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

A COMPARATIVE STUDY WITH BETAGLYCAN*

(Received for publication, March 17, 1998, and in revised form, August 26, 1998)

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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 125I-TGF-β 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 of the dominant vascular disorder known as hereditary hemorrhagic telangiectasia type I (HHT1) (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 (KD = 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 monocyteic 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

* This work was supported by Comisión Interministerial de Ciencia y Tecnología Grant CICYT-SAF97-0034, Comunidad Autónoma de Madrid (CAM), and Biomed Program of the European Community Grant BMH4-CT95-0995 (to C. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ 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 homolog 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 pCEV-EndoL vector containing the human l-endoglin isoform driven by the SV40 promoter (21) was used in transfection experiments of rat myoblasts. The pCMV-EndoLo vector, containing the human L-endoglin isoform driven by the cytomegalovirus promoter, was constructed by inserting the 2.3-kb EcoRI fragment of betaglycan cDNA (19) cloned into the pCMV vector (Invitrogen). The human betaglycan cDNA (19) cloned into the EcoRI site of pSV7d vector, was kindly provided by Dr. Kohei Miyazaki (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/EcoRI fragment of endoglin cDNA by the 0.3-kb MluI/EcoRI fragment of betaglycan (generous gift from Dr. Joan Massague, 20) for 30 min at 4 °C. After two washes with PBS, fluorescein isothiocyanate-labeled F(ab)’s, 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 CaCl₂, 1.2 mM MgCl₂, 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 sulsuccinimidyl-6-(iodotiosthiocyanato)hexaematoice (NHS-LC-SSC; Pierce Chemical Co, Rockford, IL) was added. After 15 min at 4 °C, 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 Y277-331 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 hors eradish peroxidase (Pierce) for 2 h at room temperature.

**Proliferation Assays**—Rat myoblasts were cultured in flat-bottomed 24-well plates (Costar, Cambridge, MA) at 4 × 10⁵ 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-3H]thymidine (Amersham, United Kingdom). Cells from triplicate samples were washed twice in PBS, scraped, and processed as described above. Total radioactivity in the sample was determined 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 125I-labeled receptor was revealed by autoradiography or using a PhosphorImager 410A and ImageQuant software (Molecular Dynamics).

Cells were affinity-labeled with recombinant human TGF-β1 or the rabbit polyclonal antibody 822 (specific activity 1, 200–2000 Ci/mmol; Amersham Ibérica S.A., Madrid).

**Flow Cytometry**—Cells (5 × 10⁴) were incubated with the mouse mAb 8E11 (anti-endoglin) (25), the mouse mAb 44G4 (anti-fragment Y277-331 of human endoglin) (11, 26), or the rabbit polyclonal antibody 822 together with the betaglycan (generous gift from Dr. Joan Massague) for 30 min at 4 °C. After two washes with PBS, fluorescein isothiocyanate-labeled F(ab)’s, 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.
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% CO2 atmosphere. Then, cells were lysed by washing 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 electrophoresed 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 Iberica).

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 (ICN Biomedicals, Barcelona) and preincubated in 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.

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. 1A). Immunoprecipitation analysis (Fig. 1B) revealed a 170-kDa band corresponding to the dimeric form of endoglin, specifically recognized by anti-endoglin antibodies. Also, 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-β (20). Endoglin overexpression resulted in a deficient ligand response, at concentrations of 5–500 pm TGF-β1, with respect to mock transfectants (Fig. 2A). 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. 2B 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. 2B).
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. 2D). 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-β.

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. 3A). 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 transduced into the p800 construct containing the PAI-1 promoter fused to the luciferase gene. TGF-β was added 24 h after transfection to half of the transduced cells and luciferase activity was determined 48 h after transfection. The TGF-β 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.
Chimera Beta/Endo, as indicated. TGF-
L-endoglin, the chimera Endo/Beta or the
expression vectors encoding betaglycan,
transfected with the p800 construct and
Parental myoblasts were transiently co-
TGF-
ta/Endo chimera expression on the
Given the distinct modulatory effect of endoglin and
fectants—
shows that specific binding to mock, endoglin, and betaglycan
ments performed in duplicates is shown.
not shown). These data suggest the involvement of the extra-
cellular domains of endoglin or betaglycan in their capacity to
modulate TGF-β responses. This was confirmed by prolifera-
tion 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. 4A).
The same mAb was used in immunoprecipitation analysis (Fig. 4B),
revealing a 170-kDa band corresponding to the endoglin/beta-
glycan 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 chi-
mera. As shown in Fig. 2, endoglin overexpression was associ-
ated with a deficient response to TGF-β1 with respect of mock
transfectants. Fig. 4C reveals that expression of the chimeric
protein resulted in a similar unresponsiveness to TGF-β1,
whereas betaglycan transfectants showed an increased respon-
siveness. Taken together, these results suggest that the extra-
cellular domain of endoglin is responsible for its modulatory
role in TGF-β cellular responses.

