Identification of Endoglin in Rat Hepatic Stellate Cells

NEW INSIGHTS INTO TRANSFORMING GROWTH FACTOR β RECEPTOR SIGNALING*[5]

Received for publication, May 14, 2004, and in revised form, October 8, 2004 Published, JBC Papers in Press, November 10, 2004, DOI 10.1074/jbc.M405411200

Steffen K. Meurer, Lidia Tihaa, Birgit Lahme, Axel M. Gressner, and Ralf Weiskirchen‡

From the Institute of Clinical Chemistry and Pathobiocchemistry, RWTH-University Hospital, D-52074 Aachen, Germany

The transforming growth factor family of growth factors regulates a diverse set of physiologic processes including proliferation, cellular differentiation, apoptosis, and expression of extracellular matrix genes (1–3). Signaling by the three identified mammalian TGF-β isoforms TGF-β1, TGF-β2, and TGF-β3 is initiated by binding to the high affinity cell surface receptors, the accessory TGF-β type III receptor (TβRIII) (4), the type II receptor (TβRII) (5), and type I (6) receptor (TβRI), of which TβRII and TβRII possess intracellular serine/threonine kinase domains. Upon binding of TGF-β1 or TGF-β3 to the receptor type II, the liganded receptor dimer associates with and whose constitutive active kinase transphosphorylates the type I receptor at the GS domain (7). The active type I receptor in turn phosphorylates and thereby activates the intracellular signal transducers represented by the R-Smad family members 2 and 3 (2, 7, 8).

Because TGF-β2 has a lower affinity for the type II receptor (9, 10), cells sense this ligand by either expressing a type II receptor splice variant TβRII-B (11–13) or employing an accessory receptor of the type III family (i.e. betaglycan) (14–16). Nevertheless, betaglycan has a high affinity for all three TGF-β isoforms (17–19), which has been exploited to counteract TGF-β signaling by sequestering the ligand using the soluble extracellular ligand binding domain (20, 21). Depending on the cell type, betaglycan either enhances (14, 15, 22) or inhibits TGF-β responses (23). As the ligand specificity suggests, TβRIII is not only required for TGF-β2 but also for TGF-β1 signaling (4). Although the C terminus of betaglycan lacks a protein kinase domain (24), it does not only present ligand to the type II receptor (TβRII-B (11–13) or employing an accessory receptor of the type III family (27) (i.e. endoglin (CD105), which shares 71% sequence identity at the protein level in its C terminus with betaglycan) (24, 28). Endoglin is a disulfide-linked, homodimeric transmembrane glycoprotein highly expressed in cells of the vascular system (29), fibroblasts (30), macrophages (31), and vascular smooth muscle cells (32). From the phenotype of endoglin-deficient

* This work was supported by a grant from the Deutsche Forschungsgemeinschaft. 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 1724 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY562420.

The on-line version of this article (available at http://www.jbc.org) contains four additional figures.

‡ To whom correspondence should be addressed: Institute of Clinical Chemistry and Pathobiocchemistry, RWTH-University Hospital, D-52074 Aachen, Germany. Tel.: 49-241-80-88683; Fax: 49-241-80-82512; E-mail: reiskekirchen@ukaachen.de.

5 The abbreviations used are: TGF-β, transforming growth factor-β; DTT, dithiothreitol; HBSS, Hanks’ buffered saline solution; HRP, horseradish peroxidase; HSC, hepatic stellate cell(s); HHT1 and HHT2, hereditary hemorrhagic telangiectasia type 1 and 2, respectively; HUVEC, human umbilical vein endothelial cell(s); HA, hemagglutinin; MFB, myofibroblasts; NAC, N-acetylcysteine; PBS, phosphate-buffered saline; sTβRII, soluble transforming growth factor-β receptor type III; TβRI, transforming growth factor-β receptor; MOPS, 3(N-morpholino)propanesulfonic acid; FCS, fetal calf serum; EST, expressed sequence tag; RT, reverse transcription; bis-Tris, 2-[bis(2-hydroxyethyl) amino]-2-(hydroxymethyl)propane-1,3-diol.

