Endofin, a FYVE Domain Protein, Interacts with Smad4 and Facilitates Transforming Growth Factor-β Signaling*

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Transforming growth factor-β (TGF-β) signaling is facilitated by scaffold proteins such as SARA (Smad anchor for receptor activation). Endofin, a member of the FYVE domain protein family, has been suggested to regulate membrane trafficking. In this study, we report that endofin functions as a scaffold protein to facilitate TGF-β signaling. Overexpression of endofin FYVE domain-deletion mutants inhibited TGF-β-induced expression of CAGA-luciferase. Knockdown of endogenous endofin expression by RNA interference specifically led to reduction of the transcriptional responses of TGF-β, but had no effect on BMP- or Wnt1–induced reporter expression. Furthermore, in endofin small interfering RNA-expressing stable cells, TGF-β–mediated expression of plasminogen activator inhibitor-1 and p21Cip1 was significantly reduced, and TGF-β–promoted apoptosis was also impaired. We further showed that endofin could interact with Smad4 and TGF-β type I receptors. Reduction of endogenous endofin expression resulted in a decrease of TGF-β–induced Smad2 phosphorylation and Smad2–Smad4 complex formation. Together, our findings suggest that endofin facilitates TGF-β signaling as a scaffold protein to promote the R-Smad-Smad4 complex formation by bringing Smad4 to the proximity of the receptor complex.

Members of the transforming growth factor (TGF-β)2 superfamily, such as TGF-β, activin, bone morphogenetic protein (BMP), and Nodal, regulate a variety of cellular events including cell growth, differentiation, apoptosis, and migration. They signal through specific cell-surface transmembrane serine/threonine kinase receptors, known as type I and type II receptors. Ligand binding promotes the formation of the receptor complex where the type II receptor phosphorylates the type I receptor. The activated type I receptor in turn phosphorylates the downstream effectors, the receptor-regulated Smads (R-Smads), which then form a complex with Smad4. In the nucleus, the Smad complex regulates the expression of TGF-β–targeted genes (1–6).

Several scaffolding proteins have been suggested to mediate TGF-β signal transduction through their interaction with the receptor complexes and/or the Smads (7). One example is SARA (Smad anchor for receptor activation), a FYVE domain-containing protein that interacts directly with non-activated Smad2/3 and the TGF-β receptors complex, thus forming a bridge between the receptors and R-Smad and assisting the specific phosphorylation of Smad2/3 by the type I receptor (8–10). SARA has also been suggested to target the catalytic subunit of protein phosphatase 1 to receptor complexes and negatively modulate type I receptor activity (11). Interestingly, a recent study showed that SARA is required for targeting to the spindle machinery of a subtype of endosomes containing Dpp and its receptor Tkv to ensure the equal segregation of Dpp signaling to two daughter cells (12). Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) is another FYVE-domain-containing protein and can facilitate Smad2 activation in cooperation with SARA (13), whereas both SARA and Hrs are reported to attenuate TGF-β signaling in T cells (14). The FYVE domain is a motif known to bind phosphatidylinositol 3-phosphate and can specifically recruit the FYVE-domain-containing proteins to early endosomes where they are enriched with phosphatidylinositol 3-phosphate (15). In addition, several other adaptor proteins are also implicated in facilitating TGF-β signaling by linking the receptor complex with R-Smads, Disabled2 (16), Axin (17), TRAP1 and the related protein TLP (18, 19), and Dok-1 (20). These studies collectively suggest that the scaffolding protein–facilitated interaction between TGF-β receptors and Smad proteins is an important regulatory mechanism in TGF-β signaling and it might provide an explanation for the complicated cell context-dependent specificity of TGF-β responses.

