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Role of Endoglin in Cellular Responses to Transforming Growth Factor-β

A COMPARATIVE STUDY WITH BETAGLYCAN*

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Endoglin (CD105) is the target gene for the hereditary hemorrhagic telangiectasia type I (HHT1), a dominantly inherited vascular disorder. It shares with betaglycan a limited amino acid sequence homology and being components of the membrane transforming growth factor-β (TGF-β) receptor complex. Using rat myoblasts as a model system, we found that overexpression of endoglin led to a decreased TGF-β response to cellular growth inhibition and plasminogen activator inhibitor-1 synthesis, whereas overexpression of betaglycan resulted in an enhanced response to inhibition of cellular proliferation and plasminogen activator inhibitor-1 induced expression in the presence of TGF-β. The regulation by endoglin of TGF-β responses seems to reside on the extracellular domain, as evidenced by the functional analysis of two chimeric proteins containing different combinations of endoglin and betaglycan domains. Binding followed by cross-linking with ^{125}I-TGF-β1 demonstrated that betaglycan expressing cells displayed a clear increase (about 3.5-fold), whereas endoglin expressing cells only displayed an slight increment (about 1.6-fold) in ligand binding with respect to mock transfectants. SDS-polyacrylamide gel electrophoresis analysis of radiolabeled receptors demonstrated that expression of endoglin or betaglycan is associated with an increased TGF-β binding to the signaling receptor complex; however, while endoglin increased binding to types I and II receptors, betaglycan increased the binding to the type II receptor. Conversely, we found that TGF-β binding to endoglin required the presence of receptor type II as evidenced by transient transfections experiments in COS cells. These findings suggest a role for endoglin in TGF-β responses distinct from that of betaglycan.

Endoglin (CD105), is a 180-kDa homodimeric membrane glycoprotein strongly expressed by human endothelial cells (1). The gene encoding endoglin has been identified as the target for the dominant vascular disorder known as hereditary hemorrhagic telangiectasia type 1 (HHT1)[1] (2, 3). HHT is a highly penetrant autosomal dominant vascular dysplasia associated with frequent epistaxis, gastrointestinal bleedings, telangiectases, and arteriovenous malformations in brain, lung, and liver (4, 5). The specific function of endoglin responsible for the vascular dysplasia in HHT1 is not known, but it is likely related to the transforming growth factor-β (TGF-β) system as endoglin is a functional component of the membrane TGF-β receptor complex. Endoglin binds TGF-β1 and TGF-β3 with high affinity (K_D = 50 pM) in human endothelial cells (6); the heteromeric association between endoglin and the TGF-β signaling receptors I (R-I) and II (R-II) has been suggested by co-immunoprecipitation experiments (7, 8), and overexpression of endoglin is able to modulate cellular responses to TGF-β (9). TGF-β is a member of a large family of proteins that has many biological effects including regulation of cellular proliferation, differentiation, migration, and extracellular matrix formation (10, 11). Cellular responses to TGF-β also comprise the expression of genes encoding protease inhibitors such as the increased secretion of the 45-kDa plasminogen activator inhibitor-1 (PAI-1) (12, 13). In humans, three isoforms have been identified, namely TGF-β1, TGF-β2, and TGF-β3. Thus, perturbation of one or more of these processes may cause the vascular dysplasia observed in HHT1 patients.

TGF-βs exert their function through binding to a large family of specific receptors, including receptors type I, II, betaglycan, and endoglin (14, 15). Among these, the serine-threonine kinase receptors types I and II are necessary for all tested biological responses to TGF-β and transmit the signal to downstream substrates through their kinase activity. By contrast, endoglin and betaglycan have been postulated as regulators of TGF-β access to the signaling receptors. Endoglin and betaglycan share a region of high identity in the cytoplasmic tail, but show a limited homology on the extracellular domain (16–19). On the other hand, endoglin and betaglycan markedly differ in their cellular distribution, and from the functional point of view, endoglin has been shown to inhibit TGF-β1 responses in human monocytic cells (9), whereas betaglycan seems to increase TGF-β2 signaling, leaving unaffected the TGF-β1 response in rat myoblasts (20). Unfortunately, these studies were carried out in different cellular lineages and comparative conclusions about endoglin with respect of betaglycan could not be drawn. Here, we have used a common cell type to

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[1] The abbreviations used are: HHT, hereditary hemorrhagic telangiectasia; TGF-β, transforming growth factor-β; PAI-1, plasminogen activator inhibitor 1; R-I, transforming growth factor-β receptor type I; R-II, transforming growth factor-β receptor type II; kb, kilobase(s); PCR, polymerase chain reaction; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

further analyze the role of endoglin and its homologue betaglycan in the TGF-β cellular responses, as well as the interdependence of endoglin and the signaling receptors in ligand binding.

EXPERIMENTAL PROCEDURES

Plasmids—The pcEXV-EndoL vector containing the human L-endoglin isoform driven by the SV40 promoter (21) was used in transfection experiments of rat myoblasts. The pCMV-EndoL vector, containing the human L-endoglin isoform driven by the cytomegalovirus promoter, was constructed by inserting the 2.3-kb *Eco*RI endoglin cDNA, obtained by digestion of pcEXV-EndoL, into the *Eco*RI digested pCMV vector (Invitrogen). The human betaglycan cDNA (19) cloned into the *Eco*RI site of pSV7d vector, was kindly provided by Dr. Kohei Miyazono (JFCR, Tokyo, Japan). The pCMV-Endo/Beta vector containing the extracellular and transmembrane domains of endoglin and the cytoplasmic domain of betaglycan was obtained by replacing the 0.44-kb MluI/*Eco*RI fragment of endoglin cDNA by the 0.3-kb MluI/*Eco*RI fragment of a PCR product amplified from human betaglycan. Amplification of human betaglycan cDNA (accession number S50051) was carried out using the following primers: 5′-CTCACACAGGGGAGACAGCAGG- 3′ (position 3.044 to 3.065, including a MluI site) and 5′-GAATTCTAGT-GTGGTACAGAAGCCC-3′ (position 3.395 to 3.371, including an *Eco*RI site). The pSV7-Beta/Endo vector containing the extracellular domain of betaglycan and the transmembrane and cytoplasmic domains of endoglin was obtained in a triple in-frame reaction. The 2.6-kb *Eco*RI/*Ssp*I fragment of betaglycan cDNA (19), the *Eco*RI/*Xba*I-digested pSV7d expression vector, and the 0.6-kb *Ssp*I/*Xba*I fragment of the PCR product number 3, amplified as described below, were ligated resulting in the chimeric construct pSV7-Beta/Endo. The PCR product number 3 was obtained by a three-step amplification process. First, amplification of L-endoglin cDNA (accession number J05481) was carried out using the following primers: 5′-GGCTATGCCATGCTGCTGGTGG-3′ (oligonucleotide EndoCyt2; position 1.919 to 1.940, including the stop codon), and 5′-GGTACATCTACTCTCACAC-3′ (oligonucleotide EBC-1; positions 1.793 to 1.811 with a point mutation G → T at 1.806, containing a highly homologous sequence with betaglycan), resulting in the 150-bp PCR product number 1. The parallel amplification of betaglycan was performed using the following primers: 5′-GTGTGAGAGTAGATG-TACC-3′ (oligonucleotide EBC2; positions 3.032 to 3.050 with a point mutation T → G at 3.042, containing a highly homologous sequence with endoglin), and 5′-CCATTATTGAGAATATTTGT-3′ (oligonucleotide *Ssp*I-OL; positions 2.576 to 2.597, including a *Ssp*I site), resulting in the 476-base pair PCR product number 2. Next, annealed PCR products numbers 1 and 2 were extended and amplified by *Taq* polymerase with oligonucleotides EndoCyt2 and *Ssp*I-OL, yielding the 0.6-kb recombinant PCR product number 3. This fragment was cloned into pCR2.TOPO vector (Invitrogen) and the 0.6-kb *Ssp*I/*Xba*I digestion product was ligated, as described above. Plasmids pCMV5-TβR-II containing the TGF-β receptor type II, and pCMV5-TβRI containing the Alk-5 TGF-β receptor type I were generously provided by Dr. Joan Massagué (Howard Hughes Medical Institute, New York). The plasmid pCMV5-TSR-I contains the Alk-1 TGF-β receptor type I (22). The *Eco*RI fragment of S-endoglin (21) was subcloned into pCMV5 for resulting in pCMV-EndoS. Plasmid p800 (kindly provided by Dr. Daniel B. Rifkin, NYU Medical Center, NY) encodes the PAI-1 promoter (23) fused to the luciferase reporter gene. The promoter activity of this construct has been shown to be induced by TGF-β (24). The plasmid pSV2neo (CLONTECH) contains a neomycin resistance gene.