3078 This paper is available on line at http://www.jbc.org

Downloaded from http://www.jbc.org by guest on July 18, 2018
mic, it was inferred that the receptor is indispensable for embryonic development (33–36). In the presence of the signal-
ing receptor types I and II, endoglin binds TGF-β1, TGF-β3, activin-A, BMP-2, and BMP-7 (28, 37, 38). It has been
documented that endoglin is competent to associate with diverse
TGF-β receptors (28, 38–42). Nevertheless, due to the highly
similar phenotypes caused by the human disease hereditary
hemorrhagic telangiectasia type 1 (HHT1) (43) and type 2
(HHT2) (44), the corresponding knockout mouse models (16,
36), as well as through direct association with ALK1 (40) it has
been assumed that endoglin signals most likely through the
ALK1 type I receptor. Recently, it has been shown that endog-
lin modulates TGF-β/ALK1 signaling in endothelial cells (45).
In contrast to ALK5, which activates R-Smad2 and -3, ALK1
mediates signals via the R-Smad isoforms 1, 5, and 8 (46). The
In contrast to ALK5, which activates R-Smad2 and -3, ALK1
mediates signals via the R-Smad isoforms 1, 5, and 8 (46). The

**Table I**

**Antibodies used in this study**

| Antibody       | Modification | Supplied by         | Epitope, location | Species<sup>a</sup> | Dilution |
|----------------|--------------|---------------------|-------------------|----------------------|----------|
| sc-6199        | None         | Santa Cruz<sup>b</sup> | TgRIII, carboxyl terminus | h, m, r          | 1:250    |
| sc-20632       | None         | Santa Cruz         | Endoglin (amino acids 27–326), amino terminus | h         | 1:250    |
| sc-20072       | None         | Santa Cruz         | Endoglin, amino terminus | h         | 1:500    |
| AF-242-PB      | None         | R&D Systems        | TgRIII, extracellular domain | h, m, r       | 1:250    |
| BAF242         | Biotinylated | R&D Systems        | TgRII, extracellular domain | h, m, r       | 1:250    |

<sup>a</sup> h, human; m, mouse; r, rat.

<sup>b</sup> Santa Cruz, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).
medium was aspirated, and monolayers were washed and incubated in binding buffer (50 mmol/liter HEPES (pH 7.4), 128 mmol/liter NaCl, 5 mmol/liter KCl, 5 mmol/liter MgSO₄, 13 mmol/liter CaCl₂, 0.5% (w/v) bovine serum albumin) at 37 °C for 1.5 h. Cells were then washed with ice-cold binding buffer, and ligand binding was performed at 4 °C using 2.8 ng/ml ¹²⁵I-TGF-β1 (1621 Ci/mmol; Amersham Biosciences) with or without a 100-fold excess of unlabeled TGF-β1 (Pepro Tech, Rocky Hill, NJ) for 3 h at 4 °C. Bound ligand was cross-linked to its receptor with disuccinimidyl suberate (Perbio Science) for 15 min at 4 °C. Thereafter, proteins were extracted in lysis buffer including proteinase inhibitors and subjected to immunoprecipitation with receptor-specific antibodies. Precipitated proteins were resolved by SDS-PAGE, and gels were dried and exposed for indicated times at −80 °C to X-Omat AR films (Eastman Kodak Co.).

RESULTS

Rat HSC Express a Homolog of TGF-β Type III Family Receptor Betaglycan—TGF-β is a growth factor regulating key aspects of cellular activation and transdifferentiation of HSC (55). The ligand, TGF-β, is bound by a heterooligomeric membrane receptor complex, consisting of the signaling receptors type I and II and at least one accessory receptor betaglycan. In a previous unpublished work of our laboratory, we could show that TGF-β signaling in cultured HSC could be abrogated by reducing agents like N-acetylcyesteine (NAC). As one possible mechanism, we found that NAC reduces ligand binding to a

RNA Isolation and Northern Blot Analysis—Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA samples (5 µg) were separated in 1% (w/v) denaturing agarose gel, transferred to Hybond-N membrane (Amersham Biosciences), and fixed by baking for 2 h at 80 °C. Hybridization probes specific for rat endoglin (nt 1–792; accession number AF0562420), rat TgfRIII (nt 1–819; accession number NM 017256), and human endoglin (nt 1384–1933; accession number BT007558) were prepared using the random prime labeling system (Amersham Biosciences). Hybridization and washings of the membranes were carried out as previously described (58). For internal standardization, the blots were rehybridized with a cDNA specific for glyceraldehyde-3-phosphate dehydrogenase.

