BAT3 Interacts with Transforming Growth Factor-β (TGF-β) Receptors and Enhances TGF-β1-induced Type I Collagen Expression in Mesangial Cells∗

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Joon Hyeok Kwak1, Sung Il Kim2, Jin Kuk Kim†§, and Mary E. Choi††

From the †Renal Division, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115 and the ‡Department of Internal Medicine, Bucheon Hospital, Soonchunhyang University, Bucheon 420-767, Korea

Transforming growth factor-β1 (TGF-β1) plays essential roles in a wide array of cellular processes, such as in development and the pathogenesis of tissue fibrosis, including that associated with progressive kidney diseases. Tight regulation of its signaling pathways is critical, and proteins that associate with the TGF-β receptors may exert positive or negative regulatory effects on TGF-β signaling. In the present study we employed a yeast-based two-hybrid screening system to identify BAT3 (HLA-B-associated transcript 3) as a TGF-β receptor-interacting protein. Analysis of endogenously expressed BAT3 in various tissues including the kidney reveals the existence of ~140-kDa full-length protein as well as truncated forms of BAT3 whose expression is developmentally regulated. Endogenous BAT3 protein interacts with TGF-β receptors type I and type II in renal mesangial cells. Functional assays show that expression of full-length BAT3 results in enhancement of TGF-β1-stimulated transcriptional activation of p3TP-Lux reporter, and these effects require the presence of functional TGF-β signaling receptors as demonstrated in R-1B and DR-26 mutant cells. Moreover, expression of full-length BAT3, but not C-terminal truncated mutant of BAT3, enhanced TGF-β1-induced type I collagen expression in mesangial cells, whereas knock down of BAT3 protein expression by small interfering RNA suppressed the expression of type I collagen induced by TGF-β1. Our findings suggest that BAT3, a TGF-β receptor-interacting protein, is capable of modulating TGF-β signaling and acts as a positive regulator of TGF-β1 stimulation of type I collagen expression in mesangial cells.

Transforming growth factor-β1 (TGF-β1),2 is a pleiotropic cytokine that regulates diverse biological activities including cell proliferation, apoptosis, differentiation, and development (1). TGF-β1 is also a potent inducer of extracellular matrix synthesis and is well established as a key mediator in the final common pathway of fibrosis associated with progressive kidney diseases (2, 3). Its actions are mediated by the heteromeric interactions of types I and II serine/threonine kinase receptors. TGF-β1 signaling is initiated upon binding of the ligand by the type II receptor (TβR-II), which induces the recruitment and transphosphorylation of the type I receptor (TβR-I) leading to subsequent activation of downstream intracellular signaling events (4). Activation of the Smad signaling pathway is well documented as the canonical pathway induced by the TGF-β1-receptor complex (5). However, the cellular responses to TGF-β1 are known to be variable, and the multiple TGF-β1 actions occur in cell type-specific and context-dependent fashion. An emerging body of evidence now demonstrates that TGF-β1 also induces various non-Smad signaling pathways, such as the Rho GTPase, phosphatidylinositol 3-kinase, TGF-β-activated kinase 1 (TAK1), and the mitogen-activated protein kinases ERKs, p38, and c-Jun N-terminal kinases (6–11). Thus, non-Smad signaling proteins can participate in transducing signals that contribute to physiological responses to TGF-β1 and may enable versatility of TGF-β1 actions.

The complexity of TGF-β signal transduction has been further broadened by recent identification of a number of cytoplasmic and nuclear proteins, which associate with the TGF-β receptors and signal transduction components to exert positive or negative regulatory effects on TGF-β signaling. For instance, Axin, a negative regulator in Wnt signaling, binds Smad3 and mediates cross-talk with the Wnt pathway (12). SARA (Smad anchor for receptor activation), a FYVE domain protein, anchors Smad2 to the plasma membrane and facilitates interaction with the receptor (13). The C-terminal portion of TRAP-1 (TGF-β receptor type I-associated protein-1) associates with activated TβR-I and can inhibit TGF-β1 signaling (14). TGF-β receptors can also bind to sorting nexins implicated in vesicular trafficking and sequestering TβR-I in caveolin-rich plasma membrane microdomains (15). STRAP (serine-threonine kinase receptor-associated protein) is a Tpr-Asp domain-roblastoma; ERK, extracellular signal-regulated kinase; NLS, nuclear localization sequence; SD medium, synthetic dropout medium; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Col-I, type I collagen protein; siRNA, small interfering RNA; FBS, fetal bovine serum; GFP, green fluorescent protein; MOPS, 4-morpholinepropanesulfonic acid; NK, natural killer.
containing protein shown to interact with both TβR-I and TβR-II (16). Thus, a complex scheme of signal transduction components downstream of TGF-β receptors is emerging that transduces signals from the cell surface to the nucleus and enables multifunctionality of TGF-β1.