Cells and Stable Transfectants—The rat myoblast cell line L_6E_9 was cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and gentamycin (25 μg/ml) in a 5% CO_2 atmosphere at 37 °C. Treatment of cells with recombinant human TGF-β1 (R&D Systems, Abingdon, UK) was performed at a concentration of 500 pM for the times indicated. L_6E_9 transfectants expressing betaglycan (20) were generously provided by Dr. Joan Massagué. Rat myoblast transfectants expressing human endoglin were generated by co-transfecting pcEXV-EndoL or pCMV-Endo/Beta vectors and psV2neo at a 10:1 ratio. 10 μg of plasmid DNA were mixed with 20 μg of Lipofectin (Life Technologies, Inc.) in serum-free medium according to the protocol provided by the manufacturer. Positive clones were selected in the presence of 400 μg/ml of the antibiotic G418. Parallel transfections with psV2neo alone yielded endoglin-negative mock transfectants. Pooled clones were used in biochemical and functional characterizations. No significant differences were observed between parental and mock transfectants in biochemical and functional studies.

Confluent monolayers of adherent cells in 60-mm diameter dishes were used in flow cytometry, biotinylation, and affinity labeling studies. Adherent cells to be analyzed by flow cytometry, or adherent biotin-labeled cells, were removed from culture dishes by incubation with 0.25% trypsin, 1 mM EDTA for 3 min at room temperature. Adherent cells to be analyzed after TGF-β affinity labeling were removed from culture dishes by incubation with 10 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA, pH 7.4, for 10 min at 4 °C, followed by gentle scraping with a Teflon scraper.

Flow Cytometry—Cells (5×10^5) were incubated with the mouse mAb 8E11 (anti-endoglin) (25), the mouse mAb 44G4 (anti-fragment Y277-G331 of human endoglin) (1, 26), or the rabbit polyclonal antibody 822 to the ectodomain of betaglycan (generous gift from Dr. Joan Massagué) for 30 min at 4 °C. After two washes with PBS, fluorescein isothiocyanate-labeled $F(ab')_2$ rabbit anti-mouse or fluorescein isothiocyanate-labeled pig anti-rabbit IgG (Dakopatts) were added and incubation proceeded for an additional period of 30 min at 4 °C. Finally, cells were washed twice with PBS and their fluorescence was estimated with an EPICS-CS (Coulter Científica, Móstoles, Spain), using logarithmic amplifiers.

Cell Surface Biotinylation—Cells were washed at 4 °C with Hepes buffer (150 mM NaCl, 5 mM KOH, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES, pH 7.4) and allowed to equilibrate for 30 min at 4 °C in the same buffer. Cells were washed again and fresh Hepes buffer containing 0.5 mg/ml sulfosuccinimidyl-6-(biotinamido)hexanoate (NHS-LC-biotin, Pierce Chemical Co.) was added. After incubation at 4 °C for 2 h, the reaction was stopped by washing twice with Hepes buffer. For immunoprecipitation studies, cells were lysed in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 50 μg/ml leupeptin, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride), for 40 min at 4 °C. The lysates were centrifuged for 15 min at 12,000 × *g* and the supernatants were precleared for 4 h with protein G coupled to Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) at 4 °C. Specific immunoprecipitations of the precleared lysates were carried out in the presence of either the mouse mAb 44G4 (anti-fragment Y277-G331 of human endoglin) or the rabbit polyclonal antibody 822 (anti-betaglycan ectodomain), using protein G coupled to Sepharose. After overnight incubation at 4 °C, immunoprecipitates were isolated by centrifugation and washed twice with lysis buffer at 4 °C. Immune complexes were subjected to SDS-PAGE on a 7.5% acrylamide gel under nonreducing conditions and then electrotransferred to nitrocellulose. Filters were blocked with 5% powder milk in PBS for 1 h and then incubated with 2 μg/ml streptavidin conjugated to horseradish peroxidase (Pierce) for 2 h at room temperature. Biotinylated endoglin or betaglycan were detected using an Enhanced ChemiLuminescence system (Amersham Ibérica S.A., Madrid).

Proliferation Assays—Rat myoblasts were cultured in flat-bottomed 24-well plates (Costar, Cambridge, MA) at 4×10^4 cells/well in Dulbecco's modified Eagle's medium with 10% fetal calf serum in the absence or presence of TGF-β1 for the times indicated, the last 6 h in the presence of 1 μCi per well of [*methyl*-^{3}H]thymidine (Amersham, United Kingdom). Cells from triplicate samples were washed twice in PBS, fixed in methanol for 30 min at 4 °C, and lysed in 0.1 N NaOH overnight. Lysates were neutralized with HCl and [^{3}H]thymidine incorporation into DNA was measured in a liquid scintillation counter.

Receptor Affinity Labeling—Affinity labeling assays were basically performed as described (27). For cross-linking experiments, cells were incubated in Hepes buffer containing 0.1% bovine serum albumin with 50–250 pM ^{125}I-TGF-β1 (specific activity 1, 200–2000 Ci/mmol; Amersham Ibérica S.A., Madrid) for 4 h. Cells were washed and radiolabeled TGF-β1 was cross-linked with 0.15 mM disuccinimidyl suberate (Pierce) in Hepes buffer for 15 min at 4 °C. Cells were washed 4 times and solubilized in lysis buffer. For each ligand concentration, cell associated radioactivity was estimated in a γ-counter (LKB, Bromma, Sweden). The total extracts were subjected to SDS-PAGE analysis or to specific immunoprecipitation. Immunoprecipitations were carried out with the rabbit polyclonal antibody BN (anti-endoglin), generated by infection with recombinant vaccinia virus expressing endoglin (28), or the rabbit polyclonal antibody 822 (anti-betaglycan). Detection of the ^{125}I-labeled receptors was revealed by autoradiography or using a PhosphorImager 410A and ImageQuant software (Molecular Dynamics).

