The disintegrin and metalloproteinase ADAM12 contributes to TGF-β signaling through interaction with the type II receptor

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Transforming growth factor-β (TGF-β) regulates a wide variety of biological processes through two types of Ser/Thr transmembrane receptors: the TGF-β type I receptor and the TGF-β type II receptor (TβRII). Upon ligand binding, TGF-β type I receptor activated by TβRII propagates signals to Smad proteins, which mediate the activation of TGF-β target genes. In this study, we identify ADAM12 (a disintegrin and metalloproteinase 12) as a component of the TGF-β signaling pathway that acts through association with TβRII. We found that ADAM12 functions by a mechanism independent of its protease activity to facilitate the activation of TGF-β signaling, including the phosphorylation of Smad2, association of Smad2 with Smad4, and transcriptional activation. Furthermore, ADAM12 induces the accumulation of TβRII in early endosomal vesicles and stabilizes the TβRII protein presumably by suppressing the association of TβRII with Smad7. These results define ADAM12 as a new partner of TβRII that facilitates its trafficking to early endosomes in which activation of the Smad pathway is initiated.

Introduction

TGF-β superfamily members are multifunctional cytokines that regulate a broad range of cellular functions, including cell proliferation, differentiation, and apoptosis (Massague et al., 2000; Derynck et al., 2001). TGF-β signals through a heteromeric complex of two types of transmembrane Ser/Thr kinases: TGF-β type I receptor and TGF-β type II receptor (TβRII). TGF-β binding to TβRII induces the recruitment and phosphorylation of TGF-β type I receptor, which, in turn, phosphorylates the receptor-regulated Smads (R-Smads) Smad2 and Smad3. Once phosphorylated, Smad2 and Smad3 associate with the common partner Smad, Smad4, and translocate to the nucleus, where they regulate the expression of TGF-β target genes. In contrast to R-Smads and Smad4, the inhibitory Smad, Smad7, appears to block signal transduction by preventing access of R-Smads to the TGF-β receptor or by recruiting distinct E3 ubiquitin ligases that target the receptor–Smad7 complex for degradation (Kavsak et al., 2000; Ebisawa et al., 2001; Seo et al., 2004).

Upon TGF-β stimulation, Smad2 is recruited to the receptor complex by an adaptor molecule called Smad anchor for receptor activation (SARA). At steady state, SARA-bound Smad2 is localized in early endosomes to which the receptor is internalized via clathrin-coated pits (Hayes et al., 2002; Di Guglielmo et al., 2003). The importance of the clathrin-mediated endocytic pathway in TGF-β signaling is also manifested by the recent finding that cPML (cytoplasmic form of the promyelocytic leukemia protein) mediates TGF-β signaling by facilitating recruitment of the SARA–Smad2 complex and TGF-β receptors to early endosomes (Lin et al., 2004).

In addition to clathrin, TGF-β receptors can also associate with caveolin (Razani et al., 2001), which leads to their internalization into caveolin1-positive vesicles with subsequent degradation through the proteasome pathway. Consistent with this notion, the caveolin1-positive vesicles were found to associate with Smad7 (Ito et al., 2004), which is known to mediate the association of the E3 ligases Smurf1 and Smurf2 to receptors, leading to their degradation.

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Abbreviations used in this paper: ADAM, a disintegrin and metalloproteinase; EEA1, early endosomal antigen 1; HSC, hepatic stellate cell; PAI-1, plasminogen activator inhibitor-1; RD, Rhabdomyosarcoma; SARA, Smad anchor for receptor activation; shRNA, short hairpin RNA; TβRII, TGF-β type II receptor.

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To gain more insight into the regulation of TGF-β signaling, we have performed yeast two-hybrid screens using TβRII as bait. ADAM12 (a disintegrin and metalloproteinase 12) was one of the TβRII interactors that exhibited specific and strong binding to TβRII. ADAM12 belongs to the ADAMs family, which are glycoproteins characterized by a multidomain structure comprised of pro-, metalloproteinase, disintegrin, cysteine-rich, transmembrane, and cytoplasmic domains (Primakoff and Myles, 2000; Seals and Courtneidge, 2003). ADAMs exhibit proteolytic, cell adhesion, and signaling properties, and perturbations of ADAM expression are associated with several human diseases, including cancers (Duffy et al., 2003). In the present study, we provide the first evidence that ADAM12 interacts with TβRII and enhances TGF-β signaling by controlling the localization of TGF-β receptors to early endosomes. These results reveal a new role for ADAM12 in the regulation of TGF-β receptor trafficking.

Results and discussion

Using the extracellular domain of human TβRII as bait, we performed a yeast two-hybrid screen of a human placental cDNA library. Eight different fragments of ADAM12 were found to interact with TβRII (Fig. 1 A). Two variants were previously described for ADAM12: a transmembrane glycoprotein (Yagami-Hiromasa et al., 1995) and a shorter secreted form (Gilpin et al., 1998). The common sequences shared by the overlapping fragments of the prey span the metalloproteinase and disintegrin domains common to the two variants (Fig. 1 A).