RT-PCR—For RT-PCR experiments, purified samples of total RNA (1 µg) was reverse transcribed at 42 °C for 60 min using the Superscript II reverse transcriptase (Invitrogen) and random hexamer primer according to the manufacturer’s instructions. Aliquots of first strand cDNAs were subjected to PCR with 2 µm forward and reverse primer, dNTPs (each 10 mM dATP, dCTP, dGTP, dTTP) in 1× PCR buffer, and 2.5 units of Taq DNA polymerase (Roche Applied Science). The coding region of rat endoglin cDNA was amplified with primers SM012 5’-GCCTAGGGCGGTGGAGACCC-3’ and SM017 5’-dAGGCAGCTGGCTGA-3’ for expression of TgfRIII (55). The TgfRIII forms recognized are indicated on the left. Note that the migration pattern of the proteins (P1 and P2) detected by antibody sc-6199 is not in accordance with that published for TgfRIII (betaglycan) and shows sensitivity toward reducing agents. B, immunoprecipitation (IP) of heterologously expressed HA-tagged rat TgfRIII. COS-7 cells were transfected with the expression plasmid pCMV-HA-TgfRIII or left untransfected (mock). Equal amounts of protein extracts (100 µg) were immunoprecipitated by sc-6199 (1 µg) (left) or BAF242 (1.25 µg) (right), separated by SDS-PAGE, and transferred to a nylon membrane. The membrane was probed with a monoclonal antibody (clone 3F10; Roche Applied Science) directed against the HA tag and developed as outlined under “Experimental Procedures.”

RNA isolation and Northern blot analysis—total RNA was isolated using the RNaseasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA samples (5 µg) were separated in 1% (w/v) denaturing agarose gel, transferred to Hybond-N membrane (Amersham Biosciences), and fixed by baking for 2 h at 80 °C. Hybridization probes specific for rat endoglin (nt 1–792; accession number AF0562420), rat TgfRIII (nt 1–819; accession number NM 017256), and human endoglin (nt 1384–1933; accession number BT007558) were prepared using the random prime labeling system (Amersham Biosciences). Hybridization and washings of the membranes were carried out as previously described (58). For internal standardization, the blots were rehybridized with a cDNA specific for glyceraldehyde-3-phosphate dehydrogenase.

RT-PCR—For RT-PCR experiments, purified samples of total RNA (1 µg) was reverse transcribed at 42 °C for 60 min using the Superscript II reverse transcriptase (Invitrogen) and random hexamer primer according to the manufacturer’s instructions. Aliquots of first strand cDNAs were subjected to PCR with 2 µm forward and reverse primer, dNTPs (each 10 mM dATP, dCTP, dGTP, dTTP) in 1× PCR buffer, and 2.5 units of Taq DNA polymerase (Roche Applied Science). The coding region of rat endoglin cDNA was amplified with primers SM012 5’-GCCTAGGGCGGTGGAGACCC-3’ and SM017 5’-dAGGCAGCTGGCTGA-3’, specific for rat endoglycan. Cycle conditions were set to the following: initial denaturation for 5 min at 95 °C; 40 cycles at 55 °C for 30 s, 56 °C for 30 s, 72 °C for 2 min; final elongation at 72 °C for 10 min. A fragment of human endoglin cDNA was amplified from human MFB using primers SM014 5’-dCCAGAGCTCCAATTGG-3’ and SM011 5’-dCGA TGC TGT GGT TGG TAC-3’, specific for rat endoglycan. Cycle conditions were set to the following: initial denaturation for 5 min at 94 °C; 40 cycles at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 3 min, final elongation at 72 °C for 10 min. PCR products were gel-purified, cloned into pCRII-TOPO Easy vector (Promega, Madison, WI), and sequenced using the ABI PRISM BigDye® termination reaction kit (PerkinElmer Life Sciences) as described elsewhere (58).

Immunocytochemistry—Approximately 3 × 10⁵ cells were plated on coverslips incubated in 6-well dishes and incubated for 24 h at 37 °C. Thereafter, cells were transfected with selected expression plasmids. 24 h post-transfection, the medium was exchanged, and after incubation for an additional 24 h, the cells were fixed for 15 min in 4% (w/v) paraformaldehyde buffered in phosphate-buffered saline (PBS) (pH 7.4). After permeabilization (2 min on ice) in 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100, endogenous biotin was blocked by a biotin blocking system (DAKO). Nonspecific binding was further prevented by preincubation in 1% (w/v) bovine serum albumin in PBS for 30 min at 37 °C and subsequent incubation in 0.5% bovine serum albumin plus 0.1% (v/v) fish gelatin in PBS. Then the primary antibodies sc-6199 (COS-7) or P4A4 (human MFB) as well as the corresponding normal control IgG were applied for 1 h at 37 °C in 1% (v/v) bovine serum albumin in PBS followed by incubation with an anti-goat (sc-6199) or anti-mouse (P4A4) biotin conjugate. The detection of immunocomplexes was accomplished using the streptavidin-fluorescein isothiocyanate fluorophore for laser-scanning microscopy (LS 410 inverted; Zeiss) using a standard objective (40× 1.3 oil) and an external argon laser at 488 nm. For detection of heterologously expressed endoglin, the incubation with the streptavidin-fluorescein isothiocyanate fluorophore was followed by an anti-fluorescein isothiocyanate AP conjugate and subsequent Fast-Red substrate application. The chromogenic stain was documented by light microscopy.