When required, cDNAs encoding human type I receptor (Alk-1 or Alk-5), type II receptor, L-endoglin or S-endoglin in pCMV5 were transiently transfected in different combinations into COS-7 cells. Basically, 20 μg of total plasmid DNA were mixed with 20 μg of Lipofectin and incubated with COS cells following the protocol provided by Life Technologies, Inc. After 48 h in culture, cells were affinity labeled with ^{125}I-TGF-β and processed as described above.

PAI-1 Induction Assays—Experiments to determine PAI-1 synthesis

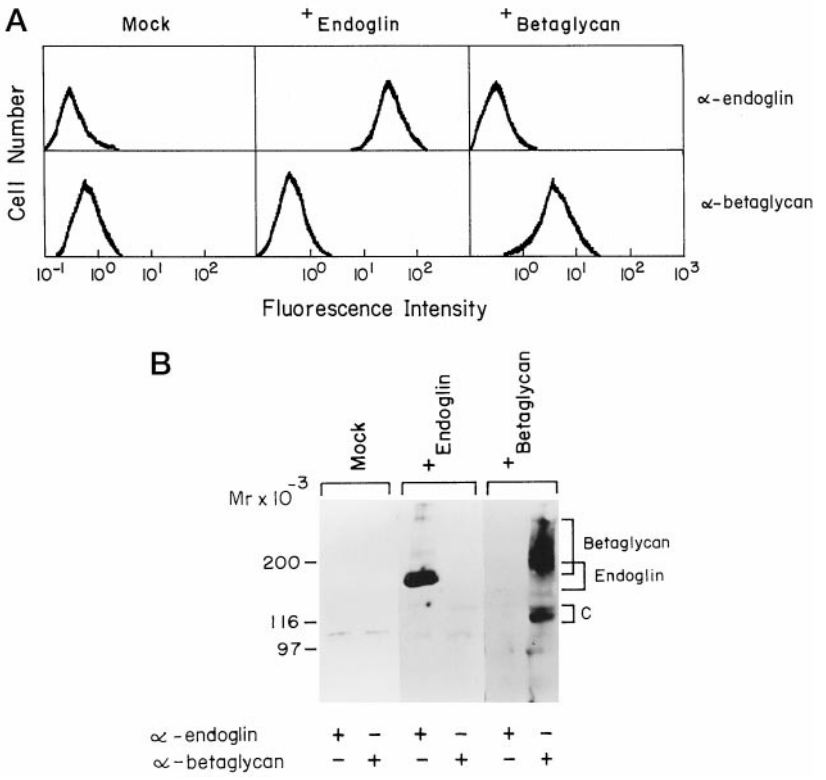

FIG. 1. **Characterization of stable myoblast transfectants expressing endoglin or betaglycan.** *A,* analysis by cytofluorometry of endoglin and betaglycan present at the cell surface. Myoblasts were stained for indirect immunofluorescence with anti-endoglin or anti-betaglycan antibodies, as indicated. A control staining of mock transfectants is also shown. *B,* immunoprecipitation analysis. Myoblasts were surface labeled with biotin, lysed, and immunoprecipitated with anti-endoglin or anti-betaglycan antibodies, as indicated. Samples were electrophoresed on a 7.5% acrylamide gel under nonreducing conditions, transferred to nitrocellulose and biotinylated polypeptides detected using a chemiluminescence assay. Bands corresponding to endoglin, betaglycan, or the putative betaglycan core (*C*) are indicated. Minor bands of approximately 100 kDa are likely unspecific as they could be detected with variable intensity in mock transfectants.

were performed basically as described (29). For Western blot analysis, subconfluent myoblasts were treated either in the absence or presence of 100 pM TGF-β1 in Dulbecco's modified Eagle's medium containing 10% fetal calf serum overnight at 37 °C under 5% CO_2 atmosphere. Then, cells were lysed by washing on ice once with PBS, three times with 10 mM Tris-HCl, pH 8, 0.5% deoxycholate, and 1 mM phenylmethylsulfonyl fluoride, twice with 2 mM Tris-HCl, pH 8, and once with PBS. Next, 80 μl of electrophoresis sample buffer containing dithiothreitol was added to each sample and dishes were scraped to recover all the matrix proteins. Samples were denatured by boiling and aliquots containing equal amounts of total protein were subjected to 10% SDS-PAGE under reducing conditions. Proteins were electrotransferred to nitrocellulose membranes (Millipore Corp., Bedford, MA). Filters were blocked with PBS containing 5% milk powder for 1 h. Specific immunodetection was carried out by incubation with rabbit IgG anti-rat PAI-1 (Molecular Innovations Inc., Royal Oak, MI) overnight, followed by peroxidase-conjugated goat anti-rabbit Ig at room temperature. The presence of PAI-1, as a characteristic band of 45 kDa, was revealed using a chemiluminescence assay (ECL detection kit, Amersham Ibérica).

For immunoprecipitation analysis, subconfluent myoblasts were treated either in the absence or presence of TGF-β1 as described above. After treatment, cells were washed three times with methionine/cysteine-free medium (ICN Biomedicals, Barcelona) and preincubated in this medium for 15 min, the medium was removed and fresh methionine/cysteine-free medium containing 50 μCi/ml [^{35}S]methionine/cysteine (Trans^{35}S-Label, ICN Biomedicals) was added. Metabolic labeling was carried out for 2 h either in the absence or presence of 500 pM TGF-β1 at 37 °C. After labeling, supernatants were collected and samples containing equal amounts of protein bound radioactivity were subjected to immunoprecipitation with a rabbit IgG anti-rat PAI-1 followed by SDS-PAGE analysis under reducing conditions. Detection of the ^{35}S-labeled PAI-1 was revealed by autoradiography.

PAI-1 promoter activity was assayed by transient transfections on myoblasts using SuperFect™ (Qiagen). Briefly, 1 μg of the p800 plasmid, encoding the PAI-1 promoter, and 1 μg of CMVβgal were mixed with 5 μg of SuperFect™ in Dulbecco's modified Eagle's medium. When required, different combinations of expression vectors encoding betaglycan, L-endoglin, the chimera Endo/Beta, or the chimera Beta/Endo, were also included. The mixture was incubated with the cells previously seeded in a 24-well plate at 5×10^4 cells/well. The following day, cells were incubated either in the absence or presence of 100 pM TGF-β1 for an additional period of 24 h. Cells were lysed and the transduced enzymatic activities were assayed. Internal normalization was done by co-transfection of the test plasmid p800 with CMVβgal, a β-galactosidase expression vector driven by the cytomegalovirus promoter. Luciferase activity was measured using a TD-20/20 Luminometer (Promega, Madison, WI) and β-galactosidase activity was determined by measuring the absorbance at 405 nm upon incubation with the substrate *ortho*-nitrophenyl galactopyranoside (Sigma).