In the present study we employed a yeast-based two-hybrid screening system to identify novel candidate proteins that may interact with TGF-β receptors. We used the TβR-1-DNA binding domain-fused protein designed to act as bait to screen a rat brain cDNA library, which were fused with the activation domain of the Saccharomyces cerevisiae transcriptional activator GAL4. From this yeast two-hybrid screen, we isolated three independent and overlapping clones that encoded a gene known as BAT3, for HLA-B-associated transcript 3. BAT3 was originally identified by chromosome walk as a gene located within human major histocompatibility complex and, hence, the name (17). BAT3 has previously been shown to interact with the N-terminal region of a TGF-β superfamily member DAN, but to date very little is known regarding its functional role (18). The human and Xenopus homologs, also known as Scythe, has been implicated in the control of apoptosis (19–21).

The BAT3 interacts with p300 protein and regulates p53 acetylation (23). Here, we provide the first evidence that the N terminus, and this region interacts with component of 26S proteasome (22). The BAT3 has a ubiquitin homologous sequence at the C-terminal amino acid residues 1119–1136 of BAT3 as an antigen (1119RSDIGKRLQPNSYPQR1136) by Invitrogen using a standard protocol. Briefly, rabbits were immunized with keyhole limpet hemocyanin-conjugated peptides in complete Freund’s adjuvant and subsequently boosted with peptide and incomplete Freund’s adjuvant. The rabbit antisera were subjected to peptide-affinity purification, and titer and specificity of the purified antibody was analyzed with enzyme-linked immunosorbent assay and absorbance.

Plasmids—Construction of TβR-I/pCDNA3.1 and TβR-II/pCDNA3.1 was previously described (24, 25). The rat TβR-I cDNA fragment was amplified by PCR from plasmid TβR-I/pCDNA3.0 using the primers TβR-I-5′FT (5′-TGG TAA AGA GGT CTT CCC AGC AAG AAT CTT CAG ATA CAT TTT GAT GCC-3′) and TβR-I-3′FT (5′-CGG GCC ACA AAG CTT GGC AGC GGG AAC ATG-3′) and cloned in the vector pDBLeu (Invitrogen) to generate pDBLeu/TβR-I. A full-length rat BAT3 cDNA was generated by PCR amplification from a neonatal rat kidney cDNA library previously described (25) using the primer pair BAT3/BglII sense primer (5′-GAA GAT CAT TCA CCT CTC GCC CAT GAG GCC TAA TG-3′) and BAT3/NotI antisense primer (5′-AAG ACG GCC GCA CAT GTC AGA AAG CTA GGG GTC-3′) and cloned into vector pCDNA3.1-HisA to obtain BAT3/pCDNA3.1-HisA. Deletion construct BM810 (deletion of 810 amino acid residues from the N terminus) was generated using the primer pair BM810 sense primer (5′-G AAG ATC TAC GCC CAG GAG CCC ACG TCC AGC-3′) and BAT3/NotI antisense primer. To generate BM810–ΔC (deletion of the C-terminal nuclear localization sequence (NLS) and BAG domain from BM810 by truncation at amino acid position 1045), the BM810 sense primer and BM810–ΔC antisense primer (5′-AAG GCG GCC GCA CTA GTG GCT CTT CTG AAT GTC CTG G-3′) were used. All of the plasmid constructs were verified by restriction analysis and DNA sequencing.

Yeast Two-hybrid Screening—The “bait” plasmid, pDBLeu/TβR-I, was constructed as described above, carrying TβR-I in-frame with the GAL4 DNA binding domain. The integrity of the rat TβR-I cDNA and production of the fusion protein were confirmed by DNA sequencing and Western blotting, respectively. Screening of a rat brain cDNA library cloned into the GAL4 activation domain vector pPC86 was carried out as previously described (26). The following media were used for propagation of the yeast cells: YPD medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose and synthetic dropout medium (SD) containing 0.6% (w/v) yeast nitrogen base without amino acids (Difco) and 2% (w/v) glucose containing the appropriate dropout solution. SD media depleted of the selective amino acid(s) was prepared as follows: SD−Ura containing X-gal (Sigma-Aldrich), SD−Leu, SD−Trp−Leu, and SD−Trp−Leu−Ura−His media (amino acids indicated in superscript were depleted from the SD medium). From 5 × 10⁶ yeast transformants, selection on SD−Trp−Leu−Ura−His media plus 25 mM 3-amino-1,2,4-triazole resulted in the isolation of seven independent cDNA clones encoding TβR-I interacting proteins. Activation of the β-galactosidase reporter gene in yeast transformants that harbored pBD-GAL4/TβR-I and/or pGAD-GAL4 cDNA clones were grown on filter papers on SD−Trp−Leu−agar plates and then incubated with X-gal and confirmed by β-galactosidase activity. Transformants harboring pBD-GAL4/TβR-I alone did not show β-galactosidase activity.