Data Base Analysis—Screening of nonredundant and expressed sequence tag as well as the Swissprot data bases was done at the National Center for Biotechnology Information using the Blast algorithm (59).

Downloaded from http://www.jbc.org/ by guest on July 18, 2018
high molecular weight receptor complex present in HSC, most likely representing a TGF-β type III receptor. To test the hypothesis that NAC acts directly on the TβRIII polypeptide in a similar manner as has been described for DTT by Philip et al. (60), we performed Western blot analysis using the commercially available TβRIII-specific antibody sc-6199, directed against a C-terminal epitope (Fig. 1A, left). In our experiments, the antibody recognizes a protein of 144 kDa under nonreducing conditions that is shifted to 74.5 kDa under reducing conditions. This migration pattern was somewhat unexpected in regard to the published molecular weight of betaglycan (61). Therefore, we decided to test a second independent antibody (AF-242-PB) directed against the extracellular domain of TβRIII (Fig. 1A, right). This antibody detects a protein of ~90 kDa in size, only slightly shifting its apparent molecular weight under reducing conditions, corresponding to the unmodified core protein of TGF-β1-labeled TβRIII from HSC (Supplement 1) confirmed the specificity of AF-242-PB toward TβRIII. In contrast, sc-6199 obviously recognizes TβRIII when overexpressed in COS-7 cells (Fig. 1B, left) but not in extracts taken from HSC (cf. Fig. 1A, left). Therefore, HSC express a protein that shares high homology with TβRIII with respect to the sc-6199 epitope, and this protein is expressed at a much higher level compared with TβRIII. Furthermore, NAC treatment of COS-7 cells transiently overexpressing TβRIII revealed that the TβRIII betaglycan is not reductant-sensitive (Supplement 2).

Cloning of Endoglin Transcripts from Rat HSC and MFB—By means of Western blot analysis, we verified that the epitope of the antibody sc-6199 maps to the TβRIII cytoplasmic domain (Supplement 3). To identify proteins with homology to the carboxyl terminus of TβRIII, we screened the Swissprot data base and found that murine TGF-β receptor endoglin matches the respective region spanning the TβRIII epitope. To elucidate whether endoglin is expressed in HSC, we next performed RT-PCR. To generate specific primers for our analysis, we screened a rat expressed sequence tag library using the murine endoglin (accession number BC029080). This search identified endoglin cDNA as the query. We identified six individual rat expressed sequence tags covering the whole coding sequence of the antibody sc-6199 maps to the TβRIII cytoplasmic domain. To elucidate whether endoglin is expressed in HSC, we next performed RT-PCR. To generate specific primers for our analysis, we screened a rat expressed sequence tag library using the murine endoglin cDNA as the query. We identified six individual rat expressed sequence tags covering the whole coding sequence of the rat cDNA (Fig. 2A). Based on this information, we designed primers flanking the start and stop codons and amplified the complete coding region (~1500 nt) of rat endoglin. The open reading frame contains 650 amino acids, predicts a protein with a calculated molecular mass of 69.9 kDa, and proves the assumed high degree of sequence identity between endoglin and betaglycan at the carboxyl terminus (Figs. 2B and 3). Rat endoglin shares 69 and 82% sequence identity with the human and murine orthologues (62, 63). The amino terminus is preceded by a putative signal peptide (amino acids 1–25) following the von Heijne prediction (64). In contrast to human endoglin, which contains an RGD tripeptide in the extracellular domain (62), both rat and mouse endoglin lack this integrin binding domain (63). Amino acids 549–573 constitute the membrane-spanning domain conserved between rats and mice. The carboxyl terminus of endoglin is about 47 amino acids and shares a potential PDZ class 1 domain with betaglycan (26).

Expression of Type III Family Receptor mRNA during Transdifferentiation of HSC—To monitor the transcript levels of
betaglycan and endoglin during transdifferentiation, we performed Northern blot analysis. For this purpose, we analyzed total RNA isolated from HSC cultured for 2 and 7 days and MFB cultured for 4 days after the first passage. Hybridization with an endoglin-specific probe resulted in the detection of two transcripts of 3 and 3.6 kb in size (Fig. 4A). The presence of two different transcripts originate most likely from the usage of two alternative polyadenylation signals rather than

**Fig. 3. Structure of rat endoglin cDNA.** The nucleotide and deduced amino acid sequence of rat endoglin cDNA is shown. Nucleotides and amino acids are numbered on the right. The translational initiation and termination codons are shown in white type on black. The signal peptide and the transmembrane region are marked by solid or intermittent lines, respectively. The nucleotide sequence has been deposited in the GenBank™ data library under accession number AY562420.