RESULTS

Endoglin Expression in Myoblasts Interferes with TGF-β Signaling—To compare the role of endoglin with that of betaglycan in the TGF-β signaling, we overexpressed by transfection endoglin (L-endoglin isoform) and betaglycan in rat myoblasts (Fig. 1). Both, endoglin and betaglycan were highly expressed on the cell surface as determined by fluorescence flow cytometry (Fig. 1*A*). Immunoprecipitation analysis (Fig. 1*B*) revealed a 170-kDa band corresponding to the dimeric form of endoglin, specifically recognized by anti-endoglin antibodies. Also, anti-betaglycan antibodies specifically immunoprecipitated a broad band of 190–300 kDa, corresponding to the mature betaglycan, along with a minor band of approximately 120 kDa corresponding to the putative betaglycan core. No specific reactivity could be detected in mock transfectant cells either by flow cytometry or immunoprecipitation analyses. Next, we analyzed the effect of TGF-β in two functions known to be modulated by this factor (Fig. 2). First, cellular proliferation of myoblasts is known to be negatively affected by TGF-β1 (20). Endoglin overexpression resulted in a deficient ligand response, at concentrations of 5–500 pM TGF-β1, with respect to mock transfectants (Fig. 2*A*). By contrast, betaglycan expression led to an increased TGF-β responsiveness, in agreement with a previous report (20). Next, we analyzed the cellular PAI-1 synthesis, which is known to be increased in the presence of TGF-β (12, 13). We then assayed synthesis of PAI-1 by Western blot analysis of rat myoblasts (Fig. 2, *B* and *C*). The levels of PAI-1 in response to TGF-β1 were found to be higher in betaglycan transfectants, or lower in endoglin transfectants, than those of parental myoblasts. As a control, immunoprecipitation analysis from metabolically labeled myoblasts revealed a characteristic 45-kDa band of PAI-1, specifically recognized upon TGF-β treatment (Fig. 2*B*).

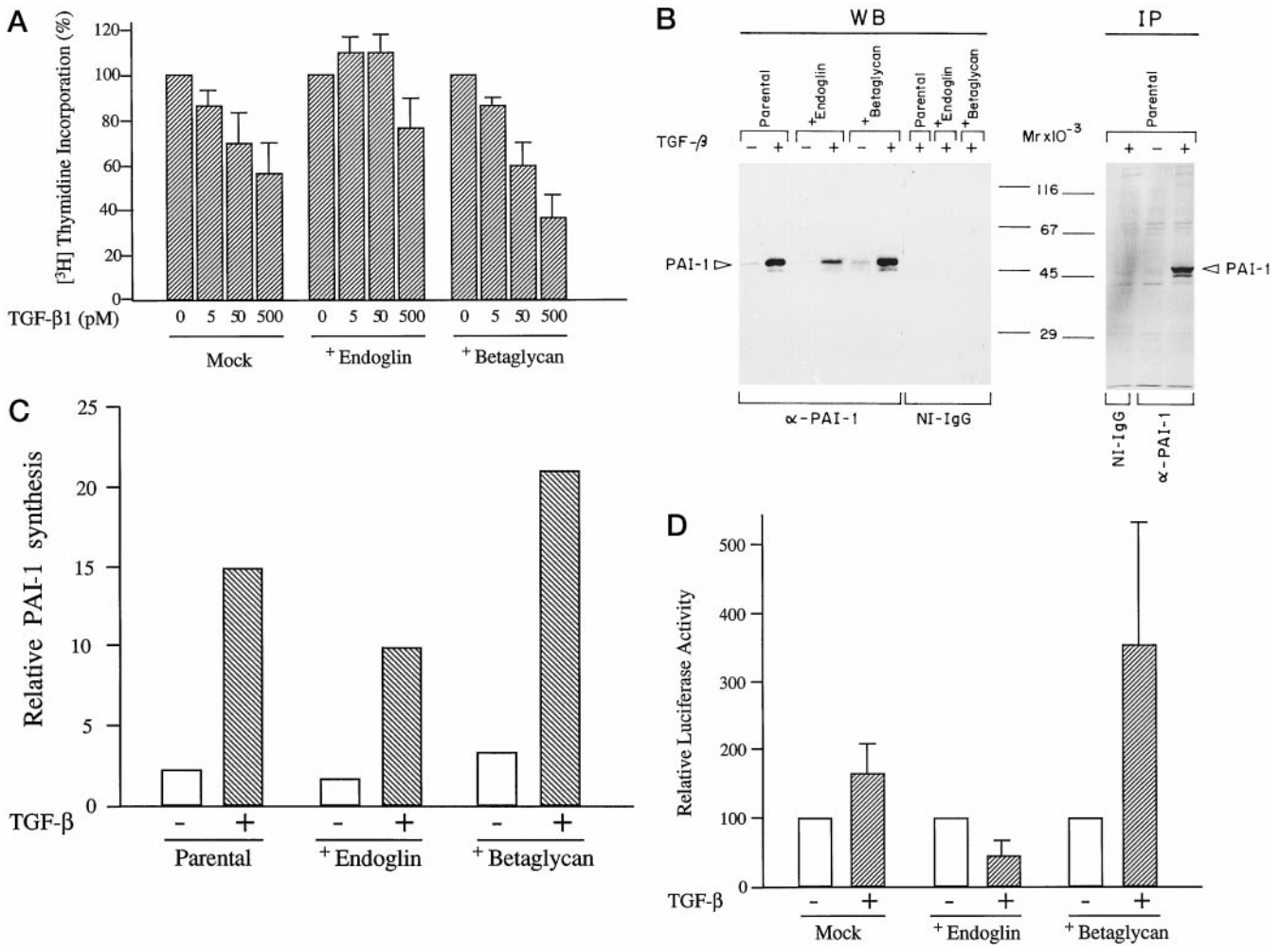

FIG. 2. **Functional characterization of stable myoblast transfectants.** *A,* effect of endoglin or betaglycan expression on the TGF-β1-induced inhibition of proliferation. Myoblast transfectants were incubated with increasing concentrations of TGF-β1 (5–500 pM) and the proliferation capacity of the cells was measured by [^{3}H]thymidine incorporation. The pattern of TGF-β1 inhibition of proliferation was the same in parental and mock transfected myoblasts. The mean of five different experiments performed in triplicate samples is shown. Standard deviations are indicated. *B,* effect of endoglin or betaglycan expression on the TGF-β1-induced PAI-1 synthesis. Myoblast transfectants (*+Betaglycan* and *+Endoglin*) and parental cells were incubated either in the absence or presence of TGF-β1 and extracellular matrix proteins solubilized as described under "Experimental Procedures." Samples containing equal amounts of total protein were subjected to SDS-PAGE under reducing conditions and analyzed by Western blot (*WB*) using a rabbit IgG anti-rat PAI-1 (*α-PAI-1*) or a non-immune rabbit IgG (*NI-IgG*). For immunoprecipitation (*IP*) studies parental myoblasts were metabolically labeled with [^{35}S]methionine/cysteine for 4 h either in the absence or presence of TGF-β1 as indicated. Radiolabeled culture supernatants were collected and samples containing equal amounts of protein bound radioactivity were immunoprecipitated with rabbit IgG anti-rat PAI-1 or non-immune rabbit IgG. Immunoprecipitates were analyzed by SDS-PAGE under reducing conditions followed by autoradiography. The TGF-β1 induction of PAI-1 was similar in parental and mock transfected myoblasts. The characteristic 45-kDa band of PAI-1 is indicated by *arrowheads. C,* the PAI-1 bands of the Western blot analysis (*panel B*) were quantitated by densitometry using a PhosphorImager 410 and ImageQuant software. *D,* TGF-β induction of PAI-1 promoter activity in myoblast transfectants. Myoblast transfectants were transiently transduced with the p800 construct containing the PAI-1 promoter fused to the luciferase gene. TGF-β1 was added 24 h after transfection to half of the transfected cells and luciferase activity was determined 48 h after transfection. The TGF-β1 inducibility of the PAI-1 promoter was similar in parental and mock transfected myoblasts. For comparative purposes, values of untreated cells were arbitrarily set at 100. The mean of three different experiments is shown.