Binding of TGF-β to Signaling Receptors in Myoblast Trans-
fectants—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. 5A
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 bind-
ing TGF-β1 were analyzed by SDS-PAGE fractionation of total
lysates (Fig. 5B). 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 ex-
pressing 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 trans-
fectants than in mock or endoglin transfectants. These high
molecular weight receptors likely correspond to betaglycan-
ligand complexes, as indicated by immunoprecipitation exper-
iments with specific antibodies (Fig. 5C). Thus, a band of mo-
lar mass >200 kDa corresponding to mature betaglycan
was immunoprecipitated from betaglycan transfectants, but
not from parental myoblasts. In addition, the putative beta-
glycan core (17, 18) could be detected as a band of approxi-
mately 120 kDa. Binding of TGF-β1 to endoglin could also be
demonstrated by immunoprecipitation with anti-endoglin an-
tibodies. Specific bands of 200 kDa (nonreducing conditions)
and 100 kDa (reducing conditions) corresponding to the dimeric
or monomeric forms of endoglin were specifically immunopre-
cipitated 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-
The results indicate that endoglin differs from betaglycan in 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. 5C 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 unsuccessfully 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 isof orm (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. 6A). Cells transfected with R-II showed a 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. 6B). 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
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-\(\beta\) 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-\(\beta\) 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-\(\beta\) isoforms. Another major difference has been found in

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**Fig. 5.** Binding of TGF-\(\beta\)1 to specific receptors on myoblast transfectants. Cells were affinity labeled by incubation at 4 °C with 25–200 pm \(\text{^{125}I}\)-TGF-\(\beta\)1 and washed as described under “Experimental Procedures.” Radiolabeled TGF-\(\beta\)1 was cross-linked to the specific receptors with disuccinimidyl suberate, cells were lysed and cell associated radioactivity was estimated in a \(\gamma\)-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 \(\text{^{125}I}\)-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. \(\bigcirc\), + betaglycan; \(\square\), + endoglin; \(\triangle\), mock.
the ability to bind TGF-β1 of these receptors. Betaglycan, readily binds TGF-β1 (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. 1A). 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. 5C); 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 potentialization 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 non-signaling 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.

**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-β 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.
Acknowledgments—We thank, Dr. Joan Massagué for reagents, Dr. Michelle Letarte for mAb 44G4 and helpful discussions, Dr. Kohei Miyazono for betaglycan cDNA, Dr. Daniel B. Rifkin for the PAI-1 promoter construct, Victoria Muñoz and Mónica Fontela for photography, and Aurelio Hurtado for delineation.