Endoglin in Hepatic Stellate Cells
the occurrence of differential splicing, because accompanying PCR experiments (not shown) gave no experimental hints for the expression of different splice variants previously reported in humans (65). During transdifferentiation of HSC into MFB, the amount of endoglin mRNA remains constant. In contrast, the expression of the 6-kb betaglycan messenger markedly declines in the course of transdifferentiation (Fig. 4B), confirming previous reports showing that the expression of different TGF-β receptors can be modified during fibrogenesis (66–68).

Detection of the Heterologously Expressed Endoglin Confirms the Specificity of the Antibody sc-6199 toward Both Type III Receptors—To confirm the hypothesis that antibody sc-6199 crossreacts with endoglin, we transiently expressed the rat endoglin cDNA in COS-7 cells and examined respective protein lysates by Western blot analysis under reducing and nonreducing conditions (Fig. 5). In this analysis, sc-6199 detects the expressed endoglin, unambiguously demonstrating that the antibody is not specific for betaglycan (Fig. 5A). Under nonreducing conditions, the antibody detects a protein of 130 kDa, representing the receptor dimer, as well as oligomeric, high molecular weight complexes. This migration pattern is in agreement with those previously observed for endoglin (38, 62, 65). The addition of reducing substances (e.g. 50 mM DTT) results in disintegration into receptor monomers possessing a relative molecular mass of 68 kDa. The slightly higher molecular mass of endogenous endoglin (cf. Fig. 1A) in HSC might be attributable to a different posttranslational receptor processing in HSC and COS-7. Furthermore, immunocytochemical staining of COS-7 cells, overexpressing endoglin, also demonstrate that antibody sc-6199 reacts with endoglin (Fig. 5B).

Expression of Type III Family Receptor Proteins during Transdifferentiation of HSC—To examine the protein expression of betaglycan and endoglin during transdifferentiation, we performed Western blot analysis (Fig. 6). We found that the content of betaglycan, especially its glycosylated form, increases during primary culture and drops at later stages (Fig. 6A). In contrast, the level of endoglin expression is constant during transdifferentiation (Fig. 6B), confirming the results of our Northern blot analysis (cf. Fig. 4). Furthermore, we found that both receptors are not expressed in hepatocytes.

Endoglin Expression and Localization in Human Myofibroblasts—We analyzed endoglin expression in human MFB ob-
tained from liver outgrowth by RT-PCR and Northern blot analysis using antibodies BAF-242-PB (A) and sc-6199 (B). Equal protein loading of individual lanes was demonstrated by Ponceau S staining. Addi-
tionally, immunodetection of β-actin with a mouse monoclonal antibody (Sigma) is shown. To display the progress in transdifferentiation during culture time, the blot in B was additionally probed with an α-smooth muscle actin (α-SMA) specific monoclonal antibody (Cymbus Biotechnology Ltd.). The positions of protein size markers are indicated on the left.

Endoglin Is Expressed in the Plasma Membrane of HSC and MFB—Having established the expression of endoglin in HSC and MFB we next analyzed if endoglin is localized in the plasma membrane, an essential prerequisite for an integral membrane receptor. Because previous reports demonstrated that only a very small fraction of endogenous, membrane localized endoglin becomes labeled in cross-linking experiments (28), even when overexpressed in culture cells (38), we used a different strategy to analyze membrane insertion. In this approach, proteins exposed at the surface of HSC and MFB were first labeled with a membrane-impermeable biotinylation agent, and rat endoglin was subsequently immunoprecipitated from lysates of biotinylated HSC and MFB (Fig. 9A, left) and HUVEC (Fig. 9A, right). Precipitated proteins were then subjected to SDS-PAGE under reducing and nonreducing conditions, and surface receptors were detected by the streptavidin-HRP conjugate.

![Fig. 6. Western blot analysis of TGF-β type III receptor proteins during transdifferentiation of rat HSC.](image)

![Fig. 7. RT-PCR, Northern blot, and Western blot analysis for the detection of endoglin expression in human MFB.](image)
we precipitated endoglin using the monoclonal antibody P4A4 from human MFB (Fig. 9B, left). To further demonstrate that the detected protein represents human endoglin, the blot membrane was stripped and reprobed with the endoglin-specific antibody sc-6199 (Fig. 9B, right).

