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Endoglin Regulates Cytoskeletal Organization through Binding to ZRP-1, a Member of the Lim Family of Proteins*

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Endoglin is a component of the transforming growth factor-β receptor complex abundantly expressed at the surface of endothelial cells and plays an important role in cardiovascular development and vascular remodeling. By using the cytoplasmic domain of endoglin as a bait for screening protein interactors, we have identified ZRP-1 (zyxin-related protein 1), a 476-amino acid member that belongs to a family of LIM containing proteins that includes zyxin and lipoma-preferred partner. The endoglin interacting region was mapped within the three double zinc finger LIM domains of the ZRP-1 C terminus. Analysis of the subcellular distribution of ZRP-1 demonstrated that in the absence of endoglin, ZRP-1 mainly localizes to focal adhesion sites, whereas in the presence of endoglin ZRP-1 is found along actin stress fibers. Because the LIM family of proteins has been shown to associate with the actin cytoskeleton, we investigated the possibility of a regulatory role for endoglin with regard to this structure. Expression of endoglin resulted in a dramatic reorganization of the actin cytoskeleton. In the absence of endoglin, F-actin was localized to dense aggregates of bundles, whereas in the presence of endoglin, expressed in endothelial cells, F-actin was in stress fibers and colocalized with ZRP-1. Furthermore, small interfering RNA-mediated suppression of endoglin or ZRP-1, or clustering of endoglin in endothelial cells, led to mislocalization of F-actin fibers. These results suggest a regulatory role for endoglin, via its interaction with ZRP-1, in the actin cytoskeletal organization.

Endoglin is a type I membrane glycoprotein expressed as a disulfide-linked homodimer of 180 kDa at the surface of endothelial cells, activated macrophages, fibroblasts, and smooth muscle cells (1–7). Experimental evidence supports an important role for endoglin in cardiovascular development and vascular remodeling. Thus, endoglin expression is regulated during heart development in humans and chicken (8, 9), and it is highly expressed at the level of endocardial cushion during valve formation and heart septation by the mesenchymal cells of the atrioventricular canal (8). Endoglin-null mice obtained by targeted disruption of the endoglin gene die at 10–11.5 days postcoitum due to vascular and cardiac anomalies (10–12). Furthermore, the gene encoding endoglin is the target for the autosomal dominant disorder known as hereditary hemorrhagic telangiectasia type 1 (13). Hereditary hemorrhagic telangiectasia type 1 is a vascular disorder whose most common clinical manifestations include telangiectases on skin and mucosa as well as arteriovenous malformations in brain, lung, and liver (14–16).

Endoglin is a component of the TGF-β[1] receptor complex that binds TGF-β_1, TGF-β_3, activin-A, BMP-2, and BMP-7 in the presence of the type I and II TGF-β signaling receptors (17–19) and modulates TGF-β_1-dependent responses (5, 19, 20). However, a TGF-β independent function has been proposed based on the fact that only 1% of the total endoglin molecules are able to bind TGF-β_1 in endothelial cells (8). In this sense, overexpression of endoglin in fibroblasts or smooth muscle cells leads to an altered cellular morphology, migration, and adhesion (6, 21), suggesting a role in the cytoskeletal organization.

ZRP-1 (zyxin-related protein 1), also called TRIP6 (thyroid receptor interacting protein 6), is a cytoplasmic protein of 476 amino acid residues (22–24). Expression of *Zrp-1* in mouse embryos begins at day 16.5 of gestation, reaching in the adult high levels on endothelial and epithelial cells and low levels on muscle cells (25). Accordingly, strong expression of human ZRP-1 transcripts was detected in highly vascularized tissues such as lung, placenta, heart, and kidney. ZRP-1 belongs to a subset of LIM domain containing proteins that include the prototypic member zyxin and lipoma-preferred partner (LPP). LIM domains are cysteine-rich motifs of 55 amino acid residues that display a double zinc finger structure and are involved in protein-protein interactions (26). Analysis of the ZRP-1 sequence shows two distinct regions: a C-terminal half containing three LIM domains, and a proline-rich N-terminal half containing a nuclear export sequence (27). Several ZRP-1 interactors have been reported to bind through the LIM domains, including the thyroid receptor (22, 23), the tyrosine phosphatase hPTP1E (24, 25), v-Rel (28), the adaptor protein RIL (25), the OpaP protein from *Neisseria gonorrhoeae* (29), and the CasL/HEF1 and $p130^{Cas}$ members of Cas family (30).

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† We regret the passing away of Dr. Banville on February 4th, 2004.

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[1] The abbreviations used are: TGF-β, transforming growth factor-β; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HHT, hereditary hemorrhagic telangiectasia; HUVEC, human umbilical vein endothelial cells; LPP, lipoma-preferred partner; RNAi, RNA interference; siRNA, small interfering RNA; FCS, fetal calf serum; EGFP, enhanced GFP; MBP, maltose-binding protein; HA, hemagglutinin; mAb, monoclonal antibody; PBS, phosphate-buffered saline.

ZRP-1 shares with zyxin and LPP not only their structural homology but also its association with the cytoskeleton. ZRP-1 and zyxin are localized along actin stress fibers and at cell-cell and cell-substrate contact regions (25, 30–32), whereas LPP is only located at focal adhesions and cell-to-cell contacts (33, 34). The similarities between zyxin and ZRP-1 at the protein structure and subcellular localization levels suggest a similar functional role for both molecules, which are postulated to serve as a docking site for the assembly of multimeric protein complexes involved in regulating cytoskeleton assembly and cell motility. Zyxin appears to play an important role in the spatial control of actin cytoskeleton assembly by interacting with the Ena/VASP-profilin complex, with α-actinin, or with Vav (35–37). In fact, delocalization of zyxin in cultured cells, by its targeting to the inner face of the plasma membrane or to mitochondria, leads to the assembly of short actin stress fibers at the new location of zyxin (36, 38). This is similar to the function displayed by ActA, a zyxin homologous protein, that is expressed at the surface of *Listeria monocytogenes* and is involved in pathogen invasion of the host cell (39–41). In addition, ZRP-1 seems to be connected to the cytoskeleton through binding to proteins involved in the actin assembly, such as the adaptor protein RIL, the tyrosine phosphatase hPTP1E, members of Cas family proteins, or the OpaP protein from *N. gonorrhoeae*. It is worth noting that the OpaP protein is located at the external surface of the bacteria and is involved in adhesion and invasion of the host cell by altering the organization of the actin cytoskeleton (29). Also, the association with members of the Cas family of adaptor proteins suggests the involvement of zyxin and ZRP-1 in cell motility, a process highly dependent on the actin cytoskeleton (30).

Here we have identified ZRP-1 as a protein able to interact with the cytoplasmic domain of endoglin. The subcellular distribution of ZRP-1 along actin stress fibers or at focal adhesion sites was found to be dependent on the expression of endoglin, suggesting a regulatory role for endoglin in the cytoskeletal organization.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—Yeast two-hybrid analysis was performed with a GAL4-based MATCHMAKER System (Clontech) with yeast strains Y190 and Y187 according to the manufacturer's instructions. The cytoplasmic domain of endoglin (amino acids 612–658) was cloned into the "bait" vector pAS2-1. A human lung cDNA library (Clontech) was screened using the endoglin bait. DNA was isolated from positive yeast clones and transformed into competent *Escherichia coli* (TOP10, Invitrogen), according to standard protocols. DNA sequencing was performed on an ABI PRISM™ 310 genetic analyzer (Applied Biosystems Inc.).

Cell Culture—COS-7 (African green monkey kidney), L_6E_9 (rat myoblasts), and 293T (human kidney) cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (FCS). L_6E_9 transfectants expressing L-human endoglin or betaglycan (19) were cultured in Dulbecco's modified Eagle's medium containing 10% FCS and 400 μg/ml of the antibiotic G418. Human dermal microvascular endothelial cells were obtained from Promocell, plated in gelatin-coated plates and grown in MCDB-131 supplemented with 10% FCS, 2 mM L-glutamine, 10 ng/ml epidermal growth factor, 1 μg/ml hydrocortisone (Sigma), and endothelial cell growth supplement/heparin (ECGS-H2; Promocell GmbH, Heidelberg, Germany). Human umbilical vein endothelial cells (HUVEC) were isolated from cannulated vessels incubated in the presence of collagenase. Detached cells were plated on gelatin-coated flasks and grown in medium 199 supplemented with 20% fetal calf serum and 50 μg/ml bovine brain extract.

Transfections—COS-7, L_6E_9, and 293T cells were transiently transfected with the use of LipofectAMINE (Invitrogen), according to the manufacturer's instructions for adherent cells. Briefly, cells were trypsinized, plated in 35-mm tissue culture plates ($1–3 \times 10^5$ cells per plate), and incubated at 37 °C in Dulbecco's modified Eagle's medium with 10% FCS for 18–24 h, until the cells reached 50–70% confluency. Cells were washed once with serum-free medium; the lipid-DNA complex was added in serum-free medium, and transfection was allowed to occur for 3 h at 37 °C. Then fresh medium with 10% FCS was added, and cells were incubated for 48 h before analysis of the recombinant protein.