Cell Culture and Transfection—COS-7 cells and mink lung epithelial cells (Mv1Lu) and mutant cell lines R-1B and DR-26, derived from the Mv1Lu cell line by chemical mutagenesis and selection for resistance to TGF-β (kindly provided by J. Masague), were cultured in Dulbecco’s modified Eagle’s medium.
containing 10% FBS. Primary cultures of mesangial cells from male C57BL/6 mice were isolated and characterized as previously described (7, 9, 10) and maintained in RPMI 1640 medium containing 10 µg/ml insulin and 15% FBS. Cells between 7 and 16 passages were used for the experiments. All cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Transient transfections were performed using cells 24 h after plating (2 × 10⁵ cells/60-mm plate) with a total of 2 µg of the indicated plasmids using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's protocol. Transfection of siRNA was carried out using DharmaFECT™ reagent obtained from Dharmacon, Inc.

**Co-immunoprecipitation and Western Blot Analysis—**Twenty-four hours after transfection cells were harvested and lysed in buffer containing 0.5% Nonidet P-40, 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA. Protein concentrations of the cell lysates were determined by Coomassie Blue dye binding assay (Bio-Rad). Cell lysates were precleared with protein A/G plus-agarose beads for 1 h, and immunoprecipitation was carried out with 200 µg of protein lysates using the indicated antibodies and protein A/G plus-agarose beads as previously described (9, 26). Immune-complexes were eluted with 2× SDS protein sample buffer containing 0.125 mM Tris-HCl, pH 7.4, 4% SDS, and 20% glycerol (Quality Biological, Inc., Gaithersburg, MD), subjected to separation on a 10% SDS-PAGE, and transferred to polyvinylidene difluoride membranes followed by Western blot analyses with the indicated antibodies, as previously described (10). In experiments involving samples not subjected first to immunoprecipitation, an equal volume of 2× SDS protein sample buffer was added to the whole cell lysate, and the protein samples (20 µg) were resolved on a 10% SDS-PAGE and then electrophoretically transferred onto polyvinylidene difluoride membranes followed by Western blotting with the indicated antibodies and visualized with the Lumi-GLO system (Cell Signaling Technology, Inc., Danvers, MA). The tissue protein samples were obtained from neonatal (1 day after birth) and adult rat tissues and mouse kidney tissues using a homogenizer, and 60 µg of protein from each sample was used for Western blot analysis. All experiments were performed at least three times with essentially the same results; representative blots are shown. Densitometry determinations for the levels of α(I) collagen were quantified as the ratio to 18S rRNA signals using Image J software (Research Services Branch, National Institutes of Health) and normalized to the expression levels in control empty vector-transfected cells without TGF-β1 stimulation.

**Luciferase Assay—**Cells were transiently transfected with the luciferase reporter plasmid p3TPLux (kindly provided by J. Massagué) using Lipofectamine Plus Reagent. The p3TPLux contains elements from the PAI-1 promoter and drives the expression of a luciferase reporter gene in response to TGF-β1 (25). Control reporter vector pRL-CMV (Promega) was included in all transfections experiment as an internal control to normalize transfection efficiency. Cells were exposed to DNA-Lipofectamine-Plus complexes in serum-free RPMI1640 media for 3 h followed by incubation in 15% FBS growth media for 16 h, then placed in 0.5% FBS media for 5 h. Cells were then incubated in RPMI1640 media with 0.5% FBS in the absence or presence of exogenous TGF-β1 (2 ng/ml) for 5 h. Luciferase activities in the cell lysates were measured using the Dual-Luciferase reporter assay system (Promega) in a TD-20/20 luminometer from Turner Designs (Sunnyvale, CA). The results were expressed as the ratio of measured relative light units of firefly luciferase activity (p3TPL-Lux) to Renilla luciferase (pRL-CMV) activity. Data represent the mean values (±S.E.) from three independent experiments, each performed in triplicate.

**RNA Isolation and Northern Blot Analysis—**Total RNA was isolated from cell lysates and tissues with TRIzol (Invitrogen) according to the manufacturer’s instructions and size-fractionated (10 µg/lane) on a 1% agarose, 20% formaldehyde gel in 20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA (pH 7.2). The mRNA was transferred and UV-cross-linked to nylon membranes. The blots were prehybridized for 2 h in Church Gilbert’s hybridization buffer (Quality Biological, Inc.) and hybridized overnight in the same solution containing 32P-labeled cDNA probes at 65 °C. The blots were then washed 2 times in solution A (0.5% bovine serum albumin, 5% SDS, 40 mM phosphate buffer (pH 7.0), and 1 mM EDTA (pH 8.0)) for 30 min each at 65 °C followed by 15-min washes with solution B (1% SDS, 40 mM phosphate buffer (pH 7.0) and 1 mM EDTA (pH 8.0)) at 65 °C. The blots were exposed to Kodak X-AR film. The human pro-α(I) collagen cDNA was obtained from ATCC and previously described (10). To control for relative equivalence of RNA loading, the same blots were hybridized with 32P-labeled oligonucleotide probes corresponding to the 18 S rRNA as previously described (10). All experiments were performed at least three times with essentially the same results, and representative plots are shown. Densitometry determinations for the levels of pro-α(I) collagen mRNA were quantified as the ratio to 18 S rRNA signals using Image J software (Research Services Branch, National Institutes of Health) and normalized to the expression levels in control empty vector-transfected cells without TGF-β1 stimulation.