To confirm the association of ADAM12 with TβRII, a fragment of ADAM12 isolated in the yeast two-hybrid screen (amino acids 142–739 that include the metalloproteinase and cysteine-rich domains; Fig. 1 A) was tagged with Flag and cotransfected into 293 cells alone or in combination with HA-TβRII. Immunoprecipitation with anti-Flag followed by immunoblotting with anti-HA revealed that TβRII can interact with ADAM12, and this interaction was not affected by TGF-β (Fig. 1 B). To provide further evidence that ADAM12 interacts with TβRII, we examined their colocalization by immunofluorescence. As expected, TβRII is localized predominantly in patched areas near the cell surface. Interestingly, we found that ADAM12 extensively colocalized with TβRII, confirming their interaction (Fig. 1 C).

To examine whether the association of ADAM12 with TβRII can occur under physiological conditions, we used hepatic stellate cells (HSCs), Rhabdomyosarcoma (RD), and C2C12 cells, three cell lines that were previously described to express detectable ADAM12 (Gilpin et al., 1998; Galliano et al., 2000; Le Pabic et al., 2003). In immunoprecipitates prepared with preimmune antisera, no TβRII was coprecipitated. However, in the anti-ADAM12 immunoprecipitates, we could clearly detect TβRII coprecipitating with ADAM12 (Fig. 1 D). Formation of the endogenous ADAM12–TβRII complex was also

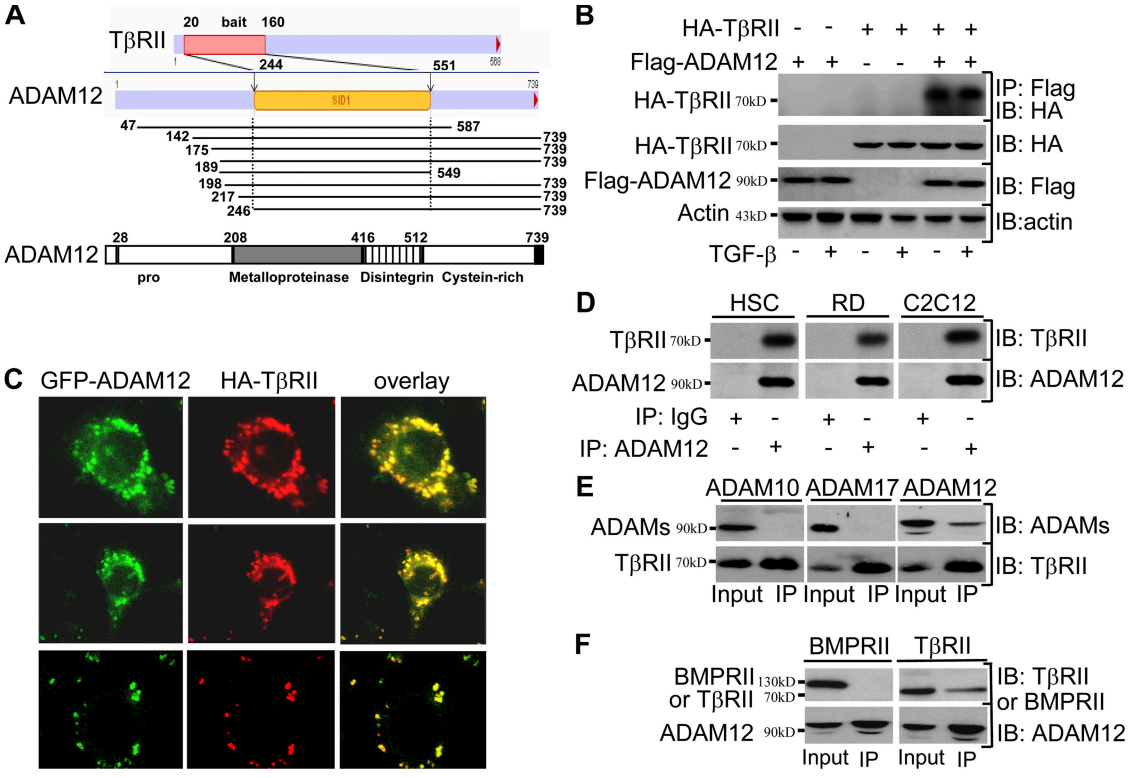

Figure 1. **TβRII interacts with ADAM12.** (A) Schematic diagram of ADAM12 fragments that interact with TβRII in two-hybrid assays. (B) 293T cells were transfected with HA-TβRII in the presence or absence of Flag-ADAM12. Cell lysates were subjected to anti-Flag immunoprecipitation (IP) followed by immunoblotting (IB) with anti-HA. In this and all of the following experiments, the expression of proteins was determined by direct immunoblotting. (C) C2C12 cells transfected with GFP-ADAM12 and HA-TβRII were immunostained with anti-HA followed by TRITC-conjugated secondary IgG. The panels represent three representative fields. (D–F) Cell extracts from HSC, RD, and C2C12 cells (D) or C2C12 cells (E and F) were immunoprecipitated with anti-ADAM12 (D and F) or anti-TβRII (E) and immunoblotted with the indicated antibodies.

demonstrated by anti-ADAM12 immunoblotting of anti-TβRII immunoprecipitates (Fig. 1 E). The interaction of ADAM12 with TβRII is specific because we were unable to detect an interaction between TβRII and ADAM10 or ADAM17 (Fig. 1 E), which share the structure organization with ADAM12. Similarly, we were unable to see an interaction between ADAM12 and the bone morphogenetic protein type II receptor (Fig. 1 F).