**DISCUSSION**

In the present study, we first demonstrate that rat and human HSC/MFB express the accessory TGF-β receptor endoglin. Endoglin is a member of the TGF-β type III receptor family (70), which includes a second receptor known as betaglycan.

We attempted to analyze the influence of TGF-β/H9252 on betaglycan and endoglin expression in HSC and MFB by Western blotting (Fig. 10). Under the chosen conditions, which are commonly used in these kinds of experiments, we found that (i) the expression of both receptors is decreased during periods of serum starvation and (ii) TGF-β/H9252 has no influence on the expression of betaglycan and endoglin in HSC. A down-regulation of TGF-β/H9252 receptors following serum starvation was previously demonstrated in rat MFB (69), most likely representing a general phenomenon. Furthermore, we found that lowering the serum content from 10 to 1% (data not shown) or 0.5 and 0.2% for 7 days resulted in a near absence of betaglycan (Fig. 10A) and endoglin (Fig. 10B) in HSC.
also interacts with and Smad3 does not occur (69). Therefore, if TGF-β signaling in activated HSC (53, 66, 67). Although both receptors share a conserved domain structure, they could be clearly distinguished by their ligand binding ability and specificity (17–19, 28, 37, 38). Therefore, it is assumed that both receptors fulfill different physiological functions. The human endoglin cDNA has been cloned (62), and mutations in the endoglin gene have been linked to the human disease HHT1 (43). In line, corresponding mouse models disrupted for the endoglin gene confirmed the importance of this receptor especially in TGF-β signaling (33–36). Here, we have cloned the rat endoglin cDNA from HSC, the cell type that, upon TGF-β stimulation, is mainly responsible in liver for excessive formation of extracellular matrix components, resulting in hepatic fibrosis (55, 71). In contrast to endothelial cells, expressing two endoglin transcript variants, denoted L- and S-endoglin, we found that rat HSC and MFB express two variants of L-endoglin most likely provoked by usage of different polyadenylation sites. The S-variant encodes a protein, which is truncated by 40 amino acids compared with the L-variant (65), missing at least the mutual class I PDZ domain (27). The deduced amino acid sequence of rat endoglin is 69 and 82% identical to the corresponding orthologs of humans and mice (62, 63) and shares the typical domain structure excluding an RGD domain found in the human counterpart (62). The carboxyl terminus of endoglin is highly homologous to betaglycan and does not possess a kinase domain (4, 24, 72) but contains a potential interface for interaction with β-arrestin 2 and GIPC (25, 26). Whether endoglin also interacts with β-arrestin 2 or GIPC is currently not known, and the expression of these components in HSC has not been analyzed. Recently, the LIM domain-containing protein zyxin has been shown to specifically interact with the C terminus of endoglin in HUVEC cells, leading to a modulation of their migration behavior (73). In liver, we have recently shown that the LIM domain containing protein CRP2 is exclusively expressed in HSC and is transiently up-regulated during the activation phase of HSC (74). Therefore, CRP2 is a putative candidate interaction partner for endoglin in activated HSC.

The comparison of the signals obtained in Western blot analysis using lysates of HUVEC cells and human myofibroblasts reveals that MFB express relatively high amounts of endoglin (cf. Figs. 6B and 7C). One potential factor that is able to increase the expression of endoglin is the receptor ligand TGF-β itself. In endothelial cells, monocytic cells, human mesangial cells, and cultured cell lines, TGF-β is able to up-regulate promoter activity and endoglin expression (75–79). Because it is well documented that activated HSC and MFB produce large amounts of TGF-β (52, 80), endoglin expression could be maintained during transdifferentiation by persistent autocrine stimulation by TGF-β. However, under the setting of our TGF-β stimulation experiments, there was no significant increase in endoglin protein expression detectable (Fig. 10B).

At least in late stage HSC and MFB, this might be explained by the inactivation of the underlying signaling pathway(s). It is postulated that the transcriptional activity of the endoglin gene is facilitated through binding of Smad3 and Smad4 to regulatory sequences within the endoglin promoter (77). In culture-activated HSC, it was demonstrated that signaling via Smad2 and Smad3 does not occur (69). Therefore, if TGF-β-dependent transcription of the endoglin gene depends on these Smad proteins, there should be at least a strong diminished effect of TGF-β on endoglin transcription.