HUVECs were transiently transfected using a Nucleofector™ (Amaxa Biosystems), following the manufacturer's instructions for adherent cells. Cells were harvested by trypsinization followed by centrifugation ($2 \times 10^5–2 \times 10^6$ cells per nucleofection sample) at $200 \times g$ for 10 min. The supernatant was completely discarded so that no residual medium covered the pellet. The pellet was resuspended in HUVEC Nucleofector™ solution (VPB-1002 kit, Amaxa Biosystems) at room temperature to a final concentration of $2 \times 10^5–2 \times 10^6$ cells/100 μl and mixed with 1–5 μg of DNA. The sample was transferred into a cuvette (Amaxa), avoiding air bubbles while pipetting. The cuvette was inserted into the Nucleofector™, and the appropriate electroporation program (U-01) was allowed to run. The cuvette was immediately removed after the program had finished, and pre-warmed culture medium containing serum and supplements was added to the cuvette, and cells were transferred using plastic pipettes to 6-well plates. Cells were incubated in a humidified 37 °C, 5% CO_2 incubator, and gene expression was analyzed 48h after nucleofection with DNA.

Gene transfer efficiency was typically monitored by flow cytometry (COS-7, L_6E_9, and 293T cells) or microscopy (HUVECs) upon transfection of the pEGFP-C1 reporter vector encoding the green fluorescent protein (GFP). Transfection efficiency was 70–80% in 293T, 30–40% in L_6E_9, 60–75% in COS, and <15% in HUVEC cells.

Plasmid Constructs—For protein expression in *E. coli*, DNA segments encoding the cytoplasmic domain of endoglin (amino acids 612–658) or betaglycan (amino acids 805–849) were generated by PCR using Taq polymerase (PerkinElmer Life Sciences) and cloned in pGEX-1λT and pGEX4T-3 vectors, respectively. Mammalian expression plasmid containing HA-tagged endoglin was constructed by subcloning the PCR-amplified full-length endoglin cDNA into the pDisplay vector (Invitrogen) (42). Constructs for the expression of extracellular domain (ECTM-Endo, amino acids 26–614) or the intracellular domain (TMCT-Endo, amino acids 573–658) of endoglin were generated by PCR and subcloned into the pDisplay vector in-frame with the HA epitope (42). In order to generate EGFP fusion proteins, full-length or partial ZRP-1 was inserted into the pEGFP-C1 (Clontech) vector. The pEGFP-C1/ZRP-1 plasmid was generated by ligating the KpnI/XbaI double digest pcDNA/FLAG-ZRP-1 vector fragment with the KpnI/XbaI digest pEGFP-C1 vector. To generate EGFP-1/274 ZRP-1 (amino acids 1–274), EGFP-234/476 ZRP-1 (amino acids 234–476), and EGFP-272/476 ZRP-1 (amino acids 272–476), the corresponding DNA fragments were generated by PCR with the use of oligonucleotides with add-on sequences that allowed cloning of these fragments into the pEGFP-C1 vector, in-frame with the sequence of EGFP protein. All constructs were verified by DNA sequence analysis.

Human ZRP-1 cDNAs, subcloned into the mammalian expression vector pAC-TAG2, tagged at the N terminus with an HA epitope, and the C-terminal portion of ZRP-1 containing the 3 LIM domains subcloned in-frame with the maltose-binding protein (MBP) into the plasmid pMal-C2, have been described (24). For RNA interference studies, the pSUPER-Endo-Ex4 vector was generated by inserting a double-stranded oligonucleotide corresponding to exon 4 of the human endoglin gene (AACACCACAGAGCTGCCATCCTT) into BglII/HindIII sites of pSUPER plasmid (Oligoengine). Similarly, the pSUPER-ZRP-1–356 vector was generated by inserting a double-stranded oligonucleotide corresponding to amino acids 356–363 of human ZRP-1 (AAGGCCTACCACCCTGGCTGCTT) into pSUPER. Upon transfection, the pSUPER-Endo-Ex4 or pSUPER-ZRP-1–356 vectors generated intracellular expression of small endoglin RNA molecules that silence human endoglin or *ZRP-1* genes, respectively (data not shown). The pSUPER-C vector (generous gift of Dr. Olga Camacho), used as a negative control, was generated by inserting an endoglin-unrelated double-stranded oligonucleotide (TGAGACAAGTCTTACAGAT) into the BglII/HindIII sites of pSUPER plasmid. Human endoglin cDNA, subcloned into the mammalian expression vector pCMV5 and tagged in its C terminus with a Myc epitope, was a gift from Dr. Douglas Marchuk (43). The expression vector peGFPC1-SV40 encoding EGFP tagged with the nuclear localization signal of SV40-T (44) was kindly provided by Dr. Gerardy-Schahn (Institute für Physiologische Chemie/Proteinstruktur, Hannover, Germany).

Immunoprecipitation and Western Blot Analyses—Cells were lysed on ice for 30 min with lysis buffer (1% digitonin, 50 mM Hepes, pH 7.4, 150 mM NaCl, 0.1 mM EGTA, 0.5 mM EDTA, 10% glycerol, protease inhibitors mixture, 1 mM Na_3VO_4, 10 mM NaF, and 1 mM Na_2MoO_4).

Lysates were centrifuged at 14,000 × *g* for 5 min. Aliquots of cleared cell lysates containing the same amount of protein were incubated with anti-GFP polyclonal antibody (Clontech), anti-hemagglutinin mAb (Roche Applied Science), anti-ZRP-1 (BD Biosciences), or anti-endoglin mAb P4A4 (45) for 3 h at 4 °C, followed by incubation with protein A- or protein G-Sepharose for 1 h. Immunoprecipitates were washed three times with lysis buffer. The immune complexes were eluted by boiling 5 min in SDS sample buffer and were analyzed by SDS-PAGE under reducing conditions. Proteins were electrotransferred to polyvinylidene fluoride membranes (Millipore Corp.) and immunoblotted with the corresponding antibodies. Expression of the transfected proteins was monitored by immunoblotting of proteins in total cell lysates.

In Vitro Pull-down Assay—For *in vitro* pull-down assays, MBP and GST fusion proteins were expressed in DH5α following the manufacturer's instructions. The proteins in the bacterial pellet were solubilized in Tris-buffered saline, 1% Nonidet P-40, containing protease inhibitors (Roche Applied Science) (buffer A), and sonicated. Lysates of each of the extracts (with the same amount of fusion protein) were mixed with 20 μl of glutathione-Sepharose beads, and the total volume was adjusted to 250 μl with Tris-buffered saline, 0.1% Nonidet P-40, containing protease inhibitors (buffer B). The precipitation was allowed to proceed for 2 h at 4 °C. The glutathione beads were separated by a brief centrifugation, washed five times with buffer B, and analyzed by Western blotting with a rabbit anti-serum anti-MBP (New England Biolabs). To perform the pull-down assays with EGFP fusion proteins, aliquots of EGFP fusion protein cell lysates were incubated for 6 h at 4 °C with 15 μg of GST or GST-Ecyt fusion proteins were immobilized on glutathione-Sepharose. The precipitates were washed three times with lysis buffer and analyzed by Western blotting.

Reverse Transcription-PCR—Total RNA from pSuper transient transfectants was extracted by using the RNAeasy kit (Qiagen). One microgram of this RNA was reverse-transcribed to cDNA using random hexamers in a total volume of 20 μl. After the RT reaction, the total volume was adjusted to 100 μl, and 5 μl was used for PCRs in the presence of 10 μM final concentration of each oligonucleotide for ZRP1 (5′-GAT CCC CGG CCT ACC ACC CTG GCT GCT TCA AGA GAG CAG CCA GGG TGG TAG GCC TTT TTG GAA A-3′ and 5′-AGC TTT TCC AAA AAT TGT TGC TCT GGA TCG AAG TCT CTT GAA GCA GCC AGG GTG GTA GGC CGG G-3′), or glyceraldehyde-3-phosphate dehydrogenase (5′-GACGCCTGGTTACCAG-3′ and 5′-CTCCTTGGAGGCGATG 3′) amplification. Samples of 10 μl were taken after 35 cycles. PCR was performed using the HotMaster Taq polymerase (Eppendorf) with hot start, and the amplification conditions were as follows: 5 min at 95 °C, followed by cycles of 1 min at 95 °C, 1 min at 52 °C, and 1 min at 68 °C with a final elongation step of 5 min at 72 °C.