**Microscopy—**Cells were transiently transfected with recombinant plasmid expressing full-length or truncated versions of BAT3 fused with green fluorescent protein (GFP)-BAT3, GFP-BM810, and GFP-BM810-ΔC. The parental vector pEGFP (Clontech Laboratories, Inc., Mountain View, CA) was used as a control. Cells were exposed to DNA-Lipofectamine-Plus complexes in serum-free Dulbecco’s modified Eagle’s medium for 3 h followed by incubation in 15% FBS growth media for 16 h, then placed in 0.5% FBS media for 5 h. Cells were then incubated in Dulbecco’s modified Eagle’s medium containing 0.5% FBS for 5 h with or without exogenous TGF-β1 (2 ng/ml). Live cell images were captured with Leica TCS SL laser scanning confocal microscope equipped with spectral detector (Leica Microsystems, Inc., Bannockburn, IL). For visualization, GFP was excited with 476- and 488-nm laser lines from an argon laser, and the emission window was set at 476–505 nm. Images were processed using the Leica Confocal software (Leica Microsystems, Inc.). All experiments were performed at least three times with essentially the same results, and representative images are shown.

**Statistical Analysis—**Statistical significance of the experimental data from three independent experiments was determined by Student’s t test for paired data. p values <0.05 were considered significant.
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RESULTS

Identification of a Novel TGF-β Receptor-interacting Protein—To identify candidate proteins that interact with TGF-β receptors, we performed a yeast two-hybrid screening of a rat brain cDNA library fused with GAL4 activation domain using the rat TGF-β receptor I fused to the DNA binding domain of GAL4 designed as bait. The nucleotide sequences of the cDNA clones isolated from this yeast two-hybrid screen were determined. Three of the cDNAs represented independent and overlapping clones (M2, M3, M4) ranging in sizes from 1.7 to 2.2 kilobases that showed a high degree of homology with BAT3. Based on these results we sought to clone the full-length rat BAT3 cDNA by PCR amplification from a neonatal rat kidney cDNA library (25). Sequence analysis of the resultant PCR-amplified product of 3.4 kilobases in length revealed complete coding sequence which was essentially identical to the rat BAT3 cDNA (accession number NP_446061) previously isolated by Ozaki et al. (18), with the exception of the extreme C-terminal region (Fig. 1A). Our rat BAT3 cDNA (designated with an asterisk in Fig. 1A) encoded 1148 deduced amino acids, which included an additional 49 amino acid residues in the C-terminal region. Comparison of the deduced amino acid sequences of the C-terminal region, containing the NLS and the BAG domain, of the rat BAT3 cDNAs and the mouse and human BAT3 cDNAs are shown in Fig. 1A. Note that this region contains almost identical amino acid sequences among mouse, human, and the cloned rat BAT3 cDNAs with the exception of two amino acid residues (designated with Δ in Fig. 1A). Overall, the rat BAT3 cDNA shares 96% amino acid sequence identity with mouse BAT3 and 90% amino acid identity with human BAT3.

Endogenously Expressed BAT3 Protein Is Developmentally Regulated—We sought to examine and characterize the endogenous expression pattern of the BAT3 protein. We first raised an antibody directed against the C-terminal peptide sequences of BAT3 (1119RSDIQKRLQEDPNYS-PQR1136). We then confirmed the specificity of the anti-BAT3 antibody to recognize endogenously expressed BAT3 proteins by Western blotting (Fig. 1B). COS-7 cells were transfected with expression vector carrying full-length BAT3 with N-terminal His tag (His-BAT3) or control empty vector (pcDNA3.1). Cell lysates were subjected to Western blotting with anti-BAT3 or anti-His antibodies, shown in Fig. 1B. In the His-BAT3-transfected cells, both antibodies detected bands of ~140 kDa corresponding to the transfected His-BAT3 protein, whereas in the empty vector-transfected cells only the anti-BAT3 antibody recognized the ~140-kDa bands corresponding to endogenous BAT3 protein. The other bands of lower molecular weight most likely represent nonspecific bands or partially degraded forms. Specificity of the anti-BAT3 antibody was also demonstrated in cells transfected with various BAT3 deletion constructs showing that the anti-BAT3 antibody recognized N terminus-deleted BAT3 mutants, but not C terminus-deleted BAT3, which does not contain the region of BAT3 against which the antibody was raised (data not shown). Using the anti-BAT3 antibody, we then examined the expression pattern of endogenous BAT3 protein in various tissues from neonatal and adult rats. Western blotting with anti-BAT3 antibody detected bands of ~140 kDa corresponding to the full-length BAT3 protein in all tissues examined (Fig. 2A). Decreased abundance of the endogenous
full-length BAT3 protein was observed in the adult rat tissues, particularly the lung, kidney, and liver, compared with the corresponding neonatal tissues, indicating that regulated expression of BAT3 protein may be important in development. In addition to the full-length BAT3, lower molecular mass bands ranging in sizes from ~50 to ~100 kDa were detected in several of the tissues examined. In the adult rat kidney tissues, the predominant form detected was ~70 kDa in size, whereas the adult liver expressed a slightly smaller sized form. In the spleen and the heart, additional bands were detected ranging from ~50 kDa to the 140-kDa full-length BAT3. Given that the anti-BAT3 antibody recognizes an epitope present on the extreme C terminus of BAT3, the lower molecular mass proteins are deduced to be endogenously expressed truncated C-terminal fragments of BAT3. As in adult rats, the predominant form detected in the adult mouse kidney tissues was ~70 kDa, whereas the predominant form expressed in cultured primary mouse mesangial cells was the ~140-kDa full-length BAT3 protein (Fig. 2B, left panels). Northern blot analysis of BAT3 mRNA expression demonstrated a single major transcript size of ~4.4 kilobases in both adult mouse kidneys and mouse mesangial cells (Fig. 2B, right panels).