Endofin (endosome-associated FYVE-domain protein) is closely related to SARA and shares about 50% identity in the carboxyl-terminal 800-amino acid region related to SARA. It was shown that endofin is targeted to early endosomes by its FYVE domain and colocalized with SARA (21). Overexpression of endofin induced aggregation of endosomes, suggesting that endofin is involved in endosome trafficking regulation (21–23). In this study, we provide evidence that either mislocalization of endofin or knockdown of endofin expression resulted in reduction of transcription responses of TGF-β. Endofin interacts with both the TGF-β type I receptor TβRI/ALK5 and Smad4.
and facilitates TGF-\(\beta\) signaling. Reduction of endogenous endofin expression attenuated TGF-\(\beta\)-induced apoptosis and impaired TGF-\(\beta\)-promoted association of Smad2 and Smad4. Therefore, these results suggest that endofin may act as a scaffold protein to promote the R-Smad-Smad4 heterocomplex formation by bringing Smad4 to the vicinity of the receptor complex.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Establishment of Stable Cell Lines**—HepG2 and Hep3B cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum (Hyclone) and antibiotics in 5% \(\text{CO}_2\) at 37 °C in a humidified atmosphere. HEK293T and HeLa cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and antibiotics. At 48 h after transfection with anti-endofin siRNA expressing plasmids, stable transfectants were selected with 0.3 \(\mu\)g/ml of puromycin (Sigma) for 14 days. Individual clones were picked and amplified with 0.2 \(\mu\)g/ml of puromycin and cells were collected for analysis of endogenous endofin mRNA levels.

**Luciferase Reporter Assay**—Cells were co-transfected with luciferase reporters and the indicated plasmids, as well as *Renilla* luciferase (20 ng) as an internal control. One day after transfection, cells were seeded into 24-well plates and treated with TGF-\(\beta\) (100 pM unless otherwise indicated) in minimal essential medium containing 0.2% fetal bovine serum for 20 h. After washing with phosphate-buffered saline, cells were harvested, and the luciferase activity of cell lysates was determined using a luciferase assay system (Promega) as described by the manufacturer. Total light emission during the initial 20 s of the reaction was measured in a luminometer (Berthold Lumat LB 9501).

**Immunoprecipitation and Immunoblotting**—HEK293T cells and Hep3B cells were transfected with the indicated constructs or empty vector by using the calcium phosphate method or lipofectamine (Roche). At 48 h post-transfection, the cells were lysed. Immunoprecipitation and immunoblotting were performed as described previously (24).

**Immunofluorescence**—Cells were grown on glass coverslips, fixed with 4% paraformaldehyde for 15 min and blocked with 10% bovine serum albumin in phosphate-buffered saline (pH 7.0) for 60 min. The cells were then incubated with primary antibodies for 3 h, followed by fluorescein isothiocyanate (FITC) or rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) for an additional 40 min. The nuclei were counterstained with 4,6-diamidino-2-phenylindole (Sigma). Images were obtained with the confocal Olympus fluorescence 500 microscope.

**Reverse Transcription (RT)-Polymerase Chain Reaction**—At 48 h post-transfection, total RNA was extracted using TRIzol reagent (Invitrogen). RNA was further treated with DNase I according to the manufacturer’s recommendations. Reverse transcription was carried out in 20-\(\mu\)l volumes containing 1.5 \(\mu\)g of total RNA using the reverse transcription system (Promega). The final cDNA volume was diluted to 100 \(\mu\)l and used in PCR amplification for endofin or \(\beta\)-actin, respectively. 1.5–2 \(\mu\)l of cDNA were amplified in a 25-\(\mu\)l reaction volume, containing 0.4 mM dNTPs, 2.5 \(\mu\)l of 10× LA Taq buffer, 1.25 units of Taq DNA polymerase (Takara), and 0.3 pM primers. Following 94 °C incubation for 2 min, 32 cycles of amplification for endofin or 24 cycles of amplification for \(\beta\)-actin was performed at 94 °C for 20 s, 56 °C for 20 s, and 72 °C for 19 s. The endofin primers were sense 5′-AATTGTACAAT-ACCTAGAG-3′ and antisense 5′-CTTTAGAAAGGTA-ACACCTCG-3′; \(\beta\)-actin, sense 5′-CGAGAAGATGACCA-GATC-3′ and antisense 5′-AGCTTCCTTAATGTCGAC-3′; plasminogen activator inhibitor-1, sense 5′-GAGAGG-GAGCTCGGATTC-3′ and antisense 5′-GGCTTCCAAAAGGT-CATTAC-3′; p21, sense 5′-TGTCCGAGAAACC-ATG-3′ and antisense 5′-TGGGAAGGTAGAGCTTGG-3′; Bax, sense 5′-ATGGACGGGTCCGGAAGCAG-3′ and antisense 5′-CATGATGGTCTGAGCTATT-3′; Bcl-x\(\_\)L, sense 5′-CTGGGTTACTACGC- GGGGGCATT-3′ and antisense 5′-TCCAGGCTTACG-GTGTTCA-3′; DAPK1, sense 5′-TCTCTCTCAGCCAAGGGT-GTT-3′ and antisense 5′-GGATGAGATGCTCCTGGTG-3′.