Immunofluorescence Microscopy—Cells were grown onto 12-mm diameter glass coverslips coated with 0.5% gelatin in 24-well cell culture plates. Cells were fixed with 3.5% formaldehyde in PBS, and after being washed, blocking was done with 2% bovine serum albumin in PBS for 1 h at 4 °C. Cells were incubated 1 h at 4 °C with mouse anti-endoglin P4A4 antibody, as indicated. When necessary, cells were permeabilized with 100 μg/ml L-α-lysophosphatidylcholine and stained with rabbit anti-ZRP-1 antibody B59 (a kind gift from Dr. M. Beckerle) or mouse anti-vinculin (Sigma). This was followed by incubation with Alexa-488 green-conjugated anti-rabbit/mouse IgG antibody or Alexa-546 red-conjugated anti mouse IgG antibody (Molecular Probes). For staining actin filaments, cells were fixed, stained, and permeabilized in a single step by the addition of a 2× cold solution containing 5 units/ml rhodamine-labeled phalloidin (Molecular Probes), 0.5 mg/ml L-α-lysophosphatidylcholine, and 3.5% formaldehyde in PBS. After 20 min at 4 °C, cells were washed twice in cold PBS. The stained cells were mounted with Mowiol 44-88 (Sigma) and observed with a spectral confocal microscope (Leica Microsystems, Heidelberg GmbH). For detection of EGFP fusion proteins, cells were fixed, and coverslips were mounted and analyzed directly. For endoglin cross-linking experiments, HUVECs were grown onto 12-mm diameter glass coverslips coated with 0.5% of gelatin in 24-well cell culture plates. After 24 h, cells were incubated with mouse anti-endoglin P4A4 or anti-CD31 (Clone HC1/6, Chemicon) antibodies at 37 °C for 30 min. Then antibody-mediated clustering was induced by incubation with Alexa-488 anti-mouse IgG antibody, and actin filaments were stained with rhodamine-labeled phalloidin as above.

RESULTS

Identification of ZRP-1 as a LIM Domain Containing Protein Interacting with the Cytoplasmic Domain of Endoglin—To identify proteins that physically interact with the cytoplasmic domain of endoglin, we conducted a human lung cDNA library

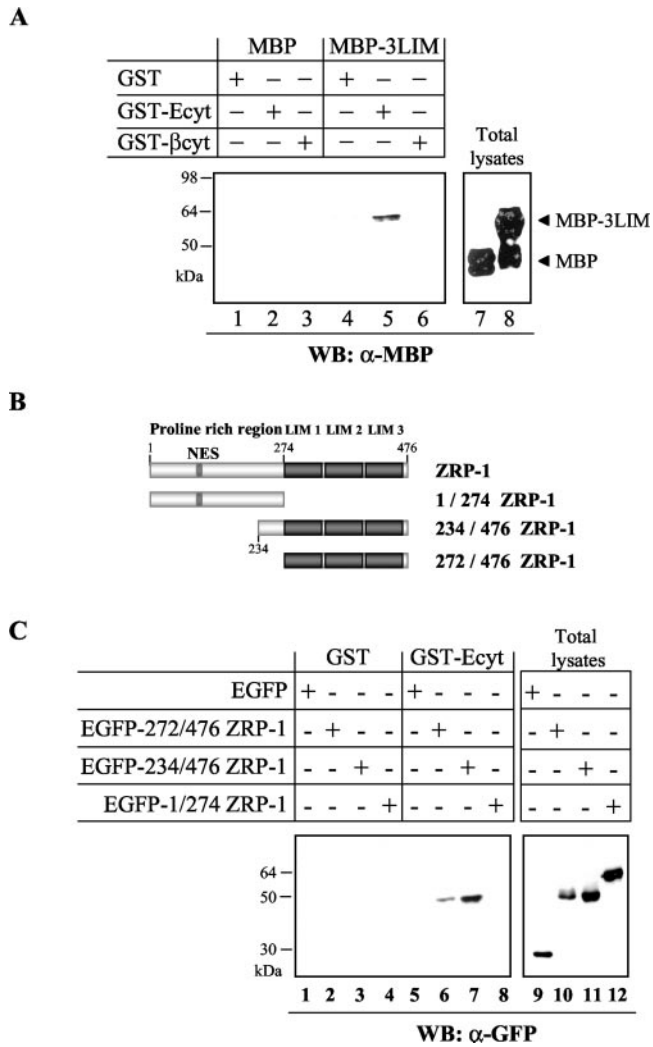

FIG. 1. **Pull-down analysis of the interaction between endoglin and ZRP-1.** *A*, the cytoplasmic domain of endoglin interacts with the LIM domains of ZRP-1. The cytoplasmic domain of endoglin fused to GST (*GST-Ecyt*), the cytoplasmic domain of betaglycan fused to GST (*GST-βcyt*), GST alone, the C-terminal portion of ZRP-1 containing all three LIM domains and fused to MBP (*MBP-3LIM*), and MBP alone were bacterially expressed. Bacterial lysates containing equal amounts of recombinant proteins were mixed, and the specific protein complex was precipitated using glutathione-Sepharose beads. The proteins bound to the beads were analyzed by Western blot (*WB*) using an anti-MBP antibody. The coprecipitation of MBP-3LIM with GST-Ecyt is shown in *lane 5*. The MBP-3LIM protein did not bind to GST or GST-βcyt (*lanes 4* and *6*). The amount of MBP (*lane 7*) and MBP-3LIM (*lane 8*) used for pull-down experiments was assessed by immunoblot. The positions of MBP and MBP-3LIM are indicated. *B*, schematic diagram of fusion proteins between different truncated forms of ZRP-1 and EGFP. The structure of full-length ZRP-1 containing the proline-rich N-terminal region and the three LIM domains is shown. *Numbers* indicate the position of the amino acid residues. The full-length (amino acids 1/476), N-terminal truncated (234/476 and 272/476), and the C-terminal truncated (1/274) forms of ZRP-1 were fused to EGFP. The position of the nuclear export sequence (*NES*) is indicated. The EGFP region of the fusion protein is not shown. *C*, the proline-rich N-terminal region of ZRP-1 is not involved in the interaction with endoglin. COS-7 cells were transiently transfected with the expression vectors coding for the indicated EGFP fusion proteins. Cell lysates were incubated with either GST alone (*lanes 1–4*) or GST-Ecyt (*lanes 5–8*) conjugated with glutathione-Sepharose beads. The bound fraction was analyzed by SDS-PAGE, followed by Western blotting with anti-GFP antibody. Constructs EGFP-272/476 ZRP-1 (*lane 6*) and EGFP-234/476 ZRP-1 (*lane 7*), but not EGFP-1/274 ZRP-1 (*lane 8*), demonstrated specific binding to GST-Ecyt. To confirm expression of transfected proteins, aliquots of total lysates were immunoblotted with anti-GFP antibody (*lanes 9–12*). The smallest 272/476 ZRP-1 construct (*lane 10*) runs with a mobility similar to the larger 234/476 construct (*lane 11*), probably because of posttranslational modifications affecting the 234–272 fragment.