BAT3 Interacts with TGF-β Receptors—To further examine the interaction of BAT3 with TβR-I identified by yeast based two-hybrid screening, the full-length BAT3 bearing an N-terminal His tag (His-BAT3) was co-expressed in COS-7 cells with TβR-I (Fig. 3A, upper group of four panels). Cell lysates subjected to immunoprecipitation using anti-TβR-I antibody were analyzed by subsequent Western blotting with anti-His antibody, or conversely, immunoprecipitation using anti-His antibody then Western blotting with anti-TβR-I antibody revealed that BAT3 interacted with TβR-I. Similarly, pulldown assays with anti-TβR-II or anti-His antibodies in COS-7 cells transfected with His-BAT3 and TβR-II demonstrated that BAT3 interacted with TβR-II (Fig. 3A, lower group of four panels). Expression of each of the transfected genes was confirmed in whole cell lysates not subjected to immunoprecipitation by Western blotting with corresponding antibodies. We next sought evidence for interactions between the endogenously expressed BAT3 proteins and endogenous TGF-β receptors in cultured primary mouse mesangial cells. Co-immunoprecipitation assays shown in Fig. 3B demonstrated that endogenous TβR-I or TβR-II pulled down with anti-TβR-I or anti-TβR-II antibodies resulted in co-precipitation of endogenous full-length BAT3 proteins. The expression of endogenous BAT3, TβR-I, and TβR-II in the mesangial cell lysates was monitored by direct immunoblotting with corresponding antibodies. The full-length BAT3 is the most abundant form expressed in cultured primary mouse mesangial cells, whereas the expression of the truncated forms are extremely low (Fig. 2B), and in our studies...
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FIGURE 3. Interaction of BAT3 with TGF-β receptors. A, co-immunoprecipitation of BAT3 with TGF-β receptors type I and type II. COS-7 cells were transiently transfected with empty vector or His-BAT3 and TβRI (upper group of four panels) or TβRII (lower group of four panels). Cell lysates were subjected to immunoprecipitation (IP) with antibodies against TβRI or TβRII or anti-His antibody followed by Western blotting (WB) with respective antibodies as indicated (upper two panels in each group). Expression of the transfected genes was confirmed in whole cell lysates not subjected to immunoprecipitation by Western blotting with corresponding antibodies (lower two panels in each group). B, association of endogenous BAT3 with endogenous TGF-β receptors in mouse mesangial cells. Cell lysates from mouse mesangial cells were subjected to immunoprecipitation (IP) with anti-TβRI or anti-TβRII antibodies as indicated followed by Western blotting (WB) using anti-BAT3 antibody. Immunoprecipitated proteins with irrelevant rabbit antibodies (IgG) were used as negative controls. Whole cell lysates (CL) not subjected to immunoprecipitation were analyzed for endogenously expressed BAT3, TβR-I, and TβR-II by Western blotting with corresponding antibodies.

we could detect only the full-length BAT3 interaction with TGF-β receptors. Our data indicate that endogenous BAT3 protein interacts with both type I and type II TGF-β receptors in mesangial cells.

BAT3 Expression Increases TGF-β1-induced Transcriptional Activation—To begin to explore its physiological functions, we investigated whether the expression of BAT3 had functional effects on TGF-β signaling as assessed by assays using the TGF-β1-inducible p3TP-Lux reporter. Wild-type Mv1Lu cells and mutant cells that lack functional TβRI (R-1B) and TβRII (DR-26) (27) were transiently co-transfected with the luciferase reporter p3TP-Lux and full-length BAT3 or empty vector pcDNA3.1. Twenty-four hours after transfection the cells were treated with exogenous TGF-β1 (2 ng/ml) for an additional 5 h, and subsequently, TGF-β1-responsive luciferase reporter activities in cell lysates were measured. As shown in Fig. 4, we observed that ectopic expression of BAT3 resulted in increased basal p3TP-Lux reporter activities in untreated wild-type and mutant Mv1Lu cells, indicating that BAT3 overexpression can activate p3TP-Lux in the absence of TGF-β1. However, the p3TP-Lux activities in response to TGF-β1 stimulation was significantly enhanced in only the wild-type Mv1Lu cells transfected with BAT3 compared with control cells transfected with empty vector pcDNA3.1. In the TGF-β receptor-deficient mutant cell lines R-1B and DR-26, ectopic expression of BAT3 failed to increase p3TP-Lux activities above basal levels upon stimulation with TGF-β1, and as expected, no TGF-β1-stimulated luciferase activities were observed after transfection of empty vector pcDNA3.1 in either the R-1B or DR-26 cells. These results suggest that the BAT3 effects on TGF-β1 response are dependent on the presence of functional TGF-β signaling receptors.