To explore the functional significance of the interaction between ADAM12 and TβRII, we investigated whether the expression of ADAM12 may influence TGF-β–mediated transcriptional esponses. For this, we made use of the TGF-β/Smad2-responsive reporter ARE_3-Lux (Labbe et al., 1998) and found that the expression of ADAM12 resulted in an approximately fivefold increase in TGF-β–induced transcription (Fig. 2 A). A similar effect of ADAM12 was observed with the TGF-β/Smad3-responsive reporter $CAGA_9$-Lux (approximately threefold in Fig. 2 B and sixfold in Fig. 2 C; Zawel et al., 1998).

Next, we attempted to confirm the role of ADAM12 in enhancing TGF-β signaling by investigating its effect on the expression of endogenous plasminogen activator inhibitor-1 (PAI-1), which contains CAGA boxes in the promoter. The results showed that the TGF-β–dependent expression of PAI-1 was increased by the expression of ADAM12 (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200612046/DC1). During the course of these analyses, we also investigated the role of endogenous ADAM12 in enhancing the transcriptional activation of collagen I (COL1A2) by TGF-β. For this, HSC cells were treated by ADAM12 antisense oligonucleotides before TGF-β stimulation, and the expression of ADAM12 or COL1A2 was analyzed. As we recently reported (Le Pabic et al., 2003), TGF-β treatment induces an accumulation of ADAM12 mRNA and protein, and this increase was reduced to the background level by ADAM12 antisense. Similarly, treatment of cells with antisense to ADAM12 attenuated the TGF-β–dependent induction of COL1A2 mRNA (Fig. S1 B). To confirm these results, we depleted HSC, RD, and C2C12 cells from ADAM12 by RNAi. When ADAM12 was targeted in these cells using a specific short hairpin RNA (shRNA), both the steady-state levels and the TGF-β–dependent accumulation of ADAM12 were reduced. Interestingly, the knockdown of ADAM12 resulted in a decrease in the TGF-β–induced expression of PAI-1 (Fig. 2 D). A similar result was obtained with JunB (Fig. 2 D), the expression of which