**Real Time PCR Analysis**—In real time PCR, 3 \(\mu\)l of cDNA was amplified in a 25-\(\mu\)l reaction volume, containing 0.4 mM dNTPs, 2.5 \(\mu\)l of 10× LA Taq buffer, 1.25 \(\mu\)l of 20× SYBR Green I buffer (OPE, Shanghai, China), 1.25 units of Taq DNA polymerase (Takara), and 0.4 pm primers. Reaction was performed in Mx3000P™ Real-time PCR system (Stratagene). PCR was performed as for RT-PCR. Each sample was run in triplicate from two biological repeats. Data were analyzed as described previously (25).

**Apoptosis Analysis**—Cells were treated with 200 pm TGF-\(\beta\)1 for 48 h in medium containing 0.2% fetal bovine serum and then collected by trypsinization. Flow cytometric analysis was performed to monitor the green fluorescence of the FITC-conjugated annexin V (530 ± 30 nm) and the red fluorescence of DNA-bound propidium iodide (630 ± 22 nm) with FACS Caliber (BD Biosciences). All data were analyzed with Cell Quest.

**RESULTS**

The FYVE Domain of Endofin Is Important for Its Subcellular Localization and TGF-\(\beta\) Signaling—SARA was suggested to promote TGF-\(\beta\) signaling and mislocalization of SARA affects its ability for regulation of TGF-\(\beta\) signaling. As endofin is closely related to SARA, we attempted to investigate whether endofin plays a role in TGF-\(\beta\) signaling. To this end, we generated a FYVE-domain deletion mutant of endofin and examined its subcellular localization by immunofluorescence. As shown in Fig. 1A, wild-type endofin was distributed in the cytoplasm as punctate spots and co-localized with EEA1, an early endosome marker, and deletion of the FYVE domain (endofin-dFYVE) led to a diffuse distribution in the cytoplasm. These data are consistent with the early report that the FYVE domain is required for the early endosomal localization of endofin (21). Then, we asked whether endofin has any effect on TGF-\(\beta\) signaling with Smad3-responsive reporter CAGA-luciferase (26). Although overexpression of wild-type endofin marginally increased TGF-\(\beta\)-induced expression of the CAGA-luciferase reporter in human hepatoma Hep3B cells, overexpression of endofin-dFYVE effectively suppressed the transcriptional response of
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FIGURE 1. The FYVE domain of endofin is important for its subcellular mislocalization and for TGF-β signaling. A, deletion of the FYVE domain in endofin caused a subcellular mislocalization of endofin. HeLa cells were transiently transfected with Myc-tagged wild-type endofin or the FYVE domain-deleted mutant. At 48 h post-transfection, the cells were subjected to indirect immunofluorescence with rabbit anti-Myc or mouse anti-EEA1 antibodies and FITC anti-mouse or rhodamine anti-rabbit secondary antibodies. The nuclei were counterstained in blue with 4,6-diamidino-2-phenylindole (DAPI). Scale bar, 10 μm. B, mislocalization of endofin attenuated cellular responses to TGF-β mediated expression of CAGA-luciferase. HepG2 cells were cotransfected with CAGA-luciferase (0.5 μg/μl) and Renilla (20 ng) together with endofin, endofin-dFYVE, and empty vector pCMV5 (as a control). Transfected cells were with TGF-β1 (100 pm) for 20 h and harvested for luciferase assay. Each experiment was performed in triplicate and the data represent the mean ± S.D. after normalized to Renilla activity.