BAT3 Enhances TGF-β1-induced Type I Collagen Expression in Mesangial Cells—In previous studies we have shown that TGF-β1 potently induces type I collagen expression in mouse mesangial cells (7, 10). To further investigate the physiological functions of BAT3, we examined the effects of constitutive expression of BAT3 on stimulation of type I collagen expression by TGF-β1. Primary mouse mesangial cells were transiently transfected with the full-length BAT3 bearing an N-terminal His tag (His-BAT3) or control empty vector pcDNA3.1. Twenty-four hours after transfection the cells were treated with exogenous TGF-β1 (2 ng/ml) for an additional 24 h, and subsequently cell lysates were analyzed by Western and Northern blotting for type I collagen expression. As shown in Fig. 5, constitutive expression of His-BAT3 resulted in increased expression of type I collagen protein (Fig. 5A) and pro-α1(I) collagen mRNA (Fig. 5B) in mouse mesangial cells, and stimulation with TGF-β1 resulted in further enhancement of collagen induction compared with the empty vector-transfected cells.

We next generated deletion constructs BM810 (deletion of 810 amino acid residues from the N terminus) and BM810-ΔC (deletion of C-terminal NLS and BAG domain from BM810) by truncation at amino acid position 1045) as illustrated schematically in Fig. 6A and compared their effects to the full-length BAT3 transfected into mouse mesangial cells. Each of the constructs carried an N-terminal His tag, and transiently expressed
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FIGURE 5. Effects of constitutive expression of BAT3 on TGF-β1-induced type I collagen expression in mouse mesangial cells. Cells were transiently transfected with empty vector pcDNA3.1 or His-BAT3 followed by treatment with (+) or without (−) exogenous TGF-β1 (2 ng/ml) for 24 h. A, expression of type I collagen protein (Col-I) was detected by Western blotting with anti-α-tubulin antibody for normalization of protein loading. Transiently expressed His-BAT3 protein was detected by Western blotting with anti-His antibody. Quantitative analysis of levels of Col-I with (closed bars) or without (open bars) TGF-β1 stimulation was determined by densitometry. Data are presented as -fold increase in Col-I, quantified as the ratio to α-tubulin, and normalized to the expression level in empty vector-transfected cells without TGF-β1 stimulation. The results represent the mean (±S.E.) of three independent experiments (*, p < 0.05 versus TGF-β1-untreated cells; **, p < 0.05 versus TGF-β1-treated empty vector-transfected cells). B, total RNA samples isolated were subjected to Northern blot analysis with 32P-labeled cDNA probe corresponding to pro-α1(I) collagen. 18 S rRNA hybridization signals served as normalization for RNA loading. Expression of transfected His-BAT3 was confirmed by Northern blotting with 32P-labeled BAT3 cDNA probe. Densitometry data are presented as -fold increase in pro-α1(I) collagen mRNA quantified as the ratio to 18 S rRNA signals and normalized to the expression level in empty vector-transfected cells without TGF-β1 stimulation. The results represent the mean (±S.E.) of three independent experiments (*, p < 0.05 versus corresponding TGF-β1-untreated cells; **, p < 0.05 versus TGF-β1-treated empty vector-transfected cells; #, p < 0.05 versus TGF-β1-untreated cells transfected with empty vector pcDNA3.1).

full-length BAT3 and deletion mutants, BM810 and BM810-ΔC, were confirmed by Western blotting using anti-His antibody (Fig. 6B, middle panel). As shown in Fig. 6B, ectopic expression of full-length BAT3 increased basal type I collagen levels and exerted an effect that significantly enhanced the induction of type I collagen expression by treatment with TGF-β1, whereas BM810-ΔC almost completely abolished the TGF-β1-stimulated type I collagen expression. The BM810 fragment, which retains the extreme C terminus, failed to block the TGF-β1-stimulated type I collagen response. These findings suggest that the C terminus is indispensable for the effects of BAT3 to enhance TGF-β1-stimulated type I collagen expression in mouse mesangial cells.

 Knockdown of BAT3 by siRNA Suppresses TGF-β1-induced Type I Collagen Expression in Mesangial Cells—To provide further support for BAT3 function to enhance TGF-β1-induced type I collagen expression in mesangial cells, we determined the effects of targeted knock-down of BAT3 by siRNA. Cells were transiently transfected with BAT3-specific siRNA (si-BAT3) or control siRNA (si-CNT), and knock down of BAT3 protein was confirmed by Western blot analysis of the cell lysates using anti-BAT3 antibody (Fig. 7). BAT3 siRNA significantly suppressed the induction of type I collagen expression by TGF-β1 stimulation but not by control siRNA.

