally immunoprecipitable complexes. The association of EMP2 and \( \beta_1 \) integrin probably is not a simple isolation artifact for two reasons. The interaction between EMP2 and \( \beta_1 \) integrin was observed even in the presence of 1% Nonidet P-40, a detergent that overcomes some types of nonspecific hydrophobic interactions observed with other isolation conditions for tetraspan proteins \((7, 8, 32, 36)\). In addition, we did not observe an association between EMP2 and CD9, a distinct tetraspan protein, using either biochemical or laser confocal assays.

Our experimental conditions do not enumerate the absolute molecular abundance of proteins in these pools, so we cannot ascertain the molecular stoichiometry of EMP2 and \( \beta_1 \) integrin in these complexes. Also, it is not clear whether the immunoprecipitation of EMP2 and the \( \beta_1 \) integrin chain reflects a direct association of these proteins or an indirect association involving additional components of a heterogeneous noncovalent complex. For example, various tetraspanins appear to form homologous and heterologous multimers as well as heterogeneous complexes containing integrins and other protein species \((4, 6–8)\). Thus, it is likely that the molecular complexes bearing EMP2 and \( \beta_1 \) integrins include other tetraspan molecules and additional classes of membrane proteins. Definition of this heterogeneity and the process of complex formation is needed to understand the structural basis of the tetraspan-integrin association.

Our laser confocal data indicate that EMP2 selectively associates with certain integrins \((\alpha_6 \beta_1 \) but not \( \alpha_5 \beta_1 \) integrins\). This selectively suggests that the EMP2-associated and -independent \( \beta_1 \) integrin pools may in part involve \( \alpha_6 \beta_1 \) and \( \alpha_5 \beta_1 \), respectively. Selective associations between certain integrins and tetraspanin family proteins are well known. \( \alpha_6 \beta_1 \) integrins have received particular attention, including interactions with CD9, CD63, CD81, CD82, CD83, and CD151 \((1, 18, 19, 32, 39, 40–42)\). However, since tetraspanins form heterologous complexes, it is uncertain which tetraspanins are directly associated with this integrin. For example, using Triton X-100 solubiliza-

**Fig. 5.** EMP2 colocalizes with \( \alpha_6 \beta_1 \) integrin. NIH3T3 and 3T3/EMP2 cells were fixed in methanol and stained with rabbit anti-EMP2 (FITC secondary) and anti-\( \alpha_6 \) integrin (clone G0H3; Texas Red secondary) \((A)\) and rabbit anti-EMP2 (FITC secondary) and anti-\( \alpha_5 \) integrin (clone 5H10–27; Texas Red secondary) \((B)\). Colocalization between EMP2 and the \( \alpha_6 \) integrin subunit appears as yellow and is visualized in the far right panels. Images were captured on a Fluoview laser-scanning confocal microscope and merged using the Fluoview image analysis software \((version 2.1.39)\). Cells are magnified \(\times 600\).

**Fig. 6.** The level of EMP2 expression alters the integrins expressed on the surface of NIH3T3 cells. Surface integrin expression was assessed in 3T3/EMP2 cells \((A, D, \) and \(G)\), 3T3/V cells \((B, E, \) and \(H)\), or 3T3/RIBO cells \((C, F, \) and \(I)\). Cells were stained with an anti-\( \alpha_6 \) (clone G0H3; \(A-C\)) or anti-\( \alpha_5 \) integrin (clone 5H10–27; \(D-F\)), or anti-\( \beta_1 \) integrin antibodies \((clone 9EG7; G-I)\). Staining was visualized using an red-phycocerythrin-conjugated anti-rat IgG \(\kappa\) light chain antibody. Secondary antibody staining alone is indicated by the white histogram. The MFI was quantitated by flow cytometry and tabulated in the top corner of each histogram.
tion, Serru et al. (7) observed a more selective association of α5β1 integrin with CD151. Like EMP2, CD151 also appears to associate with α5β1 integrins (7), and both proteins share a functional anti-tumor phenotype (13, 40). Since NIH3T3 cells do not express α5 integrins, we could not address in the present study whether EMP2 shares this reciprocal feature with CD151.

Mechanistically, an elegant biochemical study recently has provided direct evidence for binding of CD151 and α4β1 integrins (40, 43). These findings suggest that bioactive growth factors and/or stabilization of integrin isoforms to the cell surface.

Differential expression of the various tetraspanin and GAS3/PMP22 proteins) share the structural features per-

To address the potential for rapid responsiveness through pool mobilization, it is unknown how this pool is mobilized in a subunit-specific fashion upon activation through certain signaling pathways and cell cycle transitions (31, 33, 38). One possible explanation for our results may be that the association of EMP2 and αβ1 integrins with α5 versus α5 integrin chain. Finally, EMP2 may function in the trafficking of specific membrane-bound proteins from the ER-Golgi compartment to the plasma membrane. EMP2 appears to modify the association of a diverse set of proteins and glycolipids with
cavolin-independent lipid rafts.3 The mechanism of these interactions may thus provide a fresh insight into the processes affecting surface receptor expression and the consequences for cellular functional phenotype. Clarification of such EMP2-dependent regulatory mechanisms may offer new perspectives on the function of GAS3/PMP22 proteins as well as on the cell biology of integrins.

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