REFERENCES

1. Gougos, A., and Letarte, M. (1988) J. Immunol. 141, 1925–1933
2. Fernández-Ruiz, E., St-Jacques, S., Bellón, T., Letarte, M., and Bernabéu, C. (1993) Cytoomega. Cell Genet. 64, 204–207
3. MacAllister, K. A., Grogg, K. M., Johnson, D. W., Gallione, C. J., Baldwin, M. A., Jackson, C. E., Helmbold, E. A., Markel, D. S., McKinnon, W. C., Murrell, J., McCormick, M. K., Pericak-Vance, M. A., Heutink, P., Oustra, B. A., Haitjema, T., Westerner, C. J. J., Porteous, M. E., Letarte, M., and Marchuk, D. A. (1994) Nat. Genet. 8, 345–351
4. Guttmacher, A. E., Marchuk, D. A., and White, R. I. (1995) N. Engl. J. Med. 333, 918–924
5. Shovlin, C. L. (1997) Thromb. Haemostasis 78, 145–150
6. Cheifetz, S., Bellón, T., Cales, C., Vera, S., Bernabéu, C., Massagué, J., and Letarte, M. (1992) J. Biol. Chem. 267, 19027–19030
7. Yamashita, H., Ichijo, H., Grimsby, S., Moren, A., ten Dijke, P., and Miyazono, K. (1992) J. Biol. Chem. 267, 680–684
8. Lastres, P., Letamendia, A., Zhang, H., Rius, C., Almendro, N., Raab, U., López, L. A., Langó, C., Fabra, A., Letarte, M., and Bernabéu, C. (1996) J. Cell Biol. 133, 1109–1121
9. Massagué, J. (1990) Annu. Rev. Cell Biol. 6, 597–641
10. Roberts, A. B., and Sporn, M. B. (1983) Growth Factors 2, 1–9
11. Thalacker, F. W., and Nilsen-Hamilton, M. (1987) J. Biol. Chem. 262, 2283–2290
12. Laiho, M., Saksela, O., Andreassen, P. A., and Keski-Oja, J. (1986) J. Cell Biol. 103, 2403–2410
13. Derynck, R., and Feng, X.-H. (1997) Biochim. Biophys. Acta 1333, F105–F150
14. Ten Dijke, P., Miyazono, K., and Heldin, C. H. (1996) Curr. Opin. Cell Biol. 8, 139–145
15. Gougos, A., and Letarte, M. (1990) J. Biol. Chem. 265, 8361–8364
16. López-Casillas, F., Cheifetz, S., Doody, J., Andrés, J. L., Lane, W. S., and Massagué, J. (1991) Cell 67, 785–795
17. Wang, X. F., Lin, H. Y., Ng-Eaton, E., Downward, J., Lodish, H. F., and Weinberg, R. A. (1991) Cell 67, 797–805
18. Moren, A., Ichijo, H., and Miyazono, K. (1992) Biochem. Biophys. Res. Commun. 189, 356–362
19. López-Casillas, F., Wrana, J. L., and Massagué, J. (1993) Cell 73, 1435–1444
20. Bellón, T., Corbi, A., Lastres, P., Cales, C., Cehrián, M., Vera, S., Cheifetz, S., Massagué, J., Letarte, M., and Bernabéu, C. (1993) Eur. J. Immunol. 23, 2340–2345
21. Attisano, L., Carcamo, J., Ventura, F., Weis, F. M., Massagué, J., and Wrana, J. L. (1993) Cell 75, 671–680
22. van Zonneveld, A. J., Curriden, S. A., and Loskutoff, D. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5525–5529
23. Abe, M., Harpel, J. G., Metz, C. N., Nunes, I., Loskutoff, D. J., and Rifkin, D. B. (1994) Annu. Rev. Biochem. 216, 276–284
24. Lastres, P., Bellón, T., Cabañas, C., Sánchez-Madrid, F., Acevedo, A., Gougos, A., Letarte, M., and Bernabéu, C. (1992) Eur. J. Immunol. 22, 395–397
25. Pichuantes, S., Vera, S., Bourdeau, A., Poez, N., Kumar, S., Wayner, E. A., and Letarte, M. (1997) Tissue Antigens 50, 265–276
26. Massagué, J. (1987) Methods Enzymol. 146, 174–195
27. Luque, A., Cabañas, C., Raab, U., Letamendia, A., Páez, E., Herreros, L., Sánchez-Madrid, F., and Bernabéu, C. (1997) FEBS Lett. 413, 265–268
28. Laiho, M., Ronnstrand, L., Heino, J., Decaprio, J. A., Ludlow, J. W., Livingston, D. M., and Massagué, J. (1991) Mol. Cell. Biol. 11, 972–978
29. Moustakas, A., Lin, H. Y., Henis, Y. I., Plamondon, J., O’Connor-McCourt, M., and Lodish, H. F. (1993) J. Biol. Chem. 268, 22215–22218
30. Johnson, D. W., Berg, J. N., Baldwin, M. A., Gallione, C. J., Marondel, I., Yoon, S. J., Stenzel, T. T., Speer, M., Pericak-Vance, M. A., Diamond, A., Guttmacher, A. E., Jackson, C. E., Attisano, L., Kucherlapati, R., Porteus, M. E., and Marchuk, D. A. (1996) Nat. Genet. 13, 189–195
31. López-Casillas, F., Payne, H. M., Andreas, J. L., and Massagué, J. (1994) J. Cell Biol. 124, 557–568
32. Rodriguez, C., Chen, F., Weinberg, R. A., and Lodish, H. F. (1995) J. Biol. Chem. 270, 15919–15922
33. Yamashita, H., ten Dijke, P., Franzen, P., Miyazono, K., and Heldin, C.-H. (1994) J. Biol. Chem. 269, 20172–20178
34. Wrana, J. L., Attisano, L., Cárdeno, J., Zentella, A., Doody, J., Laiho, M., Wang, X. F., and Massagué J. (1992) Cell 71, 1003–1014
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J. Biol. Chem. 1998, 273:33011-33019.
doi: 10.1074/jbc.273.49.33011

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