TGF-β1 Triggers Nuclear Localization of Full-length BAT3—Our findings showed that the deletion of C-terminal region containing the NLS almost completely abrogated TGF-β1-induced type I collagen expression near completely. The NLS is thought to mediate translocation of BAT protein in the nucleus. To determine whether nuclear localization of BAT3 is regul-
Role of BAT3 in TGF-β Signaling

A growing body of evidence indicates that the TGF-β signaling pathway may be modulated by proteins that interact with TGF-β receptors and exert either positive or negative regulatory effects on TGF-β signaling. Using a yeast two-hybrid system we have identified BAT3 as a TGF-β receptor-interacting protein. The association of BAT3 and TGF-β receptors has not been previously known. Although BAT3 was originally identified by chromosome walk as a gene located within human major histocompatibility complex and, hence, the name, its functions remain largely understudied (17). To date, literally, there have been only a handful of reports investigating the functions of BAT3 protein, mostly in the Xenopus and a small number of more recent studies in mammalian cells that implicate a role in the control of apoptosis (19, 20). Studies in Xenopus egg extracts revealed that the full-length Scythe (the human and Xenopus homologs) alone did not induce apoptosis, but the C-terminal 312 amino acid fragment of Scythe was a potent inducer of apoptosis (21). The full-length Scythe required the presence of the Drosophila melanogaster apoptotic regulator known as Reaper to trigger apoptosis, and it was reasoned that Reaper binding might alter the conformation of Scythe or even lead to cleavage of the Scythe protein, allowing downstream apoptotic effectors to interact with normally inaccessible domains of Scythe. Besides Reaper, BAT3 has also been shown to interact with other apoptosis regulating factors such as Immediate early gene X-1 (28) and apoptosis-inducing factor (29). Depletion of BAT3 by siRNA in HeLa cells induces mitotic arrest of cells (30). A recent report showed that BAT3 can also bind to a cell surface receptor expressed in natural killer (NK) cells. The natural cytotoxicity receptor 3 (Nkp30) in NK cells triggers Nkp30-mediated cytotoxicity and is necessary for tumor rejection in a multiple myeloma model (31). BAT3 has also previously been shown to interact with the N-terminal region of another TGF-β superfamily member DAN (18). However, to date very little is known regarding its functional role in TGF-β signaling. In the present study we provide the first evidence that BAT3 is capable of interacting with both type I and type II TGF-β receptors in mammalian cells and exerts functional effects on TGF-β signaling. Our data demonstrate that the endogenous BAT3 protein interacts with TβR-I and TβR-II.

**FIGURE 7. Suppression of TGF-β1-induced type I collagen expression by knockdown of BAT3 protein in mouse mesangial cells.** Cells were transiently transfected with BAT3-specific siRNA (si-BAT3) or control siRNA (si-CNT) followed by treatment with (+) or without (−) exogenous TGF-β1 (2 ng/ml) for 24 h. Cell lysates were subjected to Western blot analysis with anti-BAT3 or anti-type I collagen antibodies. The same membranes were reblotted with anti-α-tubulin antibody for normalization of protein loading. Quantitation of Col-I expression in cells with (+) or without (−) exogenous TGF-β1 stimulation was determined by densitometry. Data are presented as -fold increase in Col-I expression, quantified as the ratio to α-tubulin and normalized to the expression level in si-CNT-transfected cells. **DISCUSSION**

A growing body of evidence indicates that the TGF-β signaling pathway may be modulated by proteins that interact with TGF-β receptors and exert either positive or negative regulatory effects on TGF-β signaling. Using a yeast two-hybrid system we have identified BAT3 as a TGF-β receptor-interacting protein. The association of BAT3 and TGF-β receptors has not been previously known. Although BAT3 was originally identified by chromosome walk as a gene located within human major histocompatibility complex and, hence, the name, its functions remain largely understudied (17). To date, literally, there have been only a handful of reports investigating the functions of BAT3 protein, mostly in the Xenopus and a small number of more recent studies in mammalian cells that implicate a role in the control of apoptosis (19, 20). Studies in Xenopus egg extracts revealed that the full-length Scythe (the human and Xenopus homologs) alone did not induce apoptosis, but the C-terminal 312 amino acid fragment of Scythe was a potent inducer of apoptosis (21). The full-length Scythe required the presence of the Drosophila melanogaster apoptotic regulator known as Reaper to trigger apoptosis, and it was reasoned that Reaper binding might alter the conformation of Scythe or even lead to cleavage of the Scythe protein, allowing downstream apoptotic effectors to interact with normally inaccessible domains of Scythe. Besides Reaper, BAT3 has also been shown to interact with other apoptosis regulating factors such as Immediate early gene X-1 (28) and apoptosis-inducing factor (29). Depletion of BAT3 by siRNA in HeLa cells induces mitotic arrest of cells (30).