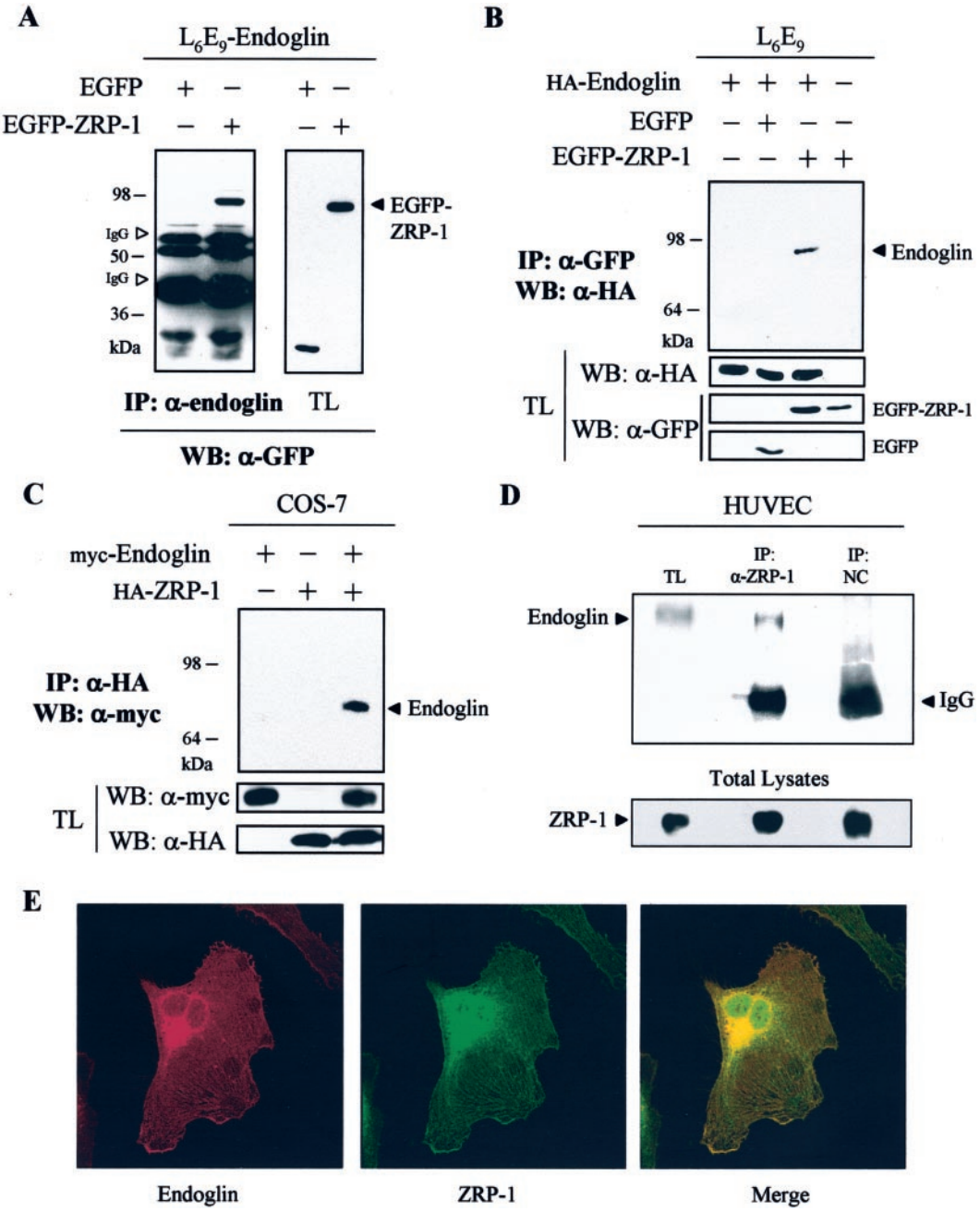

FIG. 2. **Endoglin associates with ZRP-1 in the cell.** The interaction between ZRP-1 and endoglin was analyzed either in L_6E_9 cells (*A* and *B*) or in COS-7 cells (*C*). *A*, L_6E_9 cells expressing human endoglin were transiently transfected with either EGFP-ZRP-1 or EGFP. Cell lysates were subjected to immunoprecipitation (*IP*) with anti-endoglin mAb (P4A4). Immunoprecipitates were analyzed by Western blot (*WB*) analysis with an anti-GFP antibody to allow the detection of EGFP-ZRP-1. To assess the expression of transfected proteins, aliquots of total lysates (*TL*) were immunoblotted with anti-GFP antibody (*right section*). *B*, endoglin-negative L_6E_9 cells were cotransfected with the indicated combinations of HA-tagged endoglin, EGFP, and EGFP-ZRP-1 fusion protein. Cell lysates were immunoprecipitated (*IP*) with anti-GFP antibody, and the immunoprecipitates were analyzed by Western blotting (*WB*) using an anti-HA antibody (*top section*). Expression of the recombinant proteins was confirmed by direct Western blot of total lysates (*TL*) with anti-HA or anti-GFP antibodies (*bottom section*). *C*, COS-7 cells were cotransfected with Myc-tagged endoglin and HA-ZRP-1 as indicated. Cell lysates were immunoprecipitated (*IP*) with anti-HA antibody, and the immunoprecipitates were analyzed by Western blotting (*WB*) using an anti-Myc antibody (*top section*). Expression of the recombinant proteins was confirmed by direct Western blot of total lysates (*TL*) with anti-Myc or anti-HA antibodies (*bottom section*). *D*, HUVECs were lysed and immunoprecipitated (*IP*) with an anti-ZRP-1 monoclonal antibody or an irrelevant antibody as a negative control (*NC*). Total lysates (*TL*) and immunoprecipitates were analyzed by Western blotting using anti-endoglin or anti-ZRP-1 antibodies. The position of endoglin, ZRP-1, and IgG is indicated. *E*, human endothelial cells were incubated with a mouse mAb anti-endoglin (P4A4), fixed, permeabilized, and then incubated with a rabbit polyclonal antibody to ZRP-1. Cells were washed with PBS and incubated with anti-mouse IgG coupled to Alexa-546 and anti-rabbit IgG coupled to Alexa-488. Cellular sections were analyzed by confocal microscopy in order to detect the optimal overlapping between the green fluorescence of ZRP-1 and the red fluorescence of endoglin. The *yellow* color in the merge image indicates the areas where ZRP-1 and endoglin colocalize.

screen by the yeast two-hybrid method. We used a region of amino acids 612–658, which is the full cytoplasmic domain of L-endoglin, as a bait for the screen. From 1×10^6 initial transformants, 24 clones were found to confer both the His^+ and $LacZ^+$ phenotypes. These positive clones were subjected to secondary screening to eliminate false positives and to confirm the specific interaction between the cytoplasmic domain of endoglin and the protein encoded by a cDNA library within the yeast cells. Among the positive clones, we isolated one cDNA clone, named 130, encoding the C-terminal half of the LIM

domain-containing zyxin-related protein-1, ZRP-1 (24). Human ZRP-1 is a 476-amino acid protein containing three LIM domains in its C terminus and a proline-rich N-terminal segment that belongs to a new family of LIM proteins that includes zyxin (31, 46) and LPP (34).

The LIM Containing Region of ZRP-1 Interacts with the Cytoplasmic Domain of Endoglin in Vitro—The interaction between ZRP-1 and endoglin was confirmed by pull-down experiments *in vitro*. The C-terminal portion of ZRP-1, containing the LIM domains (amino acid residues 278–476), was expressed as a maltose-binding fusion protein (MBP-3LIM); and the cytoplasmic domain of endoglin (amino acid residues 612–658) was expressed as a GST fusion protein (GST-Ecyt). These proteins were solubilized from bacterial lysates, mixed, and allowed to interact. The GST fusion protein was pelleted down using glutathione-Sepharose beads, and the bound MBP proteins were probed with a specific polyclonal antibody by Western blot analysis (Fig. 1*A*). Thus, the MBP-3LIM fusion protein was coprecipitated by glutathione-Sepharose beads along with GST-Ecyt, indicating that the two proteins interact with each other *in vitro*. By contrast, no interaction was observed with GST or with a GST fusion protein that contains the cytoplasmic domain of human betaglycan (GST-βcyt; amino acid residues 805–849), which displays a 70% sequence identity with the cytoplasmic domain of endoglin (18, 47). These findings provide independent confirmation of the results obtained in the yeast two-hybrid system and indicate that the interaction of ZRP-1 is specific for the cytoplasmic domain of endoglin, thus distinguishing endoglin from betaglycan.

To delineate in more detail the region(s) of ZRP-1 involved in its association with endoglin, we next explored if the proline-rich region of ZRP-1 was also able to interact with endoglin. EGFP fusion proteins containing different truncated forms of ZRP-1 (Fig. 1*B*) were used in pull-down assays with the fusion protein GST-Ecyt. COS-7 cell lysates expressing EGFP-1/274 ZRP-1 (the proline-rich N-terminal domain), EGFP-272/476 ZRP-1 (containing the three LIM domains), or EGFP-234/476 ZRP-1 (containing the part of the proline-rich N-terminal domain plus the three LIM domains) were incubated with the GST-Ecyt fusion protein bound to glutathione-Sepharose beads (Fig. 1*C*). The pull-down assay revealed that both EGFP-272/476 ZRP-1 and EGFP-234/476 ZRP-1, but not EGFP-1/274 ZRP-1, coprecipitated with GST-Ecyt. These results showed that the region of ZRP-1 containing the three LIM domains serves as a binding site for endoglin and excludes a significant role for the proline-rich domain in the interaction between ZRP-1 and endoglin.

Endoglin Interacts with ZRP-1 in Vivo—To confirm the observed interaction between ZRP-1 and endoglin in a more physiological context, coimmunoprecipitation studies were carried out. L_6E_9 myoblasts were selected for biochemical and cytochemical analyses because they do not express endogenous endoglin or its homologue betaglycan, and they have proved to be a useful model system to analyze the functional role of these proteins (19, 48). L_6E_9 cells expressing endoglin (19) were transiently transfected with an expression vector encoding the full-length ZRP-1 as an EGFP fusion protein (EGFP-ZRP-1). Cellular lysates were immunoprecipitated with anti-endoglin antibodies, and the presence of EGFP-ZRP-1 in the immunoprecipitates was analyzed (Fig. 2*A*). Thus, EGFP-ZRP-1, but not EGFP, was found associated with immunoprecipitated endoglin. Conversely, transient transfection of parental L_6E_9 cells with expression vectors encoding HA-endoglin, EGFP, or EGFP-ZRP-1 demonstrated the presence of endoglin coimmunoprecipitated along with EGFP-ZRP-1 but not with EGFP alone (Fig. 2*B*). These results were subsequently confirmed in a different cell type. COS-7 cells were transiently transfected with expression vectors encoding HA-ZRP-1 or Myc-endoglin, and their cellular lysates were immunoprecipitated with anti-HA. As shown in Fig. 2*C*, endoglin was found to be coimmunoprecipitated with ZRP-1. Furthermore, the endoglin-ZRP-1 association was assessed in HUVEC cells that express endogenous endoglin and ZRP-1 proteins. Again, endoglin was found to be coimmunoprecipitated with ZRP-1 (Fig. 2*D*). The inverse immunoprecipitation experiment (immunoprecipitation, anti-endoglin, and Western blot, anti-ZRP-1) could not be addressed because the electrophoretic mobility of ZRP-1 coincides with that of the immunoglobulin heavy chain. In addition, colocalization of endoglin and ZRP-1 was demonstrated by confocal microscopy analysis of human endothelial cells stained with anti-endoglin- (*red*) and anti-ZRP-1-specific (*green*) antibodies (Fig. 2*E*). Taken together, these results demonstrate that endoglin can interact with ZRP-1 *in vivo*.