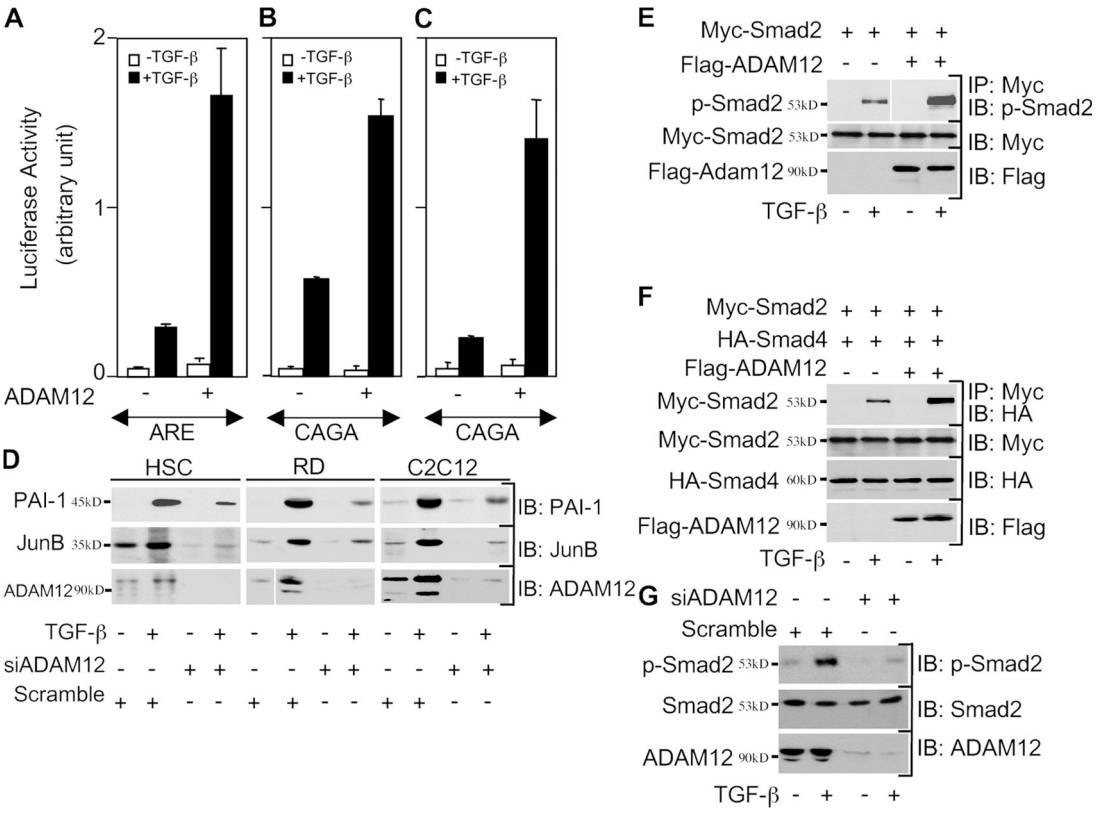

Figure 2. **ADAM12 increases TGF-β signaling.** (A–C) HepG2 (A and B) and C2C12 (C) cells transfected with ARE_3-Lux together with FAST1 (A) or $CAGA_9$-Lux (B and C) in the presence or absence of ADAM12 were treated with or without TGF-β. In these and all of the following reporter assays, luciferase activity was determined and normalized, and results are expressed as means ± SD (error bars) of triplicates from five independent experiments. (D) HSC, RD, or C2C12 cells were transfected with the indicated combinations of pEGFP, ADAM12 shRNA, or scrambled shRNA. 36 h later, GFP-transfected cells were sorted by FACS and exposed to TGF-β for 16 h. The expression of endogenous PAI-1, JunB, and ADAM12 was assessed by direct immunoblotting (IB). (E and F) 293T cells were transfected with myc-Smad2, Flag-ADAM12, and HA-Smad4 as indicated. For Smad2 phosphorylation (E), cell lysates were immunoblotted with antiphospho-Smad2. For the association of Smad2 with Smad4 (F), cell lysates were immunoprecipitated (IP) with an anti-myc before immunoblotting with anti-HA. (G) C2C12 cells were transfected with the indicated combinations of pEGFP, ADAM12 shRNA, or scrambled shRNA. 36 h later, GFP-transfected cells were sorted by FACS and exposed to TGF-β for 30 min. The phosphorylation of endogenous Smad2 was assessed by immunoblotting with antiphospho-Smad2. White lines indicate that intervening lanes have been spliced out.

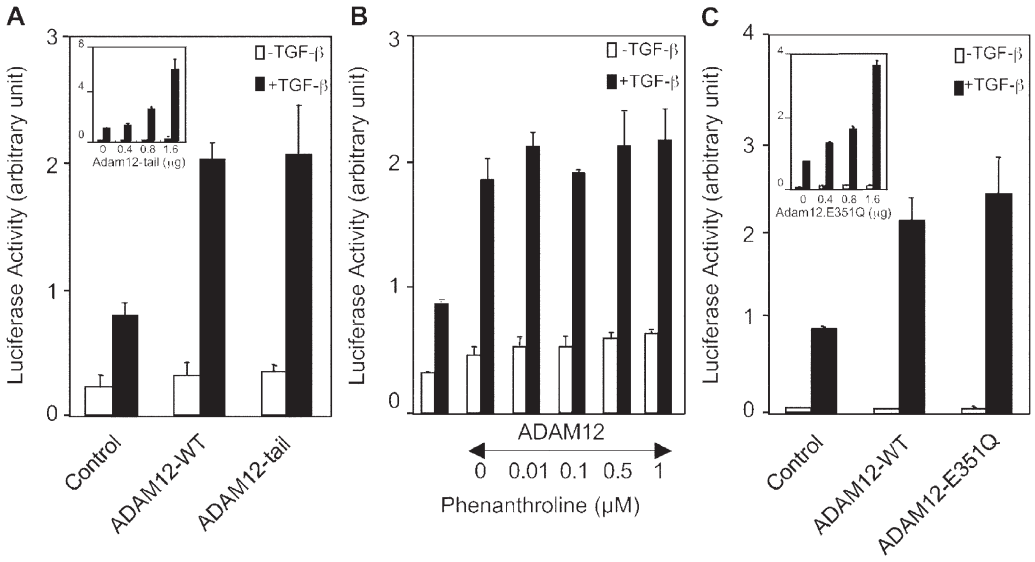

Figure 3. **Up-regulation of TGF-β signaling by ADAM12 does not involve its cytoplasmic domain or its protease activity.** (A–C) HepG2 cells were transfected with $CAGA_9$-Lux, ADAM12, ADAM12-tail, and ADAM12.E351Q as indicated. For A and C, cells were treated with or without TGF-β for 16 h before lysis. Dose effects are shown in insets. For B, cells were treated with 1.10 phenanthroline in the presence or absence of TGF-β. In all cases, luciferase activity was determined. Error bars represent SD. WT, wild type.

is up-regulated by TGF-β through a mechanism similar to that of PAI-1.

To investigate the mechanism underlying the effects of ADAM12 on TGF-β signaling, we investigated whether the expression of ADAM12 may regulate the TGF-β–dependent phosphorylation of Smad2. We observed that exposure of cells to TGF-β resulted in increased Smad2 phosphorylation, and this effect was further enhanced by the expression of ADAM12 (Fig. 2 E). Consistent with this, the expression of ADAM12 enhanced the ability of TGF-β to induce assembly of the Smad2–Smad4 complex (Fig. 2 F). In addition, the depletion of endogenous ADAM12 by RNAi suppressed Smad2 phosphorylation (Fig. 2 G). Collectively, these data suggest that ADAM12 may function to enhance TGF-β signaling by facilitating Smad2 phosphorylation and its subsequent heterodimerization with Smad4.