However, concerning the function of endoglin in HSC and MFB, we can only speculate at this time. The current view of TGF-β signaling in HSC comprises the signaling receptors type I (ALK5) and type II and betaglycan as well as the intracellular mediators R-Smad2 and -3 (53, 66–68). Several studies have been addressed to link TGF-β signaling to the process of HSC activation and fibrogenesis. Whereas the growth of quiescent and early activated HSC is inhibited by TGF-β, fully activated HSC and MFB are released from this inhibitory effect (52, 53). On the other hand, TGF-β mediates extracellular matrix gene expression in activated HSC and to a high extent in MFB through autocrine stimulation (82). These effects evoked by TGF-β have been assigned to the above mentioned simple signaling cascade, involving ALK5, type II receptor, betaglycan, and R-Smad2 and -3 as central signaling elements (53, 69).

In more detail, it was shown that Smad2 is primarily involved in early HSC, accounting for growth inhibition. Further, Smad3 is activated in early and fully activated HSC, thereby initiating the onset of extracellular matrix gene expression (83). What mechanism governs the postulated autocrine extracellular matrix gene stimulation through TGF-β in MFB is presently not known. The messenger RNA for type II receptor and betaglycan are down-regulated in the course of fibrogenesis (66–68). In line, we observed a down-regulation of betaglycan expression, whereas the endoglin mRNA was almost constant during the transdifferentiation process (Fig. 4B), and in parallel the activation of the R-Smad2 and -3 as well as a Smad3-responsive reporter construct CAGA-luciferase could not be detected in MFB (69).

However, it has recently been shown that TGF-β is able to activate an alternative signaling pathway involving a different type I receptor (ALK1) and the intracellular mediators of the R-Smad family 1, 5, and 8, formerly known to be activated by BMPs (8, 48–51). In endothelial cells, ALK1 signaling seems to be involved in the activation phase, leading to a motile and proliferative phenotype (50), a phenotype resembling transdifferentiated MFB. Current data (e.g., the human diseases HHT1 and HHT2 (43, 44) and their respective mouse knockout models of ALK1 (49) and endoglin (33–36) as well as the observed direct interaction of endoglin and ALK1 (40)) support the hypothesis that endoglin is a component of the ALK1 signaling branch. As a first prerequisite, we have demonstrated the presence of endoglin throughout the process of transdifferentiation by Northern blot (Fig. 4A), Western blot (Fig. 6B), and immunoprecipitation experiments (Fig. 9). The expression of the other elements of this putative signaling pathway in HSC/MFB is the focus of our current study. Based on data showing the expression of Smad1 (81) as well as our own unpublished data showing ALK1 expression in HSC/MFB, we are confident that this TGF-β signaling pathway is active in HSC/MFB.

Acknowledgments—We thank G. Blobe (Duke University Medical Center, Departments of Medicine and Pharmacology and Cancer Biology, Durham, NC) for kindly providing pCMV-HA-rTβRIII and P. Kiefel (Institute of Clinical Chemistry and Pathobiology, RWTH-University Hospital Aachen, Germany) for helpful discussions.

REFERENCES

1. Roberts, A. B., and Sporn, M. B. (1993) Growth Factors 8, 1–9
2. Massague, J. (1996) Annu. Rev. Biochem. 67, 753–791
3. Leask, A., and Abraham, D. J. (2004) FASEB J. 18, 816–827
4. Wang, X. F., Lin, H. Y., Ng-Eaton, E., Doward, J., Lodish, H. F., and Weinberg, R. A. (1991) Cell 67, 797–805
5. Lin, H. Y., Wang, X. F., Ng-Eaton, E., Weinberg, R. A., and Lodish, H. F. (1992) Cell 67, 775–785
6. Franzen, P., ten Dijke, P., Ichijo, H., Yamashita, H., Schulz, P., Heldin, C. H., and Miyazono, K. (1993) Cell 75, 681–692
7. Wrama, J. L., Attiasano, L., Wieser, R., Ventura, F., and Massague J. (1994) Nature 370, 341–347
8. Moustaakas, A., Souchelevtskiy, S., and Heldin, C. H. (2001) J. Cell Sci. 114, 4359–4369
9. Rodriguez, C., Chen, F., Weinberg, R. A., and Lodish, H. F. (1995) J. Biol. Chem. 270, 15919–15922
10. Lin, H. Y., Moustaakas, A., Knaus, P., Wells, R. G., Henis, Y. I., and Lodish, H. F. (1995) J. Biol. Chem. 270, 2747–2754
11. Hirai, R., and Fujita, T. (1996) Exp. Cell Res. 223, 135–141
12. Rotzer, D., Roth, M., Lutz, M., Lindemann, D., Sebald, W., and Knaus, P. (2001) EMBO J. 20, 480–490
13. del Re, E., Bahkt, J. I., Pirani A., Schnyder, A. L., and Lin, H. Y. (2004) J. Biol.
Endoglin in Hepatic Stellate Cells