TGF-β (Fig. 1B). A similar result was obtained in human hepatoma HepG2 cells (data not shown). These results suggest that the FYVE domain of endofin is critical for its localization in early endosomes and the correct subcellular localization of endofin seems to be important for cellular responses to TGF-β.

Knockdown of Endofin Expression Attenuates Cellular Responses to TGF-β—As overexpression of endofin has a limited effect on the TGF-β-induced expression of CAGA-luciferase in Hep3B and HepG2 cells, it implies that endogenous endofin in these cells might be sufficient for TGF-β signal transduction. To directly test this possibility, we first examined endofin mRNA expression in several cell lines by RT-PCR. As shown in Fig. 2A, ENDOFIN was highly expressed in the cells examined, except A549, where ENDOFIN expression was moderate. To investigate the significance of endogenous endofin in mediating TGF-β signaling, we generated two small hairpin siRNA expression constructs, Sh-1 and Sh-2, to knockdown endofin expression. To examine the efficacy and specificity of these siRNAs, we transiently transfected the empty vector pSUPER, nonspecific siRNA (Sh-NS), anti-endofin siRNA Sh-2 (Hep3B-Sh-2). The quantitative real time PCR analysis revealed a dramatic reduction of endofin expression in Hep3B-Sh-2 cells (Fig. 3A). We next performed reporter assays in Hep3B-Sh-NS and Hep3B-Sh-2 cells to examine their responses to TGF-β. Consistent with the above results, the TGF-β-induced expression of CAGA-luciferase in Hep3B-Sh-2 cells was much lower than in Hep3B-Sh-NS cells (Fig. 3B), indicating that endogenous endofin plays an important role in mediating effective TGF-β signal transduction.

Endofin Mediates TGF-β Signaling in the Physiological Condition—To further confirm the regulatory role of endofin in TGF-β signaling, we established cell lines derived from Hep3B cells that stably expressed nonspecific siRNA Sh-NS (Hep3B-Sh-NS) or anti-endofin siRNA Sh-2 (Hep3B-Sh-2). The quantitative real time PCR analysis revealed a dramatic reduction of endofin expression in Hep3B-Sh-2 cells (Fig. 3A). We next performed reporter assays in Hep3B-Sh-NS and Hep3B-Sh-2 cells to examine their responses to TGF-β. Consistent with the above results, the TGF-β-induced expression of CAGA-luciferase in Hep3B-Sh-2 cells was much lower than in Hep3B-Sh-NS cells (Fig. 3B), indicating that endogenous endofin plays an important role in mediating effective TGF-β signal transduction.

We next investigated whether endofin is important for the expression of TGF-β target genes by quantitative real time PCR. Both plasminogen activator inhibitor-1 and p21Cip1 are well known TGF-β targets (28, 29). Reduction of endofin expression apparently attenuated TGF-β-induced expression of plasminogen activator inhibitor-1 and p21Cip1 in Hep3B-Sh-2 cells (Fig. 3C), supporting the importance of endofin in TGF-β-activated transcription.

To study the role of endofin in a more physiologically relevant condition, we compared TGF-β-induced apoptosis in these two cell lines. After cells were treated with 200 pm TGF-β for 48 h, the cells were harvested for apoptosis assay. As shown in Fig. 3D, TGF-β treatment resulted in about 40% cells undergoing apoptosis in Hep3B-Sh-2, significantly lower than the ones in Hep3B-Sh-NS (50%). Furthermore, about one-half of the apoptotic cells in Hep3B-Sh-2 were in the early stage of

effect on Wnt-mediated lymphoid enhancer factor-1-luciferase expression (Fig. 2H).