At least six members of the ADAM family have been demonstrated to have proteolytic activity, including ADAM12 (Loechel et al., 2000; Shi et al., 2000). In initial experiments, we found that a truncated form of ADAM12 (ADAM12-tail), which lacks the cytoplasmic domain, retains its ability to enhance TGF-β signaling (Fig. 3 A). Therefore, we sought to investigate whether the increase in TGF-β transcriptional activity mediated by ADAM12 may involve its catalytic activity. To approach this question, we investigated the effect of phenanthroline, a specific metalloproteinase inhibitor, on the ability of ADAM12 to enhance TGF-β transcriptional responses. Surprisingly, exposure of cells to phenanthroline failed to suppress the effect of ADAM12 on TGF-β–induced $CAGA_9$-Lux (Fig. 3 B). In another approach, we used ADAM12-E351Q, a protease inactive mutant. As shown in Fig. 3 C, the expression of ADAM12-E351Q enhanced TGF-β–induced transcription with an activity similar to that of wild-type ADAM12. Together, these results indicate that ADAM12 enhances TGF-β signaling through a protease-independent mechanism.

During our immunofluorescence analyses, we observed that ADAM12 and TβRII are colocalized predominantly in patched areas near the cell surface in C2C12 cells, but a substantial fraction of both proteins can also colocalize in endosome vesicle-like structures (Fig. 1 C). This pattern of colocalization of ADAM12 and TβRII in the two compartments was also evident in Mv1Lu cells (Fig. 4 A), but their distribution is more pronounced in endosomal vesicles when compared with C2C12 cells (Fig. 1 C). Based on the findings that TβRII colocalizes with early endosomal antigen 1 (EEA1), a marker of early endosomes (Di Guglielmo et al., 2003), we sought to investigate whether ADAM12 colocalizes with TβRII in the EEA1-enriched compartment using Mv1Lu cells that exhibit extensive staining of these proteins in early endosomes (Fig. 4 A; Di Guglielmo et al., 2003). As for TβRII, there is some colocalization of ADAM12 with EEA1 in Mv1Lu cells (Fig. 4 A and Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200612046/DC1), suggesting that ADAM12 may accumulate in early endosomes to which TβRII is internalized via clathrin-coated pits.

To examine whether the localization of ADAM12 in early endosomes plays a role in TGF-β signaling, we examined the effect of inhibition of clathrin-mediated endocytosis by potassium depletion, which was reported to prevent endosome-dependent TGF-β signaling (Di Guglielmo et al., 2003). As shown in Fig. 4 B, potassium depletion decreased the ability of ADAM12 to enhance TGF-β–induced transcription. Potassium depletion also decreased TGF-β signaling in the absence of transfected ADAM12, but this effect seems to depend on ADAM12 because it was lost in cells depleted from endogenous ADAM12 by RNAi. In a control experiment, we found that potassium depletion can further decrease TGF-β–induced transcription in cells depleted from Smad3 (Fig. 4 C), supporting the hypothesis that potassium depletion may inhibit TGF-β signaling by specifically interfering with ADAM12 function. To provide further