Sankar, S., Mahooti-Brooks, N., Centrella, M., McCarthy, T. L., and Madri, J. A. (1995) J. Biol. Chem. 270, 13567–13572

Bown, C. B., Boyer, A. S., Bunyan, R. B., and Barnett, J. V. (1999) Science 283, 2080–2082

Stenvers, K. L., Tursky, M. L., Harder, K. W., Kountouri, N., Amatyakul-Chantler, S., Graal, D., Small, C., Weinberg, R. A., Sizeland, A. M., and Zhu, H. J. (2003) Mol. Cell. Biol. 23, 4371–4385

Chefetz, S., Weatherbee, J. A., Tsang, M. L., Anderson, J. K., Mole, J. E., Lucas, R., and Massague, J. (1987) Cell 48, 409–415

Chefetz, S., Hernandez, L., Haino, M., ten Dijke, P., Iwata, K. K., and Massague, J. (1989) J. Biol. Chem. 265, 20333–20338

Segers, P. R., Rosen, D. M., and Seyedin, S. M. (1989) Mol. Endocrinol. 3, 261–272

Vilchis-Landeros, M. M., Montiel, J. L., Mendoza, V., Mendoza-Hernandez, G., Lopez-Casillas, F., Wrana, J. L., and Massague, J. (1993) J. Biol. Chem. 268, 20533–20538

Schafer, S., Zorbe, O., and Gressner, A. M. (1997) Hepatology 7, 680–687

Andre, J. L., Stanley, K., Chefetz, S., and Massague, J. (1989) J. Cell Biol. 109, 3137–3145

Gougos, A., and Letarte, M. (1988) J. Biol. Chem. 263, 8361–8364

Lebrin, F., Goumans, M. J., Jonker, L., Carvalho, R. L., Valdimarsdottir, G., Thorikay, M., Mummery, C., Arthur, H. M., and ten Dijke, P. (2003) EMBO J. 22, 1041–1048

Gougos, A., and Letarte, M. (1999) Biochem. Biophys. Res. Commun. 254, 655–663

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z. Miller, and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402

Guettat, A., Hannah, R., and O’Connor-McCourt, M. (1999) Eur. J. Biochem. 261, 618–626

Brodie, D., Sevock, A. M., Coste, T., Strosberg, A. D., and Marullo, S. (1999) Hepatology 29, 1703–1738

Dooley, S., Delvoux, B., Lahme, B., Mangasser-Stephan, K., and Gressner, A. M. (2000) Hepatology 31, 1104–1106

Thorikay, M., Mummery, C., Arthur, H. M., and ten Dijke, P. (2004) J. Biol. Chem. 279, 671–680

Goumans, M. J., Lebrin, F., and Valdimarsdottir, G. (2003) Trends Cardiovasc. Med. 13, 301–307

Li, D. Y., Sorensen, L. K., Brooke, B. S., Urness, L. D., Davis, E. C., Taylor, D. G., Boak, B. B., and Wendel, D. P. (1999) FEBS Lett. 452, 11–16

Chen, W., Kirkbride, K. C., How, T., Nelson, C. D., Mo, J., Frederick, J. P., Wang, X. F., Letkowitz, R. J., and Blobe, G. C. (2003) Science 301, 1394–1397

Lee, K., Song, B. W., Park, H. J., and Lee, S. Y. (2000) J. Biol. Chem. 275, 13567–13572

Lebrin, F., Goumans, M. J., Jonker, L., Carvalho, R. L., Valdimarsdottir, G., Thorikay, M., Mummery, C., Arthur, H. M., and ten Dijke, P. (2004) EMBO J. 23, 4018–4028

Chem. 279, 23765–23772

Chem. 279, 23765–23772
Identification of Endoglin in Rat Hepatic Stellate Cells: NEW INSIGHTS INTO TRANSFORMING GROWTH FACTOR β RECEPTOR SIGNALING
Steffen K. Meurer, Lidia Tihaa, Birgit Lahme, Axel M. Gressner and Ralf Weiskirchen

J. Biol. Chem. 2005, 280:3078-3087.
doi: 10.1074/jbc.M405411200 originally published online November 10, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M405411200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2004/11/24/M405411200.DC1

This article cites 83 references, 34 of which can be accessed free at
http://www.jbc.org/content/280/4/3078.full.html#ref-list-1