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confirming our initial findings from the yeast two-hybrid screen and overexpression studies. Functional assays using the TGF-β1-inducible p3TP-Lux reporter demonstrate that BAT3 protein not only is capable of association with TβR-I and TβR-II but also modulates TGF-β signaling. We show that expression of the full-length BAT3 by transient transfection into Mv1Lu cells resulted in enhancement of TGF-β1-stimulated transcriptional activation of p3TP-Lux reporter activities. However, in the R-1B and DR-26 mutant cells that are deficient in functional TβR-I and TβR-II respectively, expression of the full-length BAT3 failed to enhance TGF-β1-stimulated p3TP-Lux reporter activities. These data suggest that BAT3 can enhance TGF-β signaling as assessed by the TGF-β-responsive reporter gene and that these effects require the presence of both functional TGF-β signaling receptors, and either type I and type II receptors alone is not sufficient.

TGF-β1 is arguably the single most potent profibrogenic cytokine in the pathogenesis of fibrosis in progressive kidney diseases. When ectopically expressed in mesangial cells, BAT3 enhanced the extracellular matrix-stimulatory effects of TGF-β1. Expression of full-length BAT3, but not the C-terminal-truncated mutant of BAT3, enhanced TGF-β1-induced type I collagen expression in mesangial cells, whereas knockdown of BAT3 protein expression by siRNA suppressed the expression of type I collagen induced by TGF-β1 in mesangial cells. These findings of BAT3 effects of enhancing TGF-β1 stimulation of type I collagen expression in mesangial cells together with its potentiating effects on TGF-β-responsive reporter gene suggest that BAT3 acts as a positive modulator of TGF-β signaling.

When we analyzed the expression pattern of endogenous BAT3 protein, we observed that the ∼140-kDa protein corresponding to full-length BAT3 protein was detected in all tissues examined including the brain, heart, lung, liver, spleen, and kidney. Furthermore, expression of the ∼140-kDa BAT3 protein was markedly decreased in the tissues from the adult rat compared with corresponding neonatal tissues. Our present data demonstrate that endogenous BAT3 protein expression is developmentally regulated, and we have previously shown that the expression of both TGF-β receptors, TβR-I and TβR-II, are differentially expressed in rat kidney development (32). Thus, there is a possibility for a potential role of BAT3 in modulating TGF-β signaling during development. The relevance of BAT3 in kidney development is further supported by findings reported in the homozygous Scythe knock-out mice. Inactivation of Scythe (the alternative name for BAT3) in the mouse led to embryonic or perinatal lethality associated with pronounced developmental defects in various tissues including the brain and the lung, and in the kidney severe hydropic kidneys and renal agenesis are observed, indicating that Scythe is critical for normal mammalian development (33).

Interestingly, the anti-BAT3 antibody, which we generated against the C-terminal peptide sequence (amino acid residues 1119–1136) of BAT3, also detected lower molecular weight-truncated forms of BAT3 protein in the various tissues we examined. The size of the major mRNA transcript detected in the adult mouse kidney is the same as the mRNA transcript in cultured mesangial cells, which expressed predominantly the full-length BAT3 protein, in contrast to the truncated C-termi-
tion, and implicates an important functional role of this region. Moreover, we observed that TGF-β1 stimulation enhanced nuclear localization of the full-length BAT3 protein, whereas BM810-ΔC remained in the cytosol. Thus, these data suggest that TGF-β1-induced nuclear translocation of BAT3 might be critical for the enhanced stimulation of type I collagen by TGF-β1. In addition, we noted that cells expressing the N-terminal-truncated BAT3 form, BM810, exhibited increased basal expression of type I collagen and was less responsive to the stimulatory effects of TGF-β1 on type I collagen expression. Interestingly, BM810 was almost exclusively localized in the nucleus, and this was largely unaffected by TGF-β1 stimulation, suggesting that the N terminus of BAT3 might negatively regulate the nuclear translocation of BAT3. It is plausible that TGF-β1 stimulation may induce conformational changes of receptor-bound BAT3, which in turn triggers nuclear translocation of BAT3. Once BAT3 is translocated to the nucleus, it can interact with transcription factors and activate transcription. Indeed, BAT3 has been previously shown to interact with transcription factor p300 and regulate p53 acetylation (22).

In summary, we show here that BAT3 is a TGF-β receptor-interacting protein. Endogenous BAT3 protein interacts with both TGF-β receptors type I and type II in renal mesangial cells. Furthermore, BAT3 can enhance TGF-β signaling as assessed by the TGF-β-responsive reporter gene, and these effects require the presence of both functional TGF-β signaling receptors. Expression of full-length BAT3, but not the C-terminal-truncated mutant of BAT3, enhanced TGF-β1-induced type I collagen expression in mesangial cells, whereas knockdown of BAT3 protein by siRNA suppressed the stimulation of type I collagen induced by TGF-β1. Collectively, our findings suggest a new function for BAT3 as a TGF-β receptor-interacting protein in mammalian cells capable of exerting positive regulatory effects on TGF-β signaling.

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