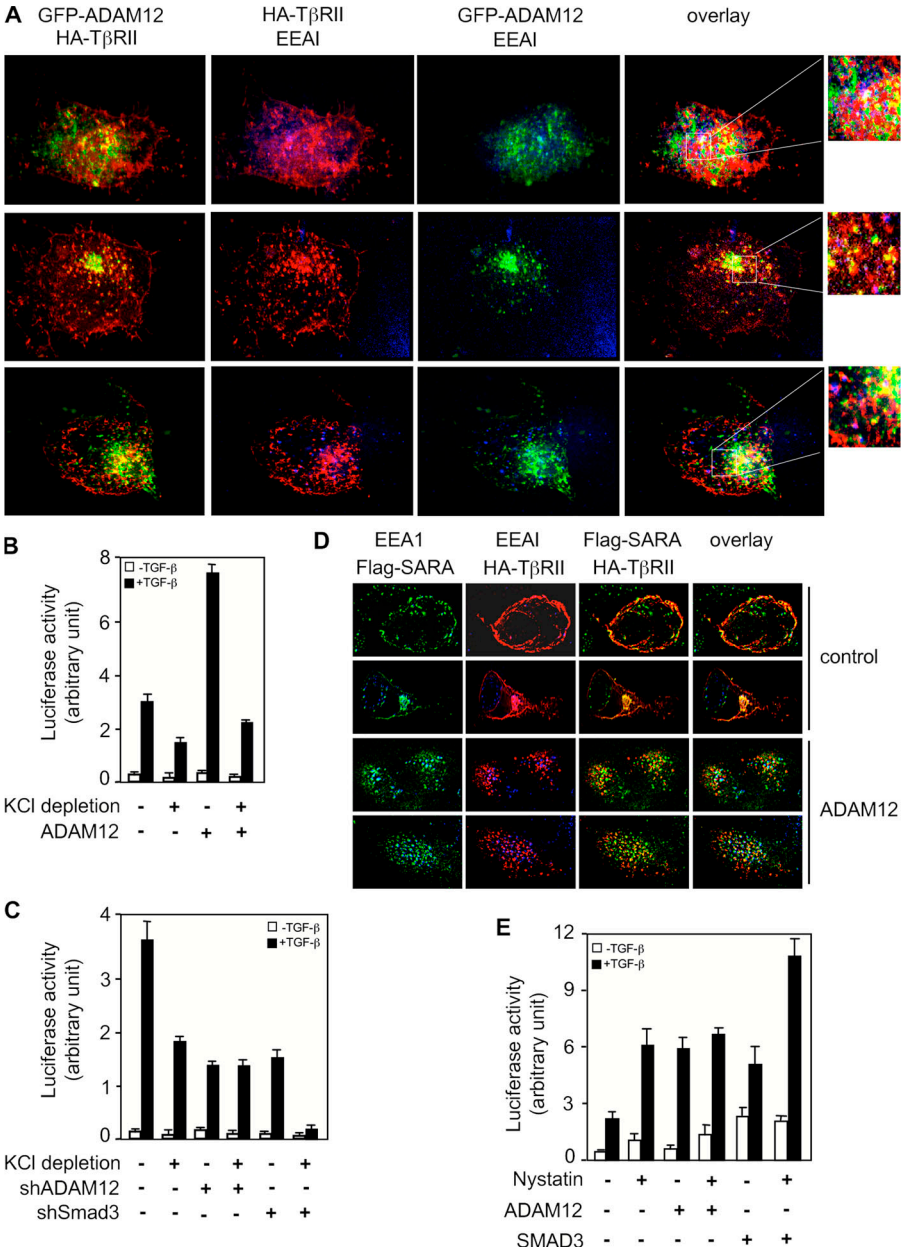

Figure 4. **ADAM12 facilitates clathrin-dependent TGF-β signaling.** (A) MvLu1 cells transfected with GFP-ADAM12 and HA-TβRII were immunostained with rabbit anti-HA and mouse anti-EEA1 followed by secondary staining with TRITC-conjugated anti–rabbit and Cy5-conjugated anti–mouse IgG. The subcellular localization of GFP-ADAM12 (green), TβRII (red), and EEA1 (blue) was analyzed by a confocal microscope. Colocalization of EEA1 with ADAM12, ADAM12 with TβRII, and EEA1 with TβRII appears as turquoise, yellow, and purple, respectively. The panels represent three independent representative fields. Representative areas from cells that display TβRII and ADAM12 in the EEA1 compartments are enlarged in the insets. (B and C) HepG2 cells were transfected with the indicated combinations of $CAGA_9$-Lux, ADAM12 shRNA, ADAM12, Smad3 shRNA, or scrambled shRNA. Cells were potassium depleted before stimulation with TGF-β, and luciferase activity was examined. (D) COS7 cells were transfected with HA-TβRII and Flag-SARA with or without ADAM12. Cells were immunostained with rabbit anti-HA and mouse anti-EEA1 followed by secondary staining with TRITC-conjugated anti–rabbit and Cy5-conjugated anti–mouse IgG. For the detection of Flag-SARA, cells were incubated with FITC-conjugated anti-Flag. Flag-SARA, TβRII, and EEA1 appear as green, red, and blue, respectively. (E) HepG2 cells transfected with $CAGA_9$-Lux and the indicated expression vectors were treated with nystatin at 10 μM for 1 h before stimulation with TGF-β, and luciferase activity was examined. Error bars represent SD.

evidence that ADAM12 functions in TGF-β signaling by facilitating the trafficking of TβRII to early endosomes, we examined the localization of SARA, which has been shown to interact with TβRII at the plasma membrane and in EEA1-positive early endosomes (Hayes et al., 2002; Itoh et al., 2002). We observed that the expression of ADAM12 caused the redistribution of the TβRII–SARA complexes from the plasma membrane into early endosomes (Fig. 4 D). This effect is likely to be direct because the expression of ADAM12 had no effect on the association of TβRII with several transmembrane proteins that could potentially prevent or enhance its trafficking (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200612046/DC1).

To provide further evidence that ADAM12 facilitates the localization of TβRII in early endosomes, we tested the effect of nystatin, a sterol-binding antibiotic that is known to induce the redistribution of TGF-β receptors into EEA1-positive endosomes by affecting the raft structures (Di Guglielmo et al., 2003). We reasoned that if we induce the majority of TβRII to accumulate in early endosomes by an alternative approach, such as the treatment of cells with nystatin, ADAM12 should have no further effect on TGF-β–mediated transcription. As shown in Fig. 4 E, exposure of cells to nystatin caused a considerable increase in the TGF-β–mediated activation of $CAGA_9$-Lux, and this increase was not affected by the expression of ADAM12. Under these experimental conditions, the expression of Smad3 can synergize with nystatin to enhance TGF-β–induced transcription, arguing against the possibility that the lack of ADAM12 effect is caused by the ability of nystatin to elicit the maximum threshold level of TGF-β signaling in this cell system. Collectively, these results suggest that ADAM12 may function as an important component in TGF-β signaling by modulating the trafficking of the TGF-β receptor.