Therefore, Sh-1 and Sh-2 siRNA can reduce endofin expression, and reduction of endogenous endofin expression leads to decreased cellular responses to TGF-β but has no effect on BMP or Wnt signaling, suggesting that endofin specifically modulates TGF-β signaling.

A similar result was obtained in HepG2 cells (Fig. 2E). Furthermore, knockdown of endofin expression by Sh-2 also decreased the TGF-β-induced expression of the Smad2-responsive reporter ARE-luciferase (Fig. 2F).
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Endofin expression was induced in Hep3B-Sh-NS and Hep3B-Sh-2 cells (Fig. 3E). Endofin Forms Complexes with Smad4 and TβRI—To elucidate the mechanism whereby endofin modulates TGF-β signaling, we tested whether endofin associates with components of the TGF-β pathway by immunoprecipitation and immunoblotting. Myc-endofin was co-transfected either alone or together with FLAG-Smads into HEK293T cells. Cell lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-Myc antibody. Endofin was found to interact with Smad4 only (Fig. 4A, top panel). As a control, SARA specifically bound to Smad2. Similar results were obtained in a reverse immunoprecipitation-immunoblotting experiment (second panel). Furthermore, when anti-Smad4 antibody was used for immunoblotting of endogenous immunoprecipitants, Smad4 was detected regardless of whether exogenous Smad4 was expressed (Fig. 4B), indicating that overexpressed endofin could also interact with endogenous Smad4.

SARA interacts with Smad2 via its Smad-binding domain (SBD) in the middle of its sequence (8, 9). Sequence alignment revealed that endofin contains a less-conserved SBD region (data not shown). To investigate whether this putative SBD is involved in Smad4 binding, we co-expressed Myc-endofin (wild-type or dSBD lacking the putative SBD) together with FLAG-Smad4. It was reported that SARA associates with the TβRI receptor via its carboxy-terminal endodomain (8). Because endofin shares a high similarity with SARA in their carboxy-terminal regions, we explored whether endofin interacts with TβRI receptors. HEK293T cells were transfected with FLAG-endofin either alone or together with HA-tagged type I receptors. Cell lysates were immunoprecipitated with an anti-FLAG antibody and laid-blotted with an anti-HA antibody. The results show that endofin strongly interacted with TβRI, BMP receptors BMPRIA and BMPRIB, activin receptor ActRIB, and TGF-β receptor ALK1, but not to ALK2 (Fig. 4D). Similar results were obtained in a reverse immunoprecipitation-immunoblotting
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![Graphs and images]

FIGURE 3. Endofin siRNA-expressing stable Hep3B cells exhibit impaired responses to TGF-β stimulation. A, the endogenous ENDOFIN mRNA levels in Hep3B-Sh-2 cells were analyzed by real time PCR analysis. The relative endofin mRNA level was normalized to β-ACTIN. B, Hep3B-Sh-NS and Hep3B-Sh-2 cells were transfected with CAGA-luciferase (0.5 μg/ml) and Renilla (20 ng) for 48 h. Then the cells were treated with TGF-β1 (100 pM) for 20 h and harvested for luciferase analysis. The experiment was performed in triplicate and the data represent the mean ± S.D. C, Hep3B-Sh-NS and Hep3B-Sh-2 cells were transfected with TGF-β1 (100 pM) for 20 h. Total RNA was isolated and subjected to real time PCR to determine the expression of plasminogen activator inhibitor-1 (PAI-1) or p21. The -fold change in mRNA level of genes was normalized to β-actin. D, Hep3B-Sh-NS and Hep3B-Sh-2 cells were treated with TGF-β1 (200 pm) for 48 h and subjected to apoptosis analysis. The cells positive for annexin V-FITC (low right square) or positive for both annexin V-FITC and propidium iodide (top right square) were presented as histograms (right panel). E, Hep3B-Sh-NS and Hep3B-Sh-2 cells were treated with TGF-β1 (100 pm) for 24 h. Total RNA was isolated and subjected to real time PCR to determine the expression of Bcl-XL, BIM, BAX, or DAPK1. The -fold change in mRNA level of genes was normalized to β-actin. All quantitative data were from three independent experiments and expressed as mean ± S.D. The asterisk (*) indicates a statistically significant difference between Hep3B-Sh-2 and Hep3B-Sh-NS cells (p < 0.05).

