Epithelial membrane protein-2 regulates surface expression of αvβ3 integrin in the endometrium

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Abstract

The four-transmembrane protein epithelial membrane protein-2 (EMP2) was recently identified as an endometrial protein necessary for blastocyst implantation, but the mechanism of this role is uncertain. In other cell types, EMP2 controls delivery of certain classes of proteins to the cell surface, including various integrin isoforms (a class of receptors implicated in endometrial–blastocyst interaction). Since αvβ3 integrin is an important endometrial molecule involved in blastocyst interaction, we evaluated the role of EMP2 in modulating integrin expression in the HEC1A endometrial cell line and endometrial epithelium in vivo. Elevation of EMP2 expression in HEC1A cells selectively increased the expression of αvβ3 integrin on the plasma membrane and was functional as judged by increased cell binding to an αvβ3 ligand, fibronectin. Conversely, reduction in EMP2 expression using an EMP2 specific ribozyme decreased the cell αvβ3 surface expression. The influence of EMP2 on αvβ3 integrin was also observed in vivo as reduction of EMP2 using ribozymes or short hairpin RNA diminished αvβ3 integrin expression in glandular and luminal uterine epithelium. Colocalization and coimmunoprecipitation studies suggested that EMP2 and αvβ3 integrin predominantly exist in a physically associated state. This study demonstrates for the first time the influence of EMP2 on αvβ3 surface expression and suggests that surface trafficking of integrin αvβ3 by EMP2 during the window of implantation may be a mechanism for its requirement in endometrial–blastocyst interaction.

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Introduction

Implantation involves a complex sequence of cell–cell interactions, cell adhesion events, and cellular activation events. For endometrial cells, hormonal and local signals instruct them to acquire receptivity during the brief implantation window. This implies the existence of a biologic process, regulated by these hormonal and local signals, that orchestrates the dramatic change in surface molecules displayed by endometrial cells during the implantation window. To date, only a few endometrial factors have been found to facilitate embryo attachment, notably integrins. Immunohistochemical studies have shown that αvβ3, α4β1, and α1β1 are present on the apical side of the luminal epithelium during the window of implantation (Basak et al., 2002; Lessey, 2002; Lessey et al., 2000). In particular, αvβ3 integrin represents a putative receptor for blastocyst attachment as it binds to ligands such as osteopontin, and neutralizing antibodies to αvβ3 prevented blastocyst implantation in mice (Illela et al., 2000, 2003).

A new molecule necessary for successful implantation is the four-transmembrane protein epithelial membrane protein 2 (EMP2). EMP2 is a member of the GAS3/PMP22 subfamily,
which together with tetraspansins and connexins comprise the three subfamilies of the large four-transmembrane (4-TM) family. EMP2 is usually restricted to the cytoplasm of the endometrial epithelium but translocates to the apical surface during the window of implantation. Moreover, inhibition of EMP2 expression in mice prevented blastocyst attachment to endometrial cells and implantation in the native endometrium, pointing to its role in endometrial competence for blastocyst interaction (Wadehra et al., submitted for publication).

Previous studies in other cell types have shown that EMP2 modulates the surface expression of integrins, MHC class I, and GPI-linked proteins and targets them to lipid raft microdomains (Wadehra et al., 2002b, 2003a, 2004). Our hypothesis is that EMP2 regulates the delivery of critical cell surface proteins required for effective endometrial–blastocyst interaction. In this fashion, EMP2 might provide an elegant biochemical switch for the properly timed uterine epithelial response required for implantation competence.

The present study examines the regulation of integrins by EMP2 in the human endometrial cell line HEC1A. EMP2 expression was modulated by ectopic overexpression or ribozyme-mediated inhibition. EMP2 overexpression selectively upregulated both the protein and steady state RNA levels of α3β1 integrin, associated with a functional augmentation of α3β1-mediated binding to the extracellular matrix fibronectin. In contrast, reduction in EMP2 expression by ribozymes or short hairpin RNA (shRNA) decreased α3β1 integrin expression both in vitro and in vivo. Colocalization and coimmunoprecipitation studies suggested that EMP2 and α3β1 integrin exist in physically associated complexes. These observations reflect an important role for EMP2 in the regulation of an integrin-mediated cellular phenotype and provide a potential mechanism for the role of EMP2 in implantation.