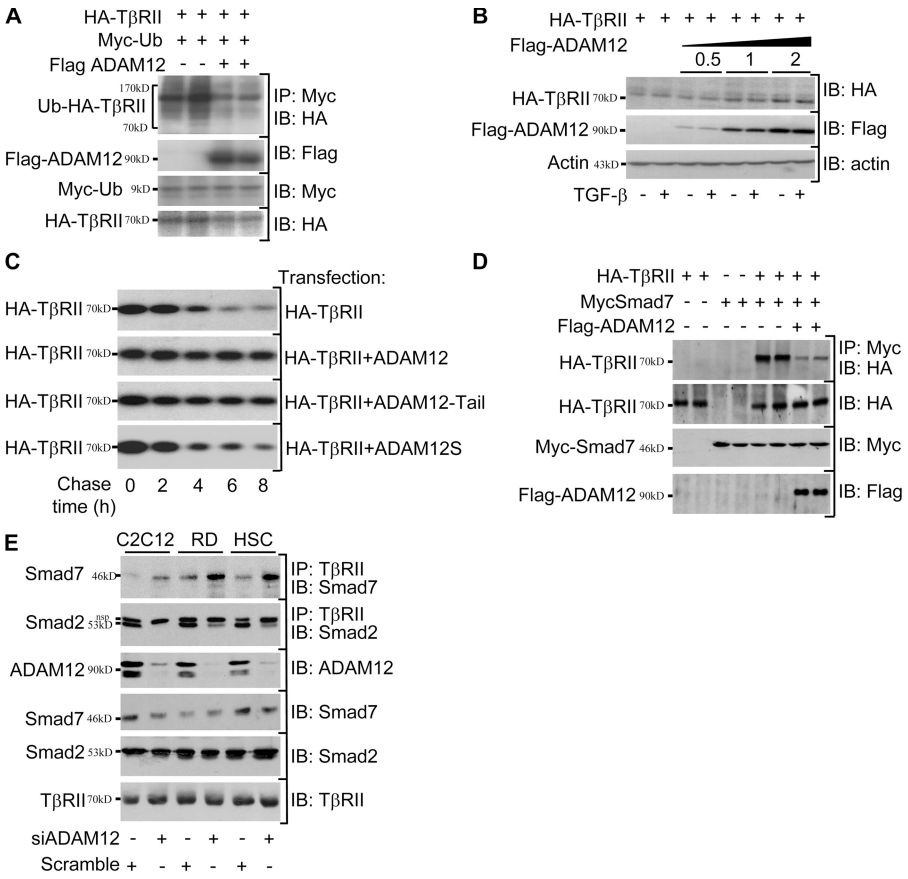

Figure 5. **ADAM12 prevents TβRII degradation.** (A) Cells were transfected with HA-TβRII and myc-Ub in the presence or absence of ADAM12. Cell lysates were normalized on the basis of TβRII expression, immunoprecipitated (two samples for each condition) with anti-myc, and immunoblotted with anti-HA. (B) 293T cells were transfected with HA-TβRII and increasing amounts of Flag-ADAM12. Cells were treated with TGF-β for 18 h before lysis, and cell lysates were immunoblotted with anti-HA, anti-Flag, or antiactin. (C) 293T cells transfected with HA-TβRII and the indicated expression vectors were pulse-chased with [^{35}S]Met/Cys, and labeled HA-TβRII was immunoprecipitated and analyzed by SDS-PAGE and autoradiography. (D) Cells were transfected with HA-TβRII, myc-Smad7, and Flag-ADAM12 as indicated. Cell lysates were normalized on the basis of TβRII expression, immunoprecipitated (two samples for each condition) with anti-myc, and immunoblotted with anti-HA. (E) C2C12 cells were transfected with the indicated combinations of pEGFP, ADAM12 shRNA, or scrambled shRNA. 36 h later, GFP-transfected cells were sorted by FACS, lysed, and normalized on the basis of TβRII expression. Then, the association of TβRII with Smad7 or Smad2 was analyzed by immunoprecipitation (IP)/immunoblotting (IB).

The clathrin-dependent internalization into early endosomes promotes TGF-β signaling, whereas the lipid raft–caveolar internalization pathway is required for receptor turnover. To obtain direct evidence that the accumulation of ADAM12 in early endosomes plays a role in the up-regulation of TGF-β signaling, we examine whether the expression of ADAM12 interferes with TβRII degradation. To approach this question, we first investigated the effect of ADAM12 on TβRII ubiquitination. We observed that the coexpression of ADAM12 resulted in a substantial decrease in the ubiquitination of TβRII (Fig. 5 A). In support of this result, the expression of ADAM12 increased the steady-state levels of TβRII (Fig. 5 B). Furthermore, in pulse-chase experiments, the expression of ADAM12 resulted in a marked decrease in the turnover of TβRII (Fig. 5 C). A similar result was obtained with the cytoplasmic truncated form ADAM12-tail, which, like the wild-type counterpart, can enhance TGF-β signaling (Fig. 5 C). As a control, we found that expression of the extracellular soluble form of ADAM12 failed to stabilize TβRII (Fig. 5 C), providing support to the theory that ADAM12 may stabilize TβRII by facilitating its intracellular redistribution from the plasma membrane to early endosomes.