Furthermore, we assayed the PAI-1 promoter activity which can be induced by TGF-β (24). As expected, mock transfectants showed an increased transcriptional activity of PAI-1, whereas betaglycan transfectants displayed an even higher response (Fig. 2*D*). By contrast, no induction of the PAI-1 promoter activity could be detected in endoglin transfectants. Taken together, these results demonstrate that, at variance with betaglycan, expression of endoglin in myoblast cells interferes with signaling responses to TGF-β1.

The Effect of Endoglin in TGF-β Responses Resides in the Extracellular Domain—Given the distinct effect of endoglin and betaglycan with respect to the cellular TGF-β responses, we engineered the chimeric constructs Endo/Beta and Beta/Endo to determine the specific endoglin domain involved (Fig. 3*A*). The Endo/Beta construct encodes a chimeric protein with the extracellular and transmembrane domains of endoglin fused to the cytoplasmic region of betaglycan; the Beta/Endo construct encodes a chimeric protein with the extracellular and transmembrane domains of betaglycan fused to the cytoplasmic region of endoglin. These constructs were transiently transfected into parental myoblasts and the TGF-β inducibility of the PAI-1 promoter was analyzed (Fig. 3*B*). As expected, parental cells showed an increased PAI-1 activity in the presence of TGF-β. Cells transfected with betaglycan or Beta/Endo chimera showed an increased PAI-1 response as compared with parental myoblasts. By contrast, the PAI-1 promoter activity of cells transfected with endoglin or Endo/Beta chimera did not respond to TGF-β. As a control, cells transiently transfected with all these constructs demonstrated the expression of the corresponding recombinant proteins at the cell surface (data

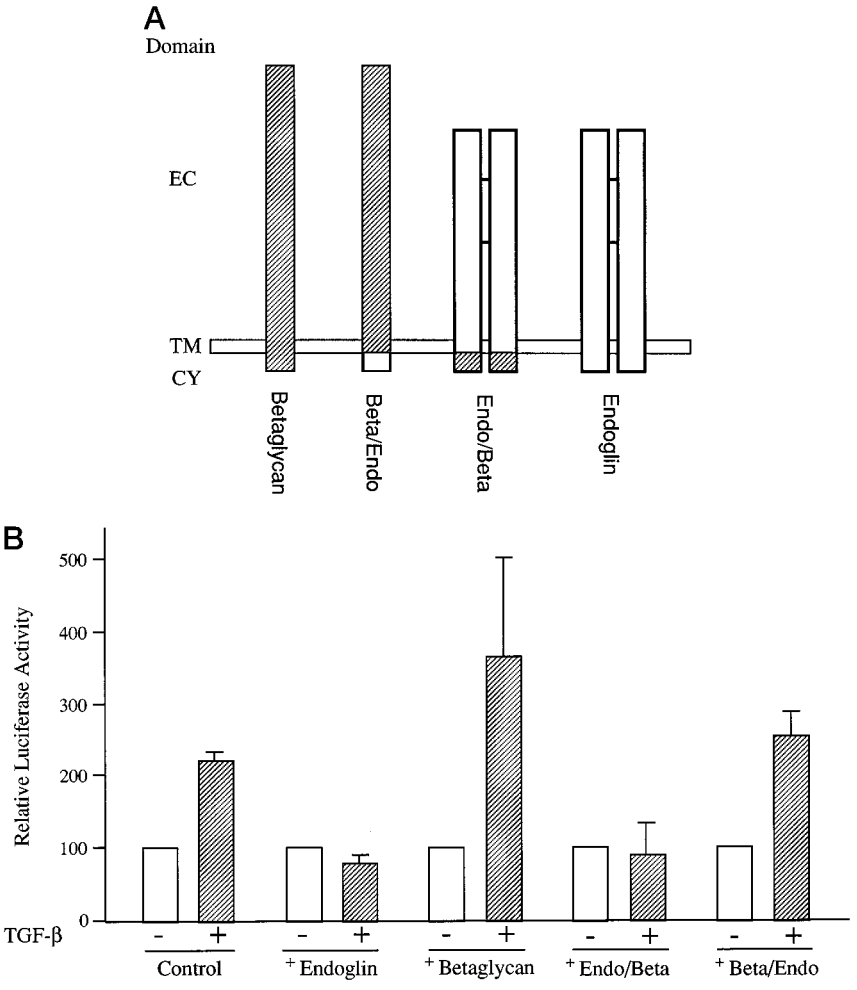

FIG. 3. **Effect of Endo/Beta and Beta/Endo chimera expression on the TGF-β inducibility of the PAI-1 promoter.** *A*, schematic representation of the protein chimeras encoded by Endo/Beta and Beta/Endo constructs. The extracellular (*EC*), transmembrane (*TM*), and cytoplasmic (*CY*) domains of the proteins are indicated. The cDNA-encoded polypeptide of human betaglycan is 849 amino acids long, whereas the one encoded by human L-endoglin contains 658 residues. The proteins are represented approximately to scale. Sequences corresponding to betaglycan or endoglin are depicted by *hatched* or *open boxes*, respectively. *B*, TGF-β inducibility of the PAI-1 promoter. Parental myoblasts were transiently cotransfected with the p800 construct and expression vectors encoding betaglycan, L-endoglin, the chimera Endo/Beta or the chimera Beta/Endo, as indicated. TGF-β1 was added 24 h after transfection to half of the transfected cells and luciferase activity was determined 48 h after transfection. For comparative purposes, values of untreated cells were arbitrarily set at 100. The mean of four different experiments performed in duplicates is shown.

not shown). These data suggest the involvement of the extracellular domains of endoglin or betaglycan in their capacity to modulate TGF-β responses. This was confirmed by proliferation studies using stable myoblast transfectants expressing the Endo/Beta chimera (Fig. 4). The mAb 44G4 to the extracellular domain of endoglin (26) demonstrated by flow cytometry the cell surface expression of the chimera in myoblasts stably transfected with the pcEXV-Endo/Beta construct (Fig. 4*A*). The same mAb was used in immunoprecipitation analysis (Fig. 4*B*), revealing a 170-kDa band corresponding to the endoglin/betaglycan chimeric protein. As expected, the polyclonal antibody 822 to the extracellular domain of betaglycan did not recognize the chimeric protein. Then, we analyzed the effect of TGF-β in the cellular proliferation of the myoblasts expressing the chimera. As shown in Fig. 2, endoglin overexpression was associated with a deficient response to TGF-β1 with respect of mock transfectants. Fig. 4*C* reveals that expression of the chimeric protein resulted in a similar unresponsiveness to TGF-β1, whereas betaglycan transfectants showed an increased responsiveness. Taken together, these results suggest that the extracellular domain of endoglin is responsible for its modulatory role in TGF-β cellular responses.