Materials and methods

Animals

All animals used in this study were maintained in accordance with the National Academy of Science Guide for the Care and Use of Laboratory Animals, with a controlled light schedule (14L:10D) and controlled temperature range. Female CF-1 mice (Harlan Sprague–Dawley, Indianapolis, Indiana) were superovulated by IP administration of 1 IU pregnant mare’s serum gonadotropin (PMSG; Calbiochem, San Diego, California) followed 48 h later by 5 IU human chorionic gonadotropin (hCG; Sigma, St. Louis, Missouri) and then housed individually with single B6D2F1/J males (Jackson Laboratory, Bar Harbor, Maine) overnight. Only females with evidence of a vaginal plug the following morning were utilized for subsequent experiments; the day of the vaginal plug was recorded as postcoital (pc) day 1.

EMP2 constructs

Ribozymes

Hammerhead ribozymes were created to cleave the mouse or human EMP2 transcripts. The murine EMP2 hammerhead ribozyme, inserted into pEGFP-N3, was designated pEGFP-mRZ1 (Wadehra et al., 2002b). The hRZ2 construct targeting human EMP2 consisted of complementary 42 bp oligonucleotides containing 22 bases of the hammerhead ribozyme conserved catalytic core and two 10 nucleotide human EMP2 recognition domains (based on nucleotides 376–396 of the human EMP2 sequence; GenBank accession no. NM001424 (Wadehra et al., submitted for publication)). Both ribozyme sequences reduced EMP2 expression in HEC1A cells under transient and stable transfection conditions. However, the hRZ1 ribozyme sequence was more potent (~2-fold) for EMP2 knockdown in transient transfection (Wadehra et al., submitted for publication), whereas the hRZ2 ribozyme sequence was similarly more potent for stable transfection. The reasons for these differences are uncertain but might reflect greater intracellular stability of the hRZ1 sequence under transient conditions and more efficient genomic integration of the hRZ1 sequence for stable transfection.

Hairpin RNAs

As a second reagent to reduce EMP2 expression, a hairpin RNA targeting construct was produced. PCR SHAging was used to generate RNA polymerase III (U6 snRNA promoter)-driven constructs expressing 29 bp shRNA targeting mouse or human EMP2 (www.cshl.org/public/SCIENCE/Hannon.html). The human U6 promoter from pGEM-Zeo-U6 (generous gift of Gregory Hannon, Cold Spring Harbor Laboratory, Cold Spring, NY) was amplified using a forward primer 5′-GATTAGTGACACTATAG-3′ (Sp6 promoter sequence) and one of two reverse primers (shRNA sequences underlined, U6 promoter sequence in bold):

Mouse EMP2 (targets nucleotides 260–288; GenBank accession no. NM007929): 5′-AAAAACACGATGCAGGAAGACAATAAT-GAAACCCAAGCTTCCTCATTGCTTCTTTCCACATCG-GTTCGTTGTCTCTCCACCAAA-3′

Human EMP2 (targets nucleotides 508–536; GenBank accession no. NM001424): 5′-AAAAAAAGACGCGCAGAATCATAAAGACGACGCCAGCTTCATGTCTGTATGATTGCGGCCTCCGGF-GTTTGCTCTTTACCAAA-3′

PCR was performed using puReTaq Ready-To-Go PCR Beads (Amer sham Biosciences, Piscataway, NJ) in a 25-μl reaction as follows: an initial denaturation step at 95°C for 5 min followed by 30 cycles at 95°C for 30 s, 52°C for 30 s, and 72°C for 20 s, and then 72°C for 5 min. The PCR product was TA-cloned into pCR II (Invitrogen, Carlsbad, CA). The resulting constructs targeting mouse or human EMP2 were designated m-shRNA and h-shRNA.

EMP2-GFP

To ectopically increase the expression of human EMP2, the complete human EMP2 open reading frame was generated using primers bearing flanking EcoRI sites (underlined), inclusive of the native transscriptional start site (bold):

5′ primer: CGGAAATTCACCACATGTGGTCTGCCCTTT
3′ primer: CGGAATTCACATTGGCGCTTCCTCAGTATCAG

PCR products were EcoRI-digested and subsequently cloned in frame with GFP in the EGFp-N3 expression vector. This human EMP2-GFP fusion protein expression vector was designated pEFGP-hEMP2.