experiment (Fig. 4E). To further examine whether the carboxyl-terminal region is important for endofin to associate with receptors, the amino-terminal region (1–886) of endofin was tested for interaction with receptors. As shown in Fig. 4F, no apparent interaction was detected between endofin-(1–886) and TβRI or BMPRIB.

Endofin Facilitates Smad Complex Formation—As endofin interacts with TβRI, we then asked whether it is involved in Smad2 activation. To this end, we examined whether endofin knockdown affects TGF-β-induced Smad2 phosphorylation. Hep3B-Sh-NS and Hep3B-Sh-2 cells were treated with different concentrations of TGF-β1, and cell lysates were immunoblotted with anti-phosphorylated Smad2 antibody. Knockdown of endofin expression by Sh-2 significantly impaired Smad2 phosphorylation when the cells were treated with 100 or 200 pm TGF-β1 although this effect is less obvious in the presence of 300 pm TGF-β1 (Fig. 5A). Reduced Smad2 phosphorylation was also observed in Hep3B-Sh-2 cells treated with TGF-β1 for various times (data not shown).

The above data that endofin can associate with both Smad4 and TβRI receptor suggests that endofin might bring Smad4 to the vicinity of the TGF-β receptor complex to facilitate R-Smad-Smad4 complex formation. To test this hypothesis, we examined the TGF-β-induced formation of endogenous Smad2-Smad4 complex in Hep3B-Sh-2 cells. Cells were harvested for anti-Smad2 immunoprecipitation and anti-Smad4 immunoblotting after a 1-h TGF-β treatment. As shown in Fig. 5B, TGF-β treatment led to the association of Smad2 with Smad4 in Hep3B-Sh-NS cells, whereas Smad complex formation was hampered in Hep3B-Sh-2 cells. These data indicate that endofin plays an important role in promoting Smad heterocomplex formation.

DISCUSSION

The essential components to transduce TGF-β signaling are relatively limited and only the receptors and Smad proteins are required for the canonical TGF-β/Smad pathway. However, this pathway is known to be modulated by many other proteins. Several scaffold or adaptor proteins have been suggested to facilitate Smad activation by the receptors. In this study, we characterized the FYVE domain-containing protein endofin in regulation of TGF-β signal transduction. Our data showed that deletion of the phosphatidylinositol 3-phosphate-binding FYVE domain in endofin caused its subcellular mislocalization from early endosomes to a diffuse distribution in the cytoplasm and reduced the TGF-β-induced...
expression of CAGA-luciferase and apoptosis. Furthermore, we showed that endofin interacted with TβRI and Smad4 and interference of endogenous endofin expression impaired Smad2 phosphorylation and Smad2-Smad4 complex formation. Our findings suggest that endofin might facilitate TGF-β signaling through recruiting Smad4 to the TGF-β receptor complex and thus assisting the association between Smad4 and the receptor-activated Smad2.

Clathrin-mediated receptor endocytosis has been suggested to contribute to Smad-mediated signal transduction of TGF-β family members (34–37) although it may be not essential (38–40). Like SARA, endofin is localized in early
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Although endofin does not interact with Smad2/3, knockdown of its expression mitigated TGF-β-induced Smad2 phosphorylation. This could be because endofin and SARA form a heterocomplex and together they coordinate R-Smad activation. Indeed, our immunoprecipitation-immunoblotting assay revealed that endofin is able to interact with SARA (data now shown). Endofin was implicated in regulation of membrane trafficking by recruiting TOM1 and clathrin to endosomes (22, 23). It waits to be determined whether endofin controls the activity or turnover of TGF-β receptors and other cell surface receptors.

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