Binding of TGF-β to Signaling Receptors in Myoblast Transfectants—Given the distinct modulatory effect of endoglin and betaglycan on TGF-β signaling, it was of interest to study ligand binding to the myoblast transfectants. TGF-β1 binding to cell transfectants was analyzed by affinity labeling followed by cross-linking at different concentrations of ligand. Fig. 5*A* shows that specific binding to mock, endoglin, and betaglycan transfectants was increased in a dose-dependent manner. Binding to betaglycan expressing cells was clearly increased (about 3.5-fold at 100–200 pM) with respect to mock cells, whereas binding to endoglin transfectants was only slightly incremented (about 1.7-fold at 100–200 pM). The species binding TGF-β1 were analyzed by SDS-PAGE fractionation of total lysates (Fig. 5*B*). Two distinct polypeptides of approximately 66 and 90 kDa corresponding to the putative TGF-β receptors type I and II, respectively, could be detected in mock transfectants at 200 pM ligand. The labeling of these two receptors was enhanced in endoglin transfectants, whereas betaglycan expressing myoblasts displayed only increased ligand binding to putative R-II. However, the presence of high molecular mass receptors (>130 kDa) were more abundant in betaglycan transfectants than in mock or endoglin transfectants. These high molecular weight receptors likely correspond to betaglycan-ligand complexes, as indicated by immunoprecipitation experiments with specific antibodies (Fig. 5*C*). Thus, a band of molecular masss >200 kDa corresponding to mature betaglycan was immunoprecipitated from betaglycan transfectants, but not from parental myoblasts. In addition, the putative betaglycan core (17, 18) could be detected as a band of approximately 120 kDa. Binding of TGF-β1 to endoglin could also be demonstrated by immunoprecipitation with anti-endoglin antibodies. Specific bands of 200 kDa (nonreducing conditions) and 100 kDa (reducing conditions) corresponding to the dimeric or monomeric forms of endoglin were specifically immunoprecipitated from endoglin transfectants, but not from parental myoblasts. These experiments also revealed the association of endoglin and betaglycan with the putative R-I and R-II. This is in agreement with previous reports demonstrating the forma-

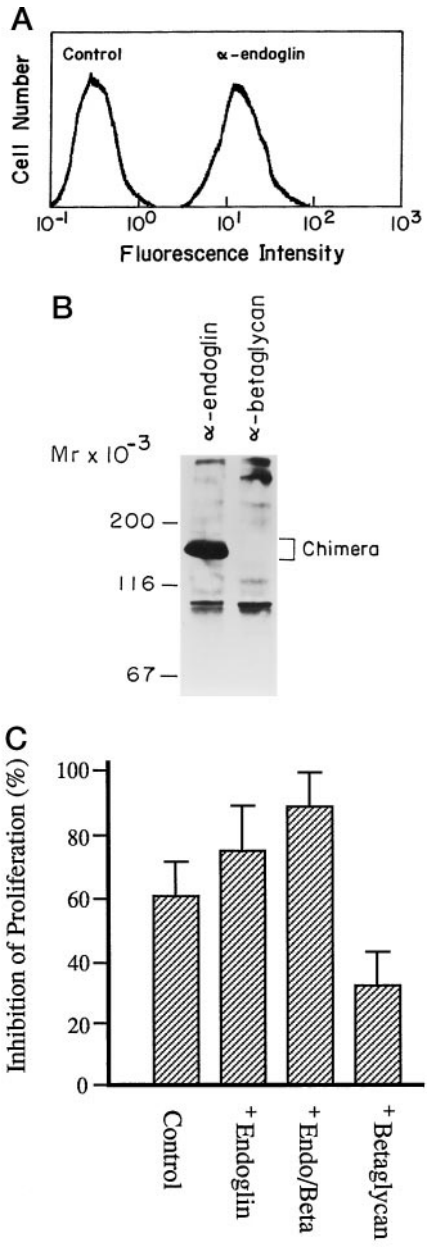

FIG. 4. **Characterization of transfectant myoblasts expressing a chimeric endoglin/betaglycan.** The rat myoblast cell line L_6E_9 was stably transfected with the pCMV-Endo/Beta vector encoding the extracellular and transmembrane domains of endoglin fused to the cytoplasmic region of betaglycan, and the expression and function of the chimeric protein was analyzed. *A,* analysis by cytofluorometry of the chimeric protein present at the cell surface. Myoblast transfectants were stained for indirect immunofluorescence with the mAb 44G4 to the extracellular domain of endoglin. *A,* control staining of mock transfectants is also shown. *B,* immunoprecipitation analysis. Myoblast transfectants were surface labeled with biotin, lysed, and immunoprecipitated with antibodies to the ectodomain of either endoglin (mAb 44G4) or betaglycan (polyclonal antibody 822). Samples were electrophoresed on a 7.5% acrylamide gel under nonreducing conditions, transferred to nitrocellulose, and the biotinylated protein detected using a chemiluminescence assay. *C,* TGF-β1-induced inhibition of proliferation. Stable myoblast transfectants expressing endoglin, betaglycan, or the Endo/Beta chimera were incubated with 500 pM TGF-β1 and the proliferation capacity of the cells was measured by [^{3}H]thymidine incorporation. Mock transfectants (*Control*) and parental myoblasts displayed a similar TGF-β1 inhibition of proliferation. The mean of four different experiments performed in triplicate samples is shown.

tion of heteromeric complexes between endoglin or betaglycan with the signaling receptors (7, 20, 30). In several immunoprecipitation experiments, we found that the contribution of the signaling receptors relative to the endoglin signal was higher than the equivalent ratio in betaglycan immunoprecipitates. This is especially evident in the endoglin immunoprecipitate at 20 pM TGF-β1 under reducing conditions of Fig. 5*C* where the labeling of R-I, but not that of endoglin, could be detected. This suggests the existence of endoglin free of ligand associated with signaling receptors loaded with TGF-β. Taken together, these results indicate that endoglin differs from betaglycan in the modulation of TGF-β binding to the cell.

Requirement for Signaling Receptors in the Binding of TGF-β1 to Endoglin—Given the positive influence of endoglin on TGF-β binding to the signaling receptors, we wondered whether the signaling receptors could influence TGF-β binding to endoglin. Initially, we tried unsuccesfully to generate stable myoblast transfectants expressing different combinations of receptors type I, type II, and endoglin. Then, we addressed this issue by transient transfection in COS cells, as this is a model system commonly used to evaluate ligand binding of transduced receptors of the TGF-β family. Plasmids encoding the L-endoglin isoform (21) and R-II were transfected in different combinations. The transduced cells were radiolabeled with TGF-β1, followed by cross-linking and analysis of the lysates by SDS-PAGE (Fig. 6*A*). Cells transfected with R-II showed an strong 90-kDa band, in agreement with previous reports demonstrating that R-II is constitutively active in binding the ligand (22). Under nonreducing conditions, cells co-transfected with L-endoglin and R-II showed a 200-kDa band likely corresponding to endoglin as it migrated as a 100-kDa band (overlapping with R-II) under reducing conditions. The fact that binding of TGF-β to endoglin is only revealed when co-expressed with R-II, suggests that R-II potentiates binding to endoglin. Similar experiments using a plasmid encoding R-I instead of R-II, did not reveal increased ligand binding to endoglin (data not shown). Specific immunoprecipitation analysis confirmed the increased ligand binding to both endoglin isoforms in the presence of R-II (Fig. 6*B*). By contrast, the R-I (Alk-1 or Alk-5) failed to induce ligand binding to endoglin. As a control, expression levels of endoglin were found to be similar in the different co-transfection protocols, as demonstrated by biotin labeling. Overexposure of immunoprecipitates from COS cells only transfected with endoglin vector revealed a specific band of radiolabeled TGF-β1-endoglin complex (data not shown), in agreement with a previous report (6). These data demonstrate the important role of the type II receptor in TGF-β binding to endoglin.