All constructs were verified by DNA sequencing (Davis Sequencing Facility, UC Davis), and plasmid DNA for transfection was column purified (Qiagen, Valencia, CA).
Cell lines

HEC1A cells (ATCC, Manassas, VA) were cultured in McCoy's 5a medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS, Hyclone, Logan, UT), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen). Stable transfectants of HEC1A were produced with pEGFP-NEMP2 for overexpression, pEGFP-hRZ2 for EMP2 knockdown, and pEGFP-N3 as a control. These transfectants were designated HEC1A-hEMP2, HEC1A-hRZ2, and HEC1A-GFP, respectively. HEC1A cells were transfected using FuGENE 6 (Roche Molecular Biochemicals). Stable clones were selected using Geneticin (800 μg/ml, Invitrogen) followed by flow sorting of GFP-positive cells. All cells were grown at 37°C in a humidified, 5% CO2 atmosphere.

Antibodies

Rabbit polyclonal anti-human and mouse EMP2 antibodies were previously described (Wadehra et al., 2003b; Wang et al., 2001). Monoclonal antibodies (mAbs) specific for mouse α6 (clone G0H3), αvβ3 (clone 23C6), α1 (clone SR84), α5 (clone 5H10–27), β1 (clone 9EG7 and 18), and β3 (clone 1) integrin isoforms were obtained from BD Biosciences (San Diego, CA). A rabbit antibody specific for mouse β3 integrin was obtained from Chemicon (Temecula, CA). R-PE-conjugated antibodies specific for rat Ig, α light chain or mouse IgG were from BD Biosciences.

Adhesion assays

A standard static adhesion assay (15–20 min) was performed as previously described (Wadehra et al., 2002b). Briefly, 96-well plates were precoated overnight with 10 μg/ml laminin, fibronectin, or poly-D-lysine (Roche Molecular Biochemicals, Indianapolis, Indiana) or with 1% fatty acid free BSA (Sigma, St. Louis, MO). For collagen I or collagen IV, plates were coated for 2 h as per manufacturer’s instructions (Becton Dickinson Labware, Bedford, MA). Cells (7 × 10^4) were added to the wells in serum-free conditions, incubated for indicated times at 37°C, and the unbound cells were then washed away. The adherent cells were stained with toluidine blue, lysed with 2% SDS (BioWhittaker, Walkersville, MD), and quantitated by the absorbance at 595 nm. Binding to each extracellular matrix protein was performed in triplicate, and the mean and standard deviation were calculated. Each experiment was repeated at least three times, and groups were compared using an unpaired Student’s t test.

In antibody blocking experiments, cells were preincubated with various dilutions of mAbs for 60 min at 4°C. Cells (7 × 10^4) were aliquoted into a 96-well plate precoated with laminin or poly-D-lysine. Each experiment was at least repeated five times.

Quantitation of EMP2 and β3 integrin levels

In order to estimate the difference in EMP2 and β3 integrin levels, HEC1A-GFP and HEC1A-hEMP2 cells were resuspended at 1 × 10^6 cells/ml in Laemmli buffer. The cell number equivalents of HEC1A-GFP and HEC1A-hEMP2 were then titrated by serial two-fold dilutions and separated by SDS-PAGE. Blots were analyzed for EMP2 and β3 levels expression. As EMP2 contains multiple glycosylation sites (Wang et al., 2001), N-linked glycans were cleaved using PNGase (New England Biolabs, Beverly, MA). Eluates were treated as per manufacturer’s instructions at 37°C for 2–3 h. Experiments were repeated at least three times independently, and results of the different experiments were used to determine means and standard errors for each group. Groups were compared using an unpaired Student’s t test.

Flow cytometry

The membrane expression of α1, α2, α4, α5, α6, and αvβ3 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 for 30 min on ice in PBS ± 2% FCS. The cells were washed two times and incubated with R-PE-conjugated anti-rat Ig, α light chain antibody or an R-PE-conjugated anti-mouse IgG antibody for 30 min on ice (BD Biosciences). R-PE was used at 0.25 μg/million cells. As a negative control, cells were incubated with secondary alone. After two consecutive washes, cells were resuspended in PBS and analyzed with a FACScan flow cytometry (BD Biosciences). Integrin expression levels were calculated as mean fluorescent intensity (MFI).