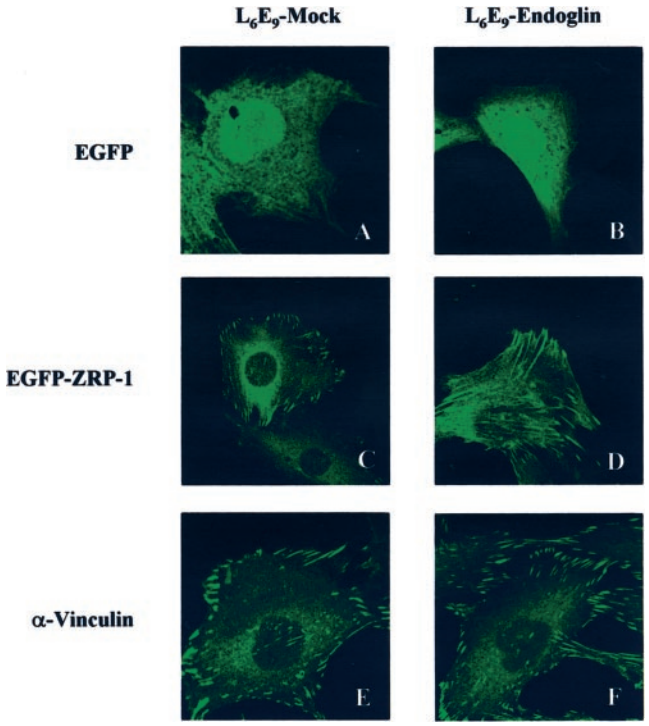

FIG. 3. **Subcellular distribution of ZRP-1 in the presence of endoglin.** Cells L_6E_9 (*L_6E_9-mock*) and L_6E_9 expressing endoglin (*L_6E_9-endoglin*) were transiently transfected with the expression vectors coding for the fluorescent proteins EGFP or EGFP-ZRP-1 and analyzed by confocal microscopy (*A–D*). As a control, untransfected cells were stained with anti-vinculin antibody (*E* and *F*) as described under "Materials and Methods." EGFP-ZRP-1 clearly targeted to focal adhesion sites in mock cells (*C*), but in the presence of endoglin it was redistributed throughout the cell along fibers (*D*). The subcellular distribution of EGFP (*A* and *B*) or vinculin (*E* and *F*) was not affected by the presence of endoglin.

Endoglin Regulates the Subcellular Localization of ZRP-1—ZRP-1 is a cytoplasmic protein associated with the cytoskeleton that localizes mainly at focal adhesion sites (25, 30, 32), whereas endoglin is a transmembrane protein distributed throughout the plasma membrane (3). Given the interaction between ZRP-1 and endoglin, we hypothesized that overexpression of endoglin would change the subcellular localization of ZRP-1 as a consequence of this interaction. To test this possibility, ZRP-1 was detected in endoglin expressing L_6E_9 cells (L_6E_9-endoglin) *versus* parental endoglin-negative cells (L_6E_9-mock). To facilitate the detection, the endogenous levels of ZRP-1 were increased by transient overexpression of the fluorescent protein EGFP-ZRP-1. Fig. 3 shows that, in the absence of endoglin, EGFP-ZRP-1 accumulates mainly at the cell substrate or focal contacts (Fig. 3*C*),

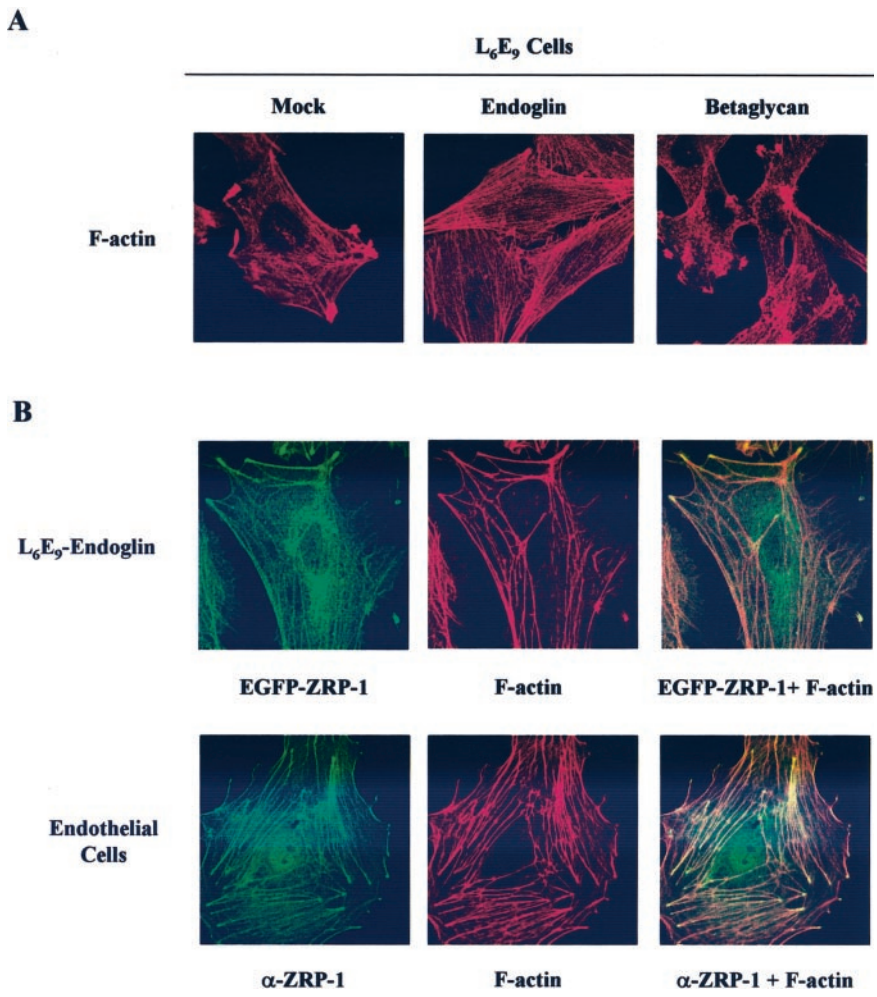

FIG. 4. **Endoglin expression is associated with increased numbers of stress fibers colocalizing with ZRP-1.** *A* , detection of actin filaments in L_6E_9 cells. L_6E_9 myoblasts stably transfected with endoglin, betaglycan, or an empty vector (*Mock*), were fixed, permeabilized, and stained with rhodamine-labeled phalloidin. The distribution of F-actin, in *red*, was analyzed by confocal microscopy. *B*, ZRP-1 colocalizes along actin fibers in cells expressing endoglin. L6E9 cells expressing human endoglin (*L_6E_9-endoglin*) were transiently transfected with the expression vector coding for the fluorescent protein EGFP-ZRP-1 (*top row*). Human dermal microvascular endothelial cells (*bottom row*) were fixed and permeabilized, and the endogenous ZRP-1 was stained with a specific antibody, followed by a fluorescently labeled secondary antibody. Both, L_6E_9-endoglin and endothelial cells were stained with rhodamine-labeled phalloidin. Cells were analyzed by confocal microscopy in order to detect the green fluorescence of ZRP-1 and the red fluorescence of F-actin. The *yellow* color in merge images indicates the areas where ZRP-1 and F-actin colocalize.

as described in other cell types. By contrast, in endoglin-expressing cells, EGFP-ZRP-1 is distributed throughout the cells along fibrillar structures reminiscent of stress fibers (Fig. 3*D*). As an internal control, the subcellular distribution of vinculin, a cytoskeleton- associated protein present at focal contacts, is not affected by the presence of endoglin (Fig. 3, *E* and *F*), demonstrating that the change of ZRP-1 localization is dependent on endoglin expression.