DISCUSSION

The cellular TGF-β receptor system is formed by several membrane receptors including receptors I and II, betaglycan, and endoglin. The core of this receptor system seems to be located in the heteromeric association between receptor I and receptor II, whose signals, mediated by their cytoplasmic domains with Ser/Thr kinase activities, are crucial in the TGF-β dependent effector functions. Here, we have used for the first time the same cellular system to analyze the role of endoglin and its homologue betaglycan in the TGF-β-dependent responses. Endoglin expression was found to inhibit the TGF-β1-dependent responses of cellular proliferation and PAI-1 expression, as opposed to an increased TGF-β1 responsiveness induced by betaglycan expression. These results agree with previous reports analyzing the role of betaglycan (20) or endoglin (9) in different cellular lineages. The negative role of endoglin in TGF-β signaling seems to be located in the extracellular domain of endoglin as evidenced by the functional analysis of two chimeric proteins containing different combinations of endoglin and betaglycan domains. This functional mapping is

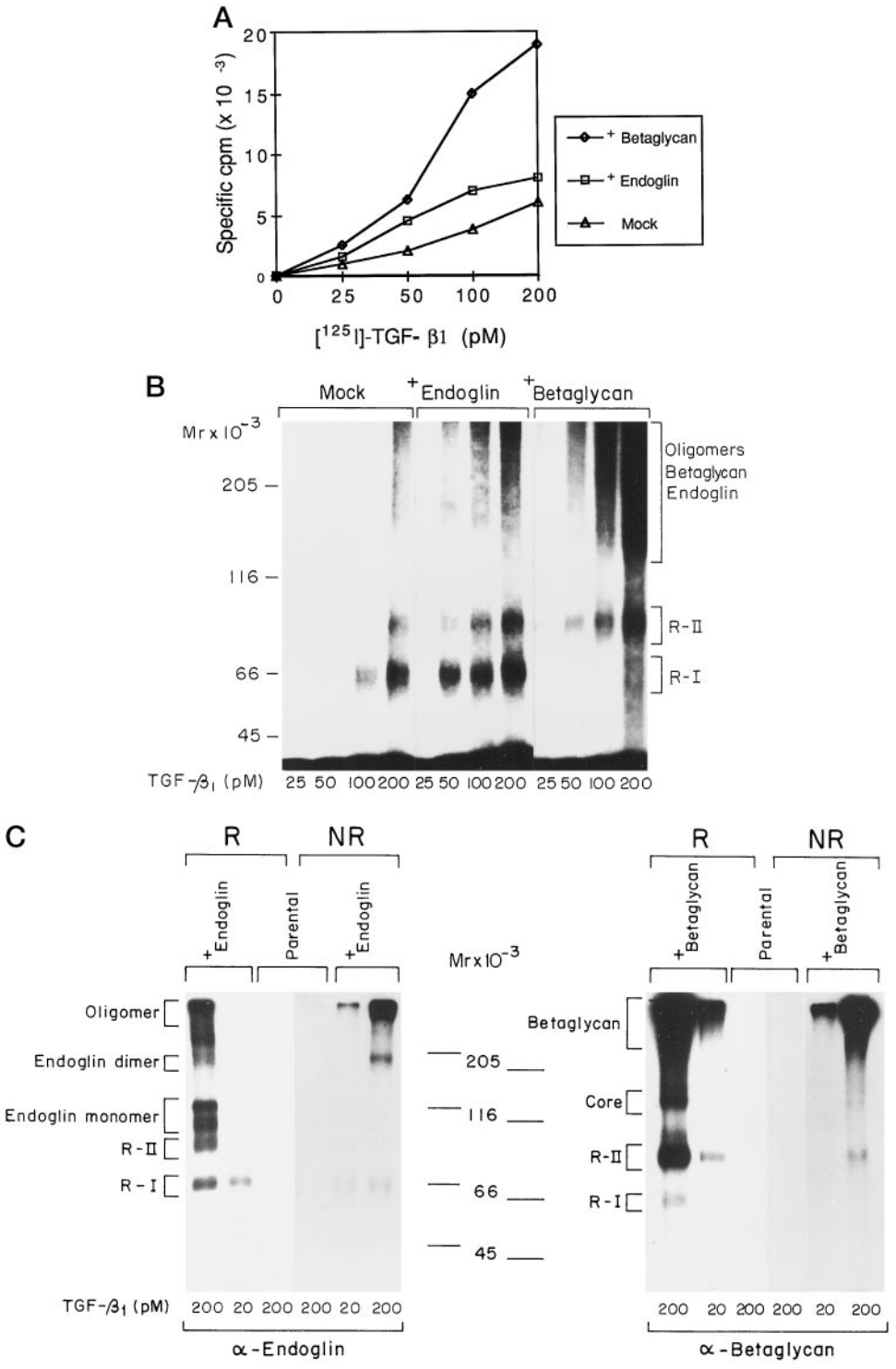

FIG. 5. **Binding of TGF-β1 to specific receptors on myoblast transfectants.** Cells were affinity labeled by incubation at 4 °C with 25–200 pM ^{125}I-TGF-β1 and washed as described under "Experimental Procedures." Radiolabeled TGF-β1 was cross-linked to the specific receptors with disuccinimidyl suberate, cells were lysed and cell associated radioactivity was estimated in a γ-counter (*panel A*). Total extracts were subjected to SDS-PAGE analysis under nonreducing conditions (*panel B*) or to specific immunoprecipitation (*panel C*). Immunoprecipitations were carried out with the rabbit polyclonal antibody BN (anti-endoglin) or the rabbit polyclonal antibody 822 (anti-betaglycan) followed by SDS-PAGE under reducing (*R*) or nonreducing (*NR*) conditions. Detection of the ^{125}I-labeled receptors in the gels was revealed by autoradiography or using a PhosphorImager. High molecular weight bands corresponding to oligomers, endoglin, or betaglycan, and bands corresponding to the putative betaglycan core and receptors type I or II are indicated. ◇, +betaglycan; □, +endoglin; Δ, mock.

compatible with the fact that the S-endoglin isoform, which has a cytoplasmic domain different from that of L-endoglin isoform used throughout this study, also displays a negative effect on TGF-β1 responses (9). Furthermore, the high homology displayed by the cytoplasmic domains of betaglycan and the L-endoglin isoform (16, 19), favors an active role of their extracellular domains as responsible for the distinct TGF-β effector functions.