Northern blot analysis

Total RNA was isolated from cells using the Qiagen RNeasy RNA isolation kit and RNeasy MiniElute RNA cleanup kit according to the manufacturer’s instructions. RNA (10 μg) was subjected to denaturing agarose gel electrophoresis and transferred to Hybond-XL nylon membrane (Amersham Biosciences) by capillary action and cross-linked by UV irradiation (Stratalinker; Stratagene, San Diego, CA). Using HEC1A cDNA, a 495 bp human beta 1 integrin probe was produced by PCR using 5'-CAGAGAAGCTCAAGCCAGAGG-3’ (sense) and 5'-CTCCAGCCAATCGATCCAC-3’ (antisense) oligonucleotides (30 cycles, annealing 50°C). A 539 bp human beta 3 integrin probe was produced under the same conditions with 5'-GTGACCTGAAGAATCTGCTG-3’ (sense) and 5'-CCTCTCGGTTCGTTGACAC-3’ (antisense). A purified GAPDH probe was produced as previously described (Wadehra et al., 2002a). Purified PCR products were labeled using a random primer labeling kit (Stratagene) with [32P]dCTP (Amersham Biosciences). Membranes were prehybridized with Rapid-Hyb buffer (Amersham Biosciences) for 1 h and hybridized with labeled probe overnight. Blots were then washed twice in 2× SSC and 0.1% SDS for 10 min at 65°C and then twice more in 0.1× SSC and 0.1% SDS for 10 min at 65°C and exposed to X-ray film.

Inhibition of uterine EMP2 expression in vivo

Liposome-mediated plasmid transfection in vivo was described previously (Wadehra et al., submitted for publication). DNA–liposomes were prepared immediately prior to transfection surgery by mixing the plasmid DNA with FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, Indiana) in a 1:3 (μg/μl) ratio per manufacturer’s instructions, incubated for 15 min, and finally volume-adjusted with sterile PBS so the desired amount of DNA could be injected in a final volume of 100 μl. In the afternoon of day 1 pc, females were anesthetized, and the distal portion of each uterine horn was identified via small flank incisions. The uterine horns each was slowly injected via a 30-G needle with 100 μl of the appropriate DNA–liposome mixture. Luminal placement of the injected fluid was confirmed by observation of slight distension of the visible portion of the uterine horn. Of note, in test experiments on CF1 mice in which the entire reproductive tract was exposed via an abdominal incision, the capacity of a uterine horn was only about 20 μl. Injected fluid above this volume leaked rapidly into the vagina. When injections were performed with Coomassie Blue dye to track the injected fluid, no dye was observed to enter the contralateral uterine horn. However, because of the possibility of crossover of the fluid into the opposite uterine horn, the first horn injected was the “treatment” horn, and the second horn was injected with the control DNA so that any crossover fluid would be washed out of the control horn. This method was chosen over using different mice for each type of DNA so that each mouse could serve as its own internal control.

To assess the efficacy of the transfected plasmids in reducing EMP2 expression, females were euthanized 4 days after transfection (day 5 postcoitum [pc]), and the uteri were processed for immunohistochemistry using mEMP2 antisera or the corresponding preimmune control. Sections from the same tissue blocks were then stained for β3 integrin as outlined below.

Immunohistochemistry

Uterine tissue was fixed in 10% formalin, embedded in paraffin, and sectioned at 5 μm. Slides were incubated in 0.1 M citrate pH 6.0 at 95°C for 20 min to improve antigen exposure. Samples were incubated with β3 antisera (1:50), EMP2 antisera (1:250), or a rabbit serum control at the same dilution and then incubated with the Vectastain ABC kit (Vector Labs, Burlingame, CA) according to manufacturer’s instructions. EMP2 expression was detected using Vector VIP (Vector Labs), with methyl green used as a counterstain. β3 integrin staining was visualized using diaminobenzidine HCl (DAB; Vector Labs), and nuclei were counterstained in Mayer’s hematoxylin (DAKO).
Immunoprecipitation

HEC1A cells ($1 \times 10^5$) were plated overnight in 100 mm dishes (Corning, Corning, NY). Cells were washed 2 times with PBS, lysed (1% Nonidet P-40 containing 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 2 μg/ml pepstatin, 10 mM iodoacetamide, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM HEPES, and 10 mM KCl) for 30 min at 4°C, and then sonicated for 15 s. Cell lysates were precleared by incubation with Protein A agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) and then incubated overnight with agarose beads bound to either anti-EMP2 rabbit polyclonal antibodies or an anti-α3 mAb. The beads were washed 3–4 times in lysis solution and finally in 50 mM Tris, pH 8 buffer. Immune complexes were eluted from the beads using Laemmli sample buffer (62.5 mM Tris–Cl, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenol blue, 2% β-mercaptoethanol). Samples were analyzed using Western blot analysis as described above.