Endoglin Interaction with ZRP-1 Regulates Organization of Actin Cytoskeleton—The actin cytoskeleton is a complex protein network that not only shapes cellular morphology but is also critical for cellular division, migration, spreading, and contraction. In endothelial cells, where endoglin is highly expressed, actin filaments play a central role in the formation of new tubes (angiogenesis) and maintain the permeability of the endothelial barrier. As indicated above, several lines of experimental evidence support the involvement of zyxin-related LIM protein family members in actin organization and assembly (25, 30, 38, 39, 41, 49). Therefore, we investigated whether the endoglin-induced relocalization of ZRP-1 was affecting the actin cytoskeleton. To this end, the actin cytoskeleton was stained with rhodamine-phalloidin in cells in the presence or absence of endoglin. As shown in Fig. 4, confocal microscopic analysis revealed that L_6E_9 cells expressing endoglin displayed numerous parallel actin fibers throughout the cytoplasm. This actin array is often ordered in a concentric fashion leaving the center of the cell free of fibers. This actin network is strikingly similar to that observed in human endothelial cells, where endoglin is highly expressed (Fig. 4*B*). By contrast, in L_6E_9 cells lacking endoglin expression, F-actin is mainly localized in peripheral bands and dense bundles at cell-substrate contacts (Fig. 4*A*). A similar actin pattern was found in L_6E_9 cells expressing betaglycan, a protein highly homologous to endoglin, indicating a very specific effect of endoglin on actin distribution. These results indicate that endoglin expression leads to a cytoskeletal reorganization with an increased staining of actin fibers.

Interestingly enough, the pattern of actin fibers (Fig. 4) resembles that of ZRP-1 (Fig. 3) in endoglin-expressing cells. Furthermore, double immunofluorescence studies of ZRP-1 (Fig. 3, *green*) and actin (Fig. 3, *red*), in endoglin-expressing endothelial and L_6E_9 cells, demonstrated that ZRP-1 is located along the numerous actin fibers (Fig. 4*B*), suggesting that ZRP-1 and actin colocalize at the actin fibers.

To assess further the role of ZRP-1-endoglin interaction in the reorganization of the actin cytoskeleton, the distribution of F-actin was analyzed in cells expressing full-length endoglin (HA-endoglin), the extracellular and transmembrane domains of endoglin (HA-ECTM-Endo), or the transmembrane and cytoplasmic domains of endoglin (HA-TMCT-Endo) (Fig. 5). In cells expressing a truncated form of endoglin lacking its cytoplasmic domain (ECTM-Endo), F-actin accumulates around the plasma membrane in a pattern known as cortical cytoskeleton (50), as well as in dense aggregates of bundles at cell-substrate contacts (Fig. 5*C2*). This is similar to the pattern displayed by cells not expressing endoglin (Fig. 5*A2*). However, staining of cells expressing the full-length endoglin or a truncated form of endoglin lacking the extracellular domain (TMCT-Endo) revealed that actin was organized along parallel actin stress fibers present throughout the cell

(Fig. 5, *B2* and *D2*). Because the cytoplasmic domain of endoglin interacts with ZRP-1 (Fig. 1), these results are compatible with a mechanism of induction of actin fibers because of the formation of the ZRP-1-endoglin complex.

Endoglin Expression or Engagement Modulates Actin Cytoskeleton—The involvement of endoglin-ZRP-1 in the reorganization of actin cytoskeleton was further substantiated by RNA interference experiments in human endothelial cells that constitutively express endoglin and ZRP-1 proteins. Transfection with pSUPER-ZRP-1–356, encoding ZRP-1-specific sequences of siRNA, results in down-regulation of ZRP-1, as evidenced by a semiquantitative RT-PCR assay (Fig. 6*A*). Similarly, endothelial cells transiently cotransfected with an expression vector encoding EGFP, targeted to the nucleus, and pSUPER-EndoEx4 encoding endoglin-specific sequences of siRNA, resulted in down-regulation of endoglin (Fig. 6*B*). Then the distribution of F-actin was analyzed in those cells where endoglin or ZRP-1 had been down-regulated. As shown in Fig. 6*C,* endoglin or *ZRP-1* gene suppression results in reorganization of the actin cytoskeleton associated with decreased number of fibers and an increase dotted distribution of F-actin. These data support the role of endoglin and ZRP-1 in the actin organization.

Next, endoglin engagement experiments were performed. Human endothelial cells were incubated with mouse monoclonal antibodies to endoglin followed by clustering with an anti-mouse antibody. As depicted in Fig. 7, engagement of endoglin resulted in the clustering of endoglin at the cell surface, as well as to an altered actin cytoskeleton characterized by the presence of packed actin bundles. Most interesting, both endoglin and actin clusters colocalized within the cell, suggesting the cytoskeletal anchoring of endoglin. As a control, clustering with anti-CD31 did not reveal significant changes in the actin cytoskeleton. These experiments indicate that endoglin is able to modulate the organization of the actin cytoskeleton.

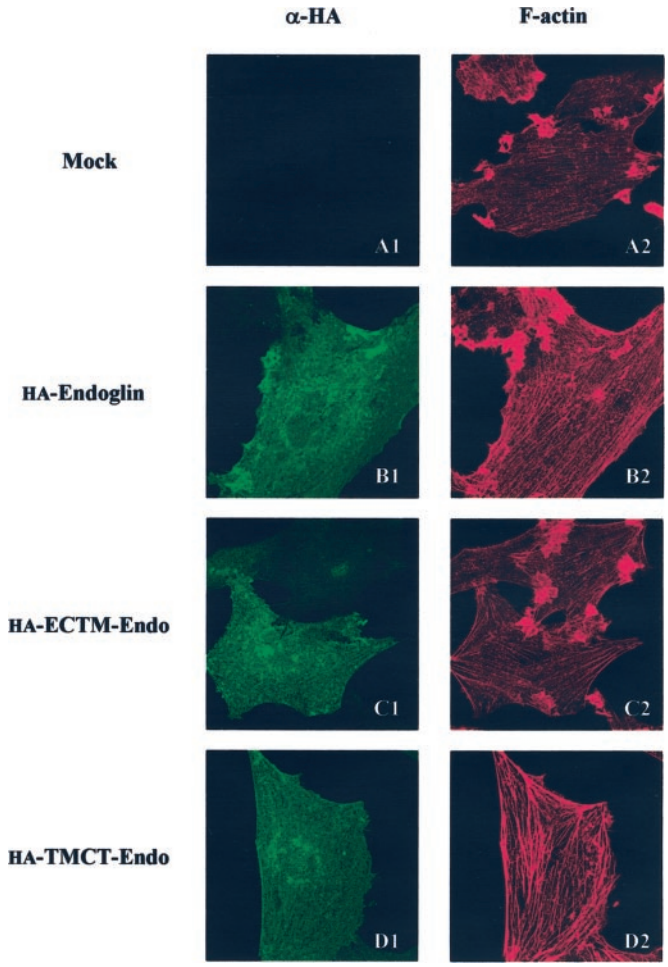

FIG. 5. **The endoglin-ZRP-1 complex regulates the formation of actin fibers.** L_6E_9 cells were transiently transfected with the expression vectors coding for the full-length HA-endoglin (*B1*, *B2*), HA-ECTM-Endo (lacking the cytoplasmic domain of endoglin; *C1*, *C2*), or HA-TMCT-Endo (lacking the extracellular domain of endoglin; *D1*, *D2*), as indicated. After 48 h, cells were fixed and stained with a mouse mAb anti-HA and rhodamine-labeled phalloidin. Cells were analyzed by confocal microscopy in order to detect the green fluorescence of endoglin constructs and the red fluorescence of F-actin. Note dense aggregates of actin bundles in cells lacking endoglin expression (*A1*, *A2*), or transfected with an endoglin construct lacking the ZRP-1-interacting cytoplasmic domain (*C2*). Cells transfected with endoglin constructs containing the ZRP-1 interacting domain show instead numerous F-actin filaments (*B2*, *D2*).

DISCUSSION

Despite the important role played by endoglin in cardiovascular development and remodeling (9–13), its cellular function is poorly understood. Endoglin has been described as an auxiliary TGF-β receptor component (18, 20, 51), but the fact that only 1% of the endoglin molecules in endothelial cells are able to bind TGF-β (18) suggests its involvement in other non-TGF-β-related functions. In this paper, we have identified ZRP-1, a component of the cytoskeleton, as an interactor of the cytoplasmic domain of endoglin. To our knowledge, this is a novel report describing the interaction of endoglin with a protein unrelated to the TGF-β system. The region of ZRP-1 involved in the interaction with endoglin has been identified as the LIM domains of the C terminus. ZRP-1 is a 476-amino acid protein that belongs to a group of LIM proteins that include zyxin (31, 46) and LPP (34). These proteins each contain a proline-rich N-terminal segment and three C-terminal LIM domains. LIM domains are multifunctional protein-protein interaction interfaces and have been found to be important for the subcellular localization of LIM-containing proteins that have a role in adhesion plaques, actin-microfilament organization, and cell motility (26, 30, 35). The specificity of the interaction between endoglin and ZRP-1 has been demonstrated by pull down (Fig. 1), coimmunoprecipitation (Fig. 2), and subcellular localization studies (Fig. 3). Further support for the existence of this interaction is the fact that the ZRP-1-related protein zyxin has also been recently identified as an interactor of endoglin (52).

The use of deletion mutants allowed a detailed characterization of the interaction between endoglin and ZRP-1. Thus, the association takes place between the cytoplasmic domain of endoglin and the LIM domains of ZRP-1, excluding the participation of the N-terminal domain of ZRP-1. Most interesting, the involvement of the LIM domains has also been demonstrated in several ZRP-1-interacting proteins (22, 24, 25, 29, 30). The interaction of endoglin-ZRP-1 is highly specific as the LIM domains of ZRP-1 are not able to interact with the cytoplasmic domain of human betaglycan, which shows a 70% identity with the cytoplasmic domain of endoglin (47, 49).