The opposite functional behavior of endoglin and betaglycan increases a list of differences between these two receptors, which include the cellular distribution, or the specificity for TGF-β isoforms. Another major difference has been found in

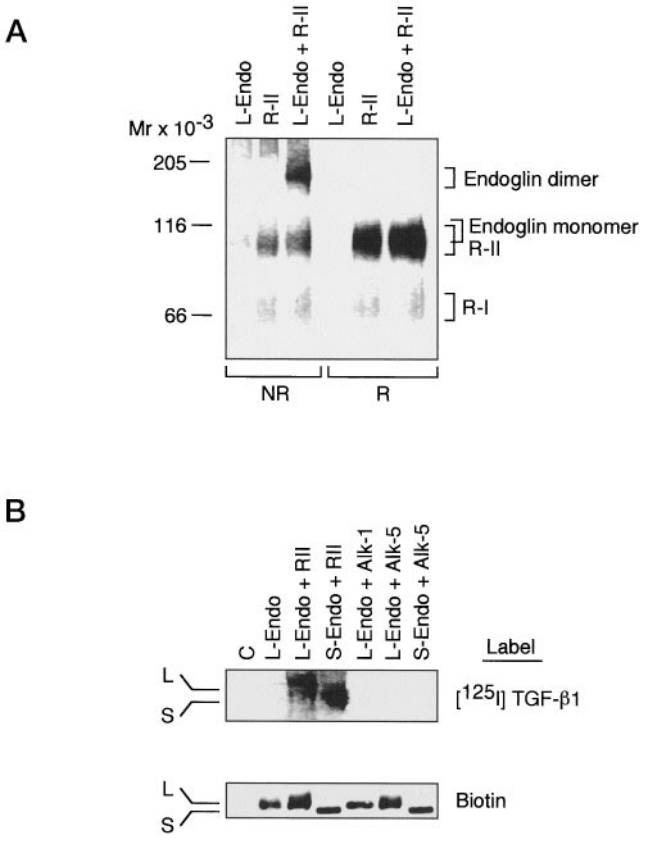

FIG. 6. **Binding of TGF-β1 to COS cells expressing endoglin, and receptor types I and II.** COS cells were transiently transfected with the pCMV5 expression vector containing cDNAs encoding R-I (Alk-1 or Alk-5), R-II, and endoglin (S-endoglin or L-endoglin), as indicated. After 2 days in culture, cells were affinity labeled with ^{125}I-TGF-β1 and cross-linked with disuccinimidyl suberate. *A,* total lysates were analyzed on a 6% acrylamide gel under either reducing or nonreducing conditions, followed by detection of the radiolabeled receptors with a PhosphorImager. The positions of endoglin, R-I, and R-II are indicated. *B,* immunoprecipitation analysis. Total lysates were subjected to immunoprecipitation with anti-endoglin antibodies, followed by SDS-PAGE analysis under nonreducing conditions. Radioactive bands were detected with a PhosphorImager (*upper panel*). As a control for endoglin expression, cells were biotinylated on their surface, lysed, and immunoprecipitated with anti-endoglin antibodies. Samples were run on SDS-PAGE under nonreducing conditions and electrotransferred to nitrocellulose membranes. Biotinylated proteins were detected with streptavidin conjugated to horseradish peroxidase using a chemiluminescence assay (*lower panel*). Only the area of the gels corresponding to labeled endoglin is shown. The positions of L-endoglin (*L*) and S-endoglin (*S*) are indicated.

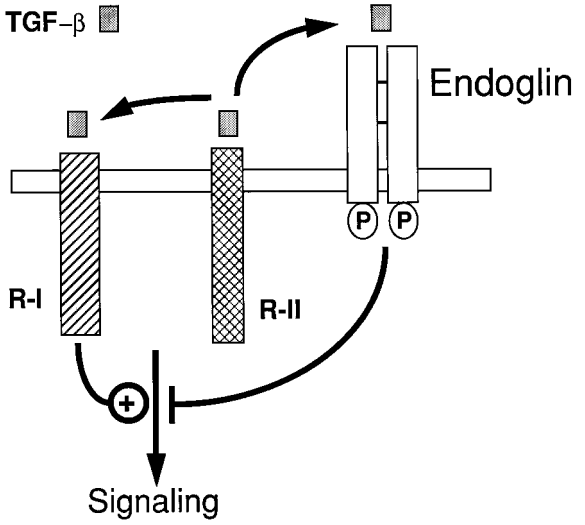

FIG. 7. **Schematic representation of a possible model of TGF-β binding and signaling.** The type II receptor (R-II) is required for binding of TGF-β to the type I receptor (R-I) leading to an increased signal transduction. By contrast, the type II receptor allows binding of TGF-β to endoglin, which is associated with a loss of signal transduction. The constitutive phosphorylation (*P*) of endoglin is indicated. For further discussion and references, see the text.

the ability to bind TGF-β1 of these receptors. Betaglycan, readily binds TGF-β (17), as confirmed here by its extensive labeling in cross-linking experiments of myoblast transfectants (Fig. 5). This contrasts with the weak labeling of endoglin, even though the levels of expression of endoglin appeared to be higher than those of betaglycan (Fig. 1*A*). Likewise, in endothelial cells, where endoglin expression is high (up to 10^6 molecules per cell), only a small number of endoglin molecules bind TGF-β1 (6). Several explanations could account for this low binding. First, there is a physiological ligand different from TGF-β. In support of this hypothesis is the fact that mutations in Alk-1, a type I receptor with unknown ligand specificity, lead to a similar phenotype as in HHT1 patients (31). Second, endoglin requires the expression of additional molecules to bind TGF-β. This agrees with: (*a*) the existence of endoglin free of ligand associated with signaling receptors loaded with TGF-β (Fig. 5*C*); and (*b*) the requirement of R-II expression for ligand binding to endoglin (Fig. 6). This is a remarkable difference with betaglycan, which binds TGF-β independently from the signaling receptors as demonstrated by the ligand binding of its soluble form (17, 32). It also remains possible that endoglin binds TGF-β1 only when associated with signaling receptor II, but its major function is not to be a component of the TGF-β receptor system. All these alternatives remain to be explored.

Overall, the cooperative effects observed in the TGF-β binding to the endoglin containing receptor complex resemble those observed with different combinations of receptors I, II, and III (20, 30, 33, 34). Despite their distinct functional effects, expression of endoglin or betaglycan was associated with a potentiation of ligand binding to the signaling receptors. It is unclear how the loss of TGF-β signaling in endoglin transfectants can harmonize with the enhanced receptor binding. However, it is worth noting that the binding assays are performed at 4 °C, far from the *in vivo* situation, and also that these assays do not unveil the final destination of the ligand bound. It has been proposed that betaglycan presents TGF-β2 to the signaling receptors, which in turn, increase the signaling (20). In principle, this model cannot be applied to endoglin. Rather, our data agree with the behavior of betaglycan transfectants which show a normal TGF-β1 response, but an increased ligand binding to the signaling receptors (20), indicating that both ligand response and binding can be uncoupled under certain conditions. On the other hand, the requirement of the R-II for the TGF-β binding to endoglin appears to be compatible with the inhibition of the TGF-β responses found in endoglin transfectants. According to the hypothetical model depicted in Fig. 7, the receptor II-induced ligand binding to endoglin might be interpreted as a deflection of the ligand from the signaling core. Similarly to endoglin, R-I has been reported to require the presence of R-II for ligand binding, although in this case the increased binding is associated with an increased TGF-β response (35). By contrast, increased binding to endoglin, a nonsignaling receptor, could lead to a sequestering of the ligand associated with a loss of cellular responses to TGF-β. Whether endoglin is a mere reservoir or scavenger receptor, or whether it is also actively involved in the modulation of the downstream signaling, remains to be determined.

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Role of Endoglin in Cellular Responses to Transforming Growth Factor-β: A COMPARATIVE STUDY WITH BETAGLYCAN

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