Results

Characterization of integrins in HEC1A cells

The human endometrial carcinoma cell line HEC1A has been shown to be permissive for blastocyst attachment (Wadehra et al., submitted for publication). As an initial assessment, we characterized the expression of integrins on HEC1A thought to be important for blastocyst attachment. The surface expression of αvβ3, αvβ5, α6, β1, β4, α5, α4, α2, and α1 was determined by flow cytometry. HEC1A cells express high levels of αvβ3, β1, and α6 integrins. Moderate to low levels were observed for αvβ5, β4, α5, and α1 integrins (Fig. 1). Expression of α4 and α2 integrins was below the limit of detection (data not shown). It should be noted that this assessment does not compare absolute integrin isoforms since the relative levels are in part affected by characteristics of anti-integrin antibodies.

EMP2 selectively modulates expression of integrin isoforms in other cell types. To test the effect of EMP2 on integrin expression in endometrial cells, stable transfectant cell lines were generated that ectopically overexpressed EMP2 (HEC1A-hEMP2), expressed a vector control (HEC1A-GFP), or expressed a ribozyme to decrease EMP2 expression (HEC1A-hRZ2). By Western analysis, EMP2 expression in HEC1A-hEMP2 and HEC1A-hRZ2 was 8.7-fold and 0.2-fold of vector

Fig. 1. Expression of integrins in HEC1A cells. HEC1A cells were fixed and stained for integrins αvβ3, αvβ5, β1, β4, α1, α4, and α5. Secondary antibody staining alone is indicated by the black line. Flow cytometry was used to quantitate the mean fluorescent intensity (MFI).
control, respectively (Fig. 2). These HEC1A sublines were stained for \( \alpha_v \beta_3 \), \( \alpha_v \beta_5 \), \( \beta_1 \), or \( \alpha_v \beta_5 \) integrins and analyzed by flow cytometry (Fig. 3). EMP2 expression specifically enhanced the surface expression of \( \alpha_v \beta_3 \) integrin, while the ribozyme decreased \( \alpha_v \beta_3 \) expression (Fig. 3A). HEC1A-hEMP2 cells expressed \( \geq 50\% \) more \( \alpha_v \beta_3 \) integrin on their surface (MFI, 64.3) compared to vector control (MFI, 43.2) or cells lacking EMP2 (MFI, 39.7). In contrast, modulation of EMP2 expression had no effect on the surface expression of integrin \( \alpha_v \beta_5 \) or the \( \beta_1 \) integrin subunit (Figs. 3B–C). Similarly, alterations in EMP2 levels did not alter the levels of \( \beta_4 \), \( \alpha_1 \), \( \alpha_5 \), or \( \alpha_6 \) integrin surface expression (data not shown).

**Modulation of EMP2 expression alters extracellular matrix binding**

One important function of integrins is to orchestrate binding to extracellular matrix proteins. To validate the functional effect of EMP2 expression levels on integrin ligand specificity, HEC1A-GFP, HEC1A-hEMP2, and HEC1A-hRZ2 were tested for their binding avidity to different extracellular matrix proteins. As shown in Fig. 4A, the expression of EMP2 regulated the ability of HEC1A cells to bind fibronectin. HEC1A-hEMP2 exhibited a 2-fold increase in fibronectin binding compared to vector control cells and a 4-fold increase in fibronectin binding compared to HEC1A-hRZ2. The differences between HEC1A-hEMP2 and HEC1A-GFP \((P < 0.05)\) and between HEC1A-GFP and HEC1A-hRZ2 \((P = 0.005)\) were significant. Moreover, the regulation of fibronectin binding by EMP2 was specific as no significant differences in laminin or collagen I binding were observed among the three cell types (Fig. 4B).

Fibronectin binding is mediated by a variety of integrin pairs and non-integrin receptors such as lectins (Belkin and Stepp, 2000). To verify that the fibronectin binding observed in HEC1A cells was mediated by integrin \( \alpha_v \beta_3 \), we tested whether adhesion to extracellular matrix proteins was blocked by anti-\( \alpha_v \beta_3 \) integrin antibodies (Fig. 4C). Cells were

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**Fig. 2. Recombinant modification of EMP2 expression levels.** Steady state protein levels of EMP2 were measured by Western blot analysis in HEC1A-GFP (control cells), HEC1A-hEMP2 cells (high EMP2), and HEC1A-hRZ2 cells (minimal EMP2). Bands corresponding to GFP-EMP2 fusion protein (48 kD) and native EMP2 (20 kD) are indicated. Experiments were performed independently at least three times with similar results.