ZRP-1 interacts with proteins from different cellular compartments such as the thyroid hormone receptor, the tyrosine phosphatase PTP1E, the adaptor protein RIL, the v-Rel oncoprotein, the Cas protein family, or the lysophosphatidic acid receptor (24, 25, 28, 30, 53). The ubiquitous interaction of ZRP-1 with the integral plasma membrane protein endoglin, the cytoplasmic tyrosine phosphatase PTP1E, members of the Cas family, and nuclear proteins, such as v-Rel and thyroid hormone receptor, resembles the behavior of a number of transcription factors, including Rel/NF-κB proteins, that show a highly regulated cytoplasmic/nuclear shuttling. In this regard, the ZRP-1 region containing the LIM domains alone is targeted to the cell nucleus (see Ref. 27 and data not shown). By con-

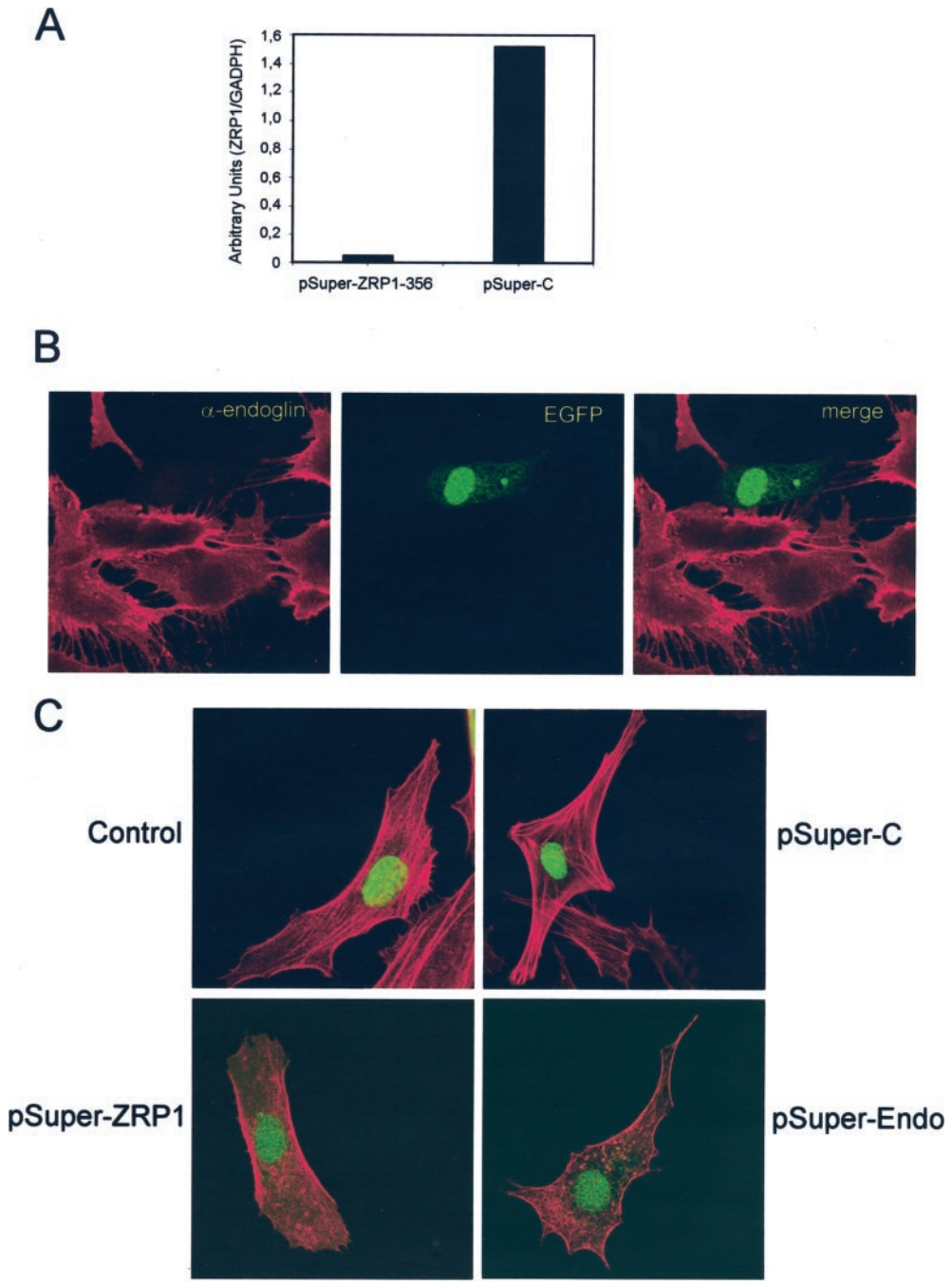

FIG. 6. **Suppression of endoglin or *ZRP-1* gene expression alters the actin organization.** *A*, transfection of pSUPER-ZRP-1–356 suppresses ZRP-1 expression. 293T cells were transiently transfected with pSUPER-ZRP-1–356 encoding a ZRP-1-specific sequence of siRNA or pSUPER-C (used as a negative control), as indicated. After 24 h, cells were lysed, and RNA was extracted and processed for RT-PCR with specific ZRP-1 or GAPDH primers. PCRs were separated on a 3% Nu-Sieve gel, and bands were quantified by densitometry and then plotted in the bar graph. A representative experiment is shown. *B*, transfection of pSUPER-Endo/Ex4 suppresses endoglin expression. HUVECs were transiently cotransfected with peGFPC1-SV40, encoding EGFP tagged with the nuclear localization signal (EGFP-NLP), and pSUPER-Endo/Ex4, encoding an endoglin-specific sequence of siRNA. For immunofluorescence microscopy, HUVECs were stained with a mouse monoclonal antibody to endoglin, then washed and incubated with a secondary anti-mouse IgG coupled to Alexa-456 (*red* fluorescence), and analyzed by confocal microscopy. Transfected cells were identified by the green fluorescence of EGFP constructs, and analysis of their red fluorescence demonstrated absence of anti-endoglin staining. *C*, HUVECs were transiently transfected with peGFPC1-SV40 encoding EGFP tagged with the nuclear localization signal (EGFP-NLP) in the absence (*Control*) or in the presence of pSUPER-Endo/Ex4 encoding an endoglin-specific sequence of siRNA (*pSUPER-Endo*), pSUPER-ZRP-1–356 encoding a ZRP-1-specific sequence of siRNA (*pSUPER-ZRP1*), or pSUPER-C (used as negative control), as indicated. For immunofluorescence microscopy, HUVECs were stained with rhodamine-labeled phalloidin and analyzed by confocal microscopy. Transfected cells were identified by the green fluorescence of EGFP constructs, and their red fluorescence (F-actin) was analyzed. Down-regulation of endoglin or ZRP-1 expression (*lower panels*) results in disruption of F-actin filaments, as compared with control cells (*upper panels*).

trast, the N-terminal ZRP-1 segment, including the nuclear export signal (27), is primarily found in the cytoplasm. Furthermore, expression of the EGFP-ZRP-1 construct encoding the full-length ZRP-1 protein (and therefore containing the nuclear export signal) appears to be excluded from the nucleus (Fig. 3, *C* and *D*, and Fig. 4*B*, *upper row*). This is at variance with the nuclear staining observed in endothelial cells using a polyclonal antibody specific for the endogenous ZRP-1 (Fig. 4*B*, *lower row*), in agreement with a previous report (30). These differences are likely explained by the distinct detection system