**Fig. 3.** The expression level of EMP2 alters surface levels of \( \alpha_v \beta_3 \) integrin in HEC1A cells. HEC1A cells expressing EMP2 at elevated (HEC1A-hEMP2; A and D), vector control (HEC1A-GFP; B and E), or minimal (HEC1A-hRZ2; C and F) levels were stained with an anti-\( \alpha_v \beta_3 \) (A–C) or anti-\( \alpha_6 \) (D–F). Staining was visualized using an R-PE-conjugated anti-rat Ig, \( \kappa \) light chain antibody. The MFI was quantitated by flow cytometry and tabulated in the top corner of each histogram.
preincubated with antibodies to either αvβ3, α5 (negative control), or α6 (negative control) integrin and allowed to adhere to a 96-well fibronectin-coated plate for 25 min. We observed that only αvβ3 integrin antibodies specifically and completely abolished the binding of HEC1A-GFP and HEC1A-hEMP2 cells to fibronectin-coated plates. These findings indicate that integrin αvβ3 serves as the predominant fibronectin-binding receptor in both wildtype and EMP2-overexpressing HEC1A cells. Thus, the increase in fibronectin binding caused by EMP2 was αvβ3-dependent.

Coordinate expression of EMP2 and β3 integrin during the window of implantation

As both EMP2 and β3 integrin are necessary for successful implantation, we hypothesized that the two proteins might colocalize. Mice were hyperstimulated, mated, and uteri were collected every 24 h until day 6 pc. The expression pattern of EMP2 at day 1 pc was perinuclear, while β3 integrin expression was generally negative in luminal epithelium (Fig. 5A, left). By day 3, β3 integrin was up-expressed, and its distribution relative to EMP2 was fully concordant. Thus, the cellular localization of EMP2 and β3 integrin on day 3 pc was diffuse, with staining throughout the cytosol (Fig. 5A, middle). By day 5 pc, both proteins had established a prominent apical surface distribution on the luminal epithelium (Fig. 4A, right). No staining was observed in tissue stained with control serum (data not shown).

Biochemical association of EMP2 and β3 integrin

Since EMP2 and β3 integrin colocalized in native epithelium, we tested the idea that they might exist in a biochemical association by coimmunoprecipitation. HEC1A cell lysates were prepared in non-ionic detergent (1% Nonidet P-40), sedimented at 300 × g to remove nuclei, immunoprecipitated with anti-EMP2 or anti-β3 integrin, and detected by Western immunoblots for the cognate or reciprocal proteins (Fig. 5B). As expected, anti-EMP2 pulled down EMP2; comparison with direct assay of equivalent cell lysate indicated that the immunoprecipitation was quantitative. However, anti-EMP2 also quantitatively pulled down β3 integrin (Fig. 5B, left). Similarly, anti-β3 integrin quantitatively pulled down both β3 integrin and EMP2 (Fig. 5B, right). Notably, this association occurred in the presence of 1% Nonidet P-40, which is a relatively strong non-ionic detergent that does not sustain nonspecific hydrophobic interactions observed with 4-TM proteins in the presence of detergents like Brij 97 or CHAPS (Knobeloch et al., 2000; Lagaudriere-Gesbert et al., 1997; Levy and Shoham, 2005; Wadehra et al., 2004). These findings suggest that the EMP2 can associate with the β3 integrin subunit and that a large fraction of these proteins may exist in a complex containing both molecular species.

EMP2 expression increases total β3 integrin protein levels

The preceding sections showed that EMP2 associates with β3 integrin and that modified EMP2 expression alters levels of αvβ3 integrin surface expression. To determine if EMP2 influences total protein levels of this integrin isoform, Western blot analysis was performed. Lysates were analyzed for EMP2, αvβ3 integrin, β3 integrin, β1 integrin, and β-actin expression. EMP2 overexpression selectively increased total β3 integrin levels but did not alter β1 or β-actin protein expression (Fig. 6A). The commercially available antibody for αvβ3 integrin was unsuitable for Western analysis (data not shown), so β3 integrin was used as a surrogate for this integrin species.