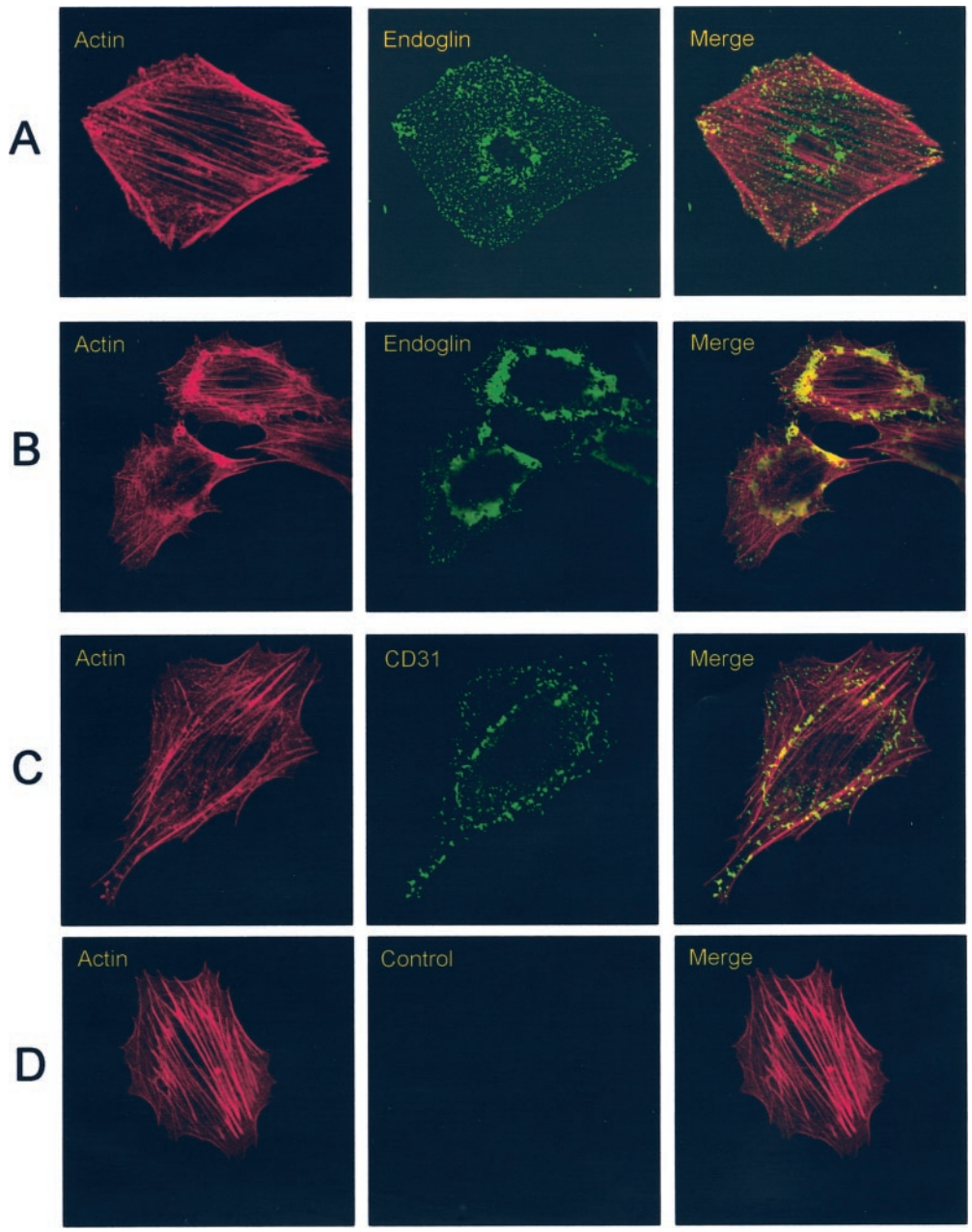

FIG. 7. **Endoglin engagement alters the actin organization.** HUVECs were incubated in the absence (*row D*) or in the presence of mouse anti-endoglin P4A4 (*rows A* and *B*) or anti-CD31 (*row C*) antibodies at either 37 (*rows B–D*) or 4 °C (*row A*). Antibody-mediated clustering was induced by incubation with an Alexa-488 green-conjugated anti-mouse IgG antibody at 37 (*rows B–D*) or 4 °C (*row A*). For immunofluorescence analysis, HUVECs were permeabilized, stained with rhodamine-labeled phalloidin, and analyzed by confocal microscopy. Clustering of endoglin at 37 °C (*green* fluorescence) in *row B* was associated with a similar clustering of actin fibers (*red* fluorescence). Cross-linking at 4 °C did not yield any effect on actin fibers (*row A*).

used for ZRP-1 (direct fluorescence of EGFP constructs *versus* indirect immunofluorescence) and/or the existence of post-translational processing of the endogenous and exogenous ZRP-1 proteins. Whether the ZRP-1 LIM domains can be physiologically cleaved from the N terminus and involved in transcriptional gene regulation (54) deserves to be investigated.

As a consequence of the interaction with endoglin, ZRP-1 changes its cellular distribution. Thus, in the absence of endoglin expression, ZRP-1 localizes to focal adhesion sites. By contrast, in the presence of endoglin, ZRP-1 is found along the actin stress fibers of the cell. As a control, vinculin remained localized at focal adhesion sites either in the absence or in the presence of endoglin. The specificity of the subcellular redistribution of ZRP-1 in the presence of endoglin was further documented using mutant constructs lacking the domains involved in the ZRP-1-endoglin interaction (LIM domains of ZRP-1 or cytoplasmic domain of endoglin). Therefore, it can be postulated that endoglin interacts with ZRP-1 at focal adhesion sites and redirects ZRP-1 localization to the plasma membrane where endoglin is anchored. Supporting this view, endoglin and ZRP-1 were found to colocalize in endothelial cells (Fig. 2*E*). The association of ZRP-1 with endoglin seems to involve the simultaneous interaction of ZRP-1 with actin, as well as important changes in the actin cytoskeleton. Thus, in cells lacking endoglin or expressing the homologous protein betaglycan, the actin cytoskeleton is poorly organized with F-actin distributed in discrete bundles at cell-substrate contacts (where ZRP-1 also localizes), and minor thin peripheral bands. However, cells expressing endoglin, such as endothelial cells, show a well organized cytoskeleton with a large number of long actin fibers

clearly defining the contour of the cell. It is worth noting the perfect colocalization between ZRP-1 along actin fibers (Fig. 4). Also, when endoglin or ZRP-1 expression is suppressed, or when endoglin is engaged, endothelial cells show important alterations of the F-actin network (Fig. 6). Furthermore, when endoglin deletion constructs lack the interacting domain, both ZRP-1 and F-actin accumulate at focal adhesion sites, similarly to cells not expressing endoglin (Fig. 5). These results indicate that F-actin accumulates at sites where ZRP-1 is localized, confirming the important role that ZRP-1 plays in the control of assembly and organization of the actin cytoskeleton. This is strikingly similar to the behavior of the homologous protein zyxin that plays a role in adhesion plaques and actin-microfilament organization (24, 26, 35, 30, 40). As a result of its interaction with ZRP-1, endoglin appears to also be involved in the organization of the cytoskeleton. This functional role of endoglin could account for the altered cellular morphology and migration observed in fibroblasts, epithelial, and smooth muscle cells upon endoglin overexpression (6, 21, 55). In agreement with these findings, overexpression of ZRP-1 leads to decreased cell migration (23); zyxin expression correlates with cell spreading (56), and mislocalization of zyxin produces dramatic cytoskeletal rearrangements that also perturb cell motility (36, 38). Most interesting, endoglin-dependent modulation of cell motility appears to be mediated by the presence of zyxin (52).

In addition to the experimental evidence shown here, a different line of research has provided further support for ZRP-1 function in actin remodeling. Thus, ZRP-1 has been identified in a two-hybrid screening for proteins that interact with *N. gonorrhoeae* OpaP, a member of a family of outer membrane proteins involved in gonococcal adherence to and invasion of human cells (29). Reorganization of the actin cytoskeleton is an important step in the invasion and spreading of bacteria in eucaryotic cells, and OpaP is likely to have a role in this process. Likewise, the similarity between the ZRP-1 homologues zyxin and ActA, the protein of *Listeria monocytogenes* that facilitates ghost cell actin remodeling during infection and spread (40), may be more than pure coincidence. Furthermore, both zyxin and ZRP-1 have been reported to interact with $p130^{Cas}$ and Casl/HEF1, members of the Cas family, which are recruited by integrins at focal adhesion sites, where they dock regulatory components such as focal adhesion kinase, Src, and Crk (30).

The mechanism by which ZRP-1 modulates the organization of the actin cytoskeleton is unknown, but it could be acting in a way analogous to the homologous proteins zyxin and LPP. In this sense, members of the Ena/VASP protein family, involved in the actin filament assembly (57–60), are able to interact with zyxin and LPP (33, 36). It has been shown that when zyxin changes its subcellular localization it simultaneously associates with members of the Ena/VASP family, leading to an important remodeling of the actin cytoskeleton (36, 39, 41). Thus, it can be speculated that the endoglin-induced change in the subcellular localization of ZRP-1 would also affect the composition of protein complexes involved in actin polymerization and focal adhesion sites.

Many cellular processes depend on cytoskeletal rearrangements involving actin filaments, for example, cell shape and locomotion. Moreover, cellular responses to extracellular signals often involve extensive rearrangement of actin cytoskeleton, including localized increases in actin polymerization. Endoglin is expressed by endothelial cells where all these processes are supposed to have important implications. Endothelial cells display a highly organized cytoskeleton with fibers orderly distributed throughout the cell. The assembly and dynamics of this network is crucial for important physiological processes such as angiogenesis, vascular morphology and permeability, inflammatory response, or sensing and responding to shear stress. Previous reports (24, 28) have shown that ZRP-1 transcripts are abundant in vascularized tissues, but the specific cell lineage responsible for this expression was not known. Here we have demonstrated for the first time the presence of ZRP-1 in endothelial cells, showing a distribution along actin fibers, similar to cells expressing ectopic endoglin. Taken together, our results suggest that endoglin, via its association with ZRP-1, could be involved in the organization and maintenance of the actin network in endothelial cells.

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