To quantitate the ratio of β3 integrin to EMP2 expression, titrations of equivalent cell lysates by Western blot analysis were performed (Fig. 6A, and data not shown). HEC1A-
EMP2 cells showed a 5.3-fold (±1.2, SEM) increase in β3 integrin compared to vector control cells. This correlated with an increase in EMP2 (GFP-EMP2 fusion protein plus native EMP2) of 8.7-fold (±0.7 SEM) in HEC1A-EMP2 cells versus native EMP2 in vector control cells (Fig. 2). These results indicated the quantitatively proportional changes in EMP2 and β3 integrin levels. Since EMP2 and αvβ3 integrin appear to exist as physical complexes (see preceding section), this proportionality suggests that αvβ3 integrin levels may be limited by the EMP2 available to form ternary complexes.

EMP2 expression alters β3 integrin mRNA levels

We have shown that EMP2 expression selectively altered the total protein levels for β3 integrin, but not β1 integrin. In order to determine if EMP2 expression altered the mRNA steady state levels of integrin subunits, Northern blots were performed on populations of HEC1A, HEC1A-EMP2, and HEC1A-hRZ2 cells. Analysis revealed no significant differences in the
transcription of β1 integrin or GAPDH (Fig. 6B). However, reduction in EMP2 expression by ribozyme cleavage reduced the steady state mRNA levels of β3 integrin by two-fold over cells expressing EMP2 (HEC1A and HEC1A-hEMP2). No significant differences were observed between control HEC1A cells and HEC1A-hRZ2. These data suggested that the increase in β3 integrin protein levels in HEC1A-hEMP2 cells might result from decreased turnover of the protein, whereas the decrease in integrin β3 levels in HEC1A-hRZ2 cells might result from a decrease in either the stability or transcription of integrin β3 mRNA. However, further study is necessary to establish the mechanism of these EMP2 effects on β3 expression.

EMP2 expression controls αvβ3 integrin expression in vivo

αvβ3 integrin expression appears to be regulated by EMP2 in vitro. To determine if this control occurred in vivo, we utilized ribozyme- and RNA interference-mediated techniques to knock down expression of EMP2 in mouse uterine horns and then examined the effect of decreasing EMP2 on the expression of αvβ3 integrin in the same tissue. This approach allowed us to examine the relationship between EMP2 and αvβ3 integrin expression in the setting of the living mouse uterus, in the presence of physiologically relevant amounts of hormones, cytokines, and growth factors that normally control endometrial protein expression. Mouse uterine horns were transfected on day 1 pc with expression plasmids encoding a mouse EMP2-specific ribozyme (pEGFP-mRZ1), shRNA targeting mouse EMP2 (m-shRNA), or control vectors (h-shRNA for human EMP2 shRNA; or pEGFP for GFP alone). Previous results demonstrated that the pEGFP-mRZ1 and m-shRNA plasmids were effective in reducing EMP2 expression in vivo by day 5 pc (Wadehra et al., submitted for publication). Similarly, αvβ3 integrin expression on day 5 pc was significantly reduced when uteri were treated with the pEGFP-mRZ1 and m-shRNA plasmids (Figs. 7E–H). In contrast, uteri treated with a vector control (Figs. 7A–B) or h-shRNA, which does not target mouse EMP2 (Figs. 7C–D), exhibited intense membrane αvβ3 integrin staining in both luminal and glandular epithelium. Inhibition of αvβ3 integrin expression appeared to be EMP2 dose-dependent as only partial inhibition was observed in uteri treated with 5 µg of pEGFP-mRZ1 or m-shRNA. No staining was observed in tissue transfected with pEGFP-mRZ1 or m-shRNA and then processed using control serum (data not shown).

Discussion

Endometrial expression of EMP2 is required for blastocyst implantation, and EMP2 is known to control surface expression of certain integrin isoforms in model cell types. Since endometrial αvβ3 integrin is thought to play a role in blastocyst implantation, this study tested the prediction that EMP2 controlled expression of this integrin isoform in endometrial cells. We observed that among several integrin isoforms, αvβ3 expression was selectively dependent on EMP2 levels. That is, recombinant up- and down-regulation of EMP2 resulted in up- and down-expression of αvβ3. This phenotype was observed both in an endometrial cell line and in native endometrium, indicating that this role of EMP2 is a physiologic feature of the endometrial cell.

One issue raised by these findings is the level of integrin regulation affected by EMP2. Recombinant manipulation of EMP2 (a 40-fold range spanning ribozyme knockdown and EMP2 overexpression) showed that levels of β3 integrin total protein changed in direct proportion to EMP2 levels. Somewhat in contrast, levels of αvβ3 integrin at the cell surface coordinately changed, but with a much smaller
dynamic range (3-fold span). The present study shows that EMP2 and αvβ3 integrin largely reside in an overlapping post-Golgi intracellular compartment and exist as physical complexes by biochemical criteria. These observations suggest that EMP2 influences αvβ3 integrin levels in the intracellular compartment, perhaps because the stability of αvβ3 integrin may be limited by the EMP2 available to form ternary complexes.

A similar conclusion was made regarding EMP2 and α6 integrin in mouse NIH3T3 fibroblasts (Wadehra et al., 2002b, 2004). Curiously, enforced alteration of EMP2 expression did not affect α6 integrin surface levels in HEC1A cells. This might reflect distinctions in species-specific (human vs. mouse) or tissue-specific (endometrial vs. fibroblast) properties of the α6 integrin isoform or other molecules involved in the net trafficking fate of this isoform.

Recombinant manipulation of EMP2 also caused quantitatively similar changes in levels of β3 integrin mRNA. This suggests that EMP2 might control αvβ3 expression at the transcriptional level. This would be surprising since EMP2 is a membrane protein mainly localized to plasma membrane and post-Golgi compartments. 4-TM proteins including EMP2 are thought to form the nidus of multi-receptor signaling complexes required for efficient integration of receptor-mediated triggering and control of signal pathway activity (Knobeloch et al., 2000; Lagaudriere-Gesbert et al., 1997; Levy and Shoham, 2005; Little et al., 2004; Wadehra et al., 2004). Accordingly, it is possible that modulation of EMP2 might impair the efficiency of certain signaling pathways that maintain β3 integrin transcription in the endometrium. However, diverse mechanisms govern integrin expression, including regulation of protein levels by transcriptional or posttranscriptional events, mRNA alternate splicing, and mobilization of preexisting stores (Hemler, 1998; Porter and Hogg, 1998; Shimaoka et al., 2002). In particular, α integrin subunit levels are rate-limiting in the integrin heterodimer expression, and reduced αvβ3 expression results accelerate β3 mRNA decay (Retta et al., 2001). Thus, altered β3 mRNA levels may be a consequence rather than cause of EMP2-induced changes in αvβ3 protein. Accordingly, the mechanism(s) of EMP2 control of αvβ3 expression will benefit from more detailed study.

A second issue is whether these EMP2-induced changes in integrin levels alter αvβ3 integrin function in endometrial cells. Quantitative changes in integrin levels do not simply relate to integrin function since integrin surface delivery is tightly regulated from the much larger cytoplasmic integrin pool and because of the conversion and stability of the activated conformation state for high avidity binding and efficient signaling (Dalton et al., 1992; Sastry et al., 1996). At a functional level, these post-translational factors might augment or compensate for the changes in αvβ3 integrin total protein levels induced by EMP2. However, using fibronectin binding as a readout of αvβ3 integrin function, we found that function was indeed correlated with quantitative changes in αvβ3 integrin levels. Endometrial binding to fibronectin, vitronectin, and osteopontin is thought to play an important role in implantation (Nejjarri et al., 1999; Sorokin et al., 2000). So, EMP2-induced control of αvβ3 integrin in endometrial cells may in part account for the requirement of EMP2 in endometrial implantation competence.

Endometrial implantation competence is dictated by an integrated set of cell functions, of which αvβ3 integrin is only one component. In addition to αvβ3 and its counterligands, several receptor–ligand pairs are involved with blastocyst–endometrial interaction (including L-selectin, HB-EGF, and LPA3) (Dey et al., 2004; Genbacev et al., 2003; Liu and Armand, 2004; Raab et al., 1996; Ye et al., 2005; Yoo et al., 1997). In addition, some of these interaction molecules act pleiotropically. For example, HB-EGF and EGF produced by the endometrium act both on the blastocyst and as paracrine molecules for decidualization, proliferation, and survival of endometrial epithelium and stroma. Accordingly, endometrial EMP2 is one new factor in a larger biologic palette of endometrial function involved in implantation. Clues to the integration of these elements are beginning to emerge, such as the regulation by HB-EGF and EGF receptors of αvβ3 integrin expression, and the regulation by 4-TM proteins of EGF and HB-EGF expression and cognate receptor signaling (Chobotova et al., 2005; Lessey et al., 2002, 2003; Raab et al., 1996; Tan et al., 2004). The present paper highlights a pattern of expression and regulation that links EMP2 and αvβ3 integrin in the endometrium, and the integration of αvβ3 integrin into the larger palette may in part account for the role of EMP2 in implantation competence.

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