In contrast to clathrin-enriched vesicles, TβRII enriched in caveolin1-positive vesicles was found to associate with Smad7, which is known to mediate the association of Smurf1/2 to receptors, leading to their degradation. To confirm that ADAM12 can interfere with the ubiquitin-dependent degradation of TβRII, we examined its effect on the association of TβRII with Smad7. We observed that the expression of ADAM12 induced a reduced assembly of the TβRII–Smad7 complex (Fig. 5 D). Further evidence that ADAM12 can modulate the interaction of Smad7 with TβRII was obtained by experiments showing a considerable increase in accumulation of the endogenous Smad7–TβRII complex in cells depleted from endogenous ADAM12 (Fig. 5 E). As Smad7 can restrict the access of Smad2 to TGF-β receptor, we also investigated whether endogenous ADAM12 regulates the association of endogenous Smad2 with endogenous TβRII. In fact, we found that the depletion of ADAM12 can interfere with the association of Smad2 with TβRII (Fig. 5 E). These results suggest that ADAM12 may counteract the internalization of TβRII into caveolin1-positive vesicles and may counteract its subsequent degradation.

Concluding remarks

Overall, our data describe a new function for ADAM12 in the positive regulation of TGF-β signaling by modulating receptor trafficking. At present, a small number of proteins that interact with TGF-β receptors are described to regulate the trafficking and turnover of these receptors. Thus, identification of ADAM12 as a novel partner of TβRII provides new insight into the initiation of TGF-β signaling, which takes place in early endosomes.

Materials and methods

Yeast two-hybrid screening

A fragment corresponding to the extracellular domain (20–160 amino acids) of human TβRII was cloned into pBTM116. The human cDNA libraries from placenta were constructed in pGADGH. A total of 10×10^6

independent colonies were screened as previously described (Colland et al., 2004). The prey fragments of the positive clones were PCR amplified and sequenced.

Cell culture and transfection

The human embryonic kidney cell line 293T, HSCs, human RD cells, mouse C2C12 cells, monkey kidney COS7 cells, and mink lung MvLu1 cells were transfected using LipofectAMINE-Plus reagent (Invitrogen) according to the manufacturer's instructions. For experiments with ADAM12 antisense, cells were incubated with 2 μM of antisense oligonucleotides to ADAM12 (CTCTCTTTTATGCCTTCT and CCCCATTCCTTTCTCC) or random control oligonucleotides (ACTACTACACTAGACTAC and GCTCTATGACTCCCAG) as previously described (Lafuste et al., 2005). For RNAi experiments, cells were transfected with 0.5 μg of expression vector encoding the indicated shRNA.

Plasmids

ARE_3-Lux, $GAGA_9$-Lux, FAST1, HA-Smad4, myc-Smad2, and myc-Smad7 were previously described (Dumont et al., 2003; Seo et al., 2004). The expression vector for HA-TβRII was provided by J. Wrana (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada). Expression constructs for wild type or mutants of ADAM12 and ADAM12 fused to EGFP were prepared as previously described (Hougaard et al., 2000). The expression vector encoding ADAM12 shRNA or scrambled shRNA was constructed using the BLOCK-IT U6 RNA System (Invitrogen) according to the manufacturer's instructions. The expression vector for Flag-ADAM12 was obtained by fusing the Flag epitope to the N terminus of the ADAM12 fragment (amino acids 142–739) isolated in the yeast two-hybrid screen.

Transcriptional reporter assays

HepG2, C2C12, or 293T cells were transfected by LipofectAMINE, and, 30 h later, they were treated for 18 h with 2 ng/ml human TGF-β1 (Sigma-Aldrich). Cell extracts were assayed for luciferase activity using the Dual Luciferase Reporter Assay System (Promega), and luciferase activities were normalized on the basis of Renilla luciferase expression from the pRL-TK control vector. For potassium depletion experiments, transfected cells were incubated in medium and water (1:1) for 5 min at 37°C followed by incubation in medium depleted or not depleted in KCl for 1 h at 37°C before stimulation with TGF-β.

Immunoprecipitation and immunoblotting

After transfection, cells were lysed in lysis buffer (Dumont et al., 2003), and cell lysates were subjected to immunoprecipitation with the appropriate antibody for 2 h followed by adsorption to Sepharose bead–coupled protein G for 1 h. Immunoprecipitates were washed five times with lysis buffer containing 0.5% NP-40. For the association of endogenous TβRII with endogenous ADAMs, immunoprecipitates were washed three times with lysis buffer containing 0.5% NP-40 and two times with lysis buffer containing 1% NP-40. Then, samples were separated by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. The following antibodies were used: anti-ADAM12 Rb 122 (Gilpin et al., 1998), anti-Flag M2 (Sigma-Aldrich), anti-HA and anti–myc-9E10 (Boehringer Manheim), antiphospho-Smad2 (Cell Signaling Technologies), anti-Smad2 (Zymed Laboratories), anti-ADAM10 (ProSci), anti-ADAM17 (Chemicon), and antiactin, anti-TβRII, anti–bone morphogenetic protein RII, anti-Smad7, anti–PAI-1, and anti-JunB (Santa Cruz Biotechnology, Inc.).

Immunolocalization

Cells were fixed in 3% PFA, permeabilized with 0.1% Triton X-100, and incubated for 60 min at room temperature with the appropriate primary antibody followed by the appropriate secondary antibody. The coverslips were washed, mounted in PBS containing 50% glycerol and 1 mg/ml 1,4-diazabicyclo[2.2.2]octane, and viewed on an automated microscope (DMRXA2; Leica) equipped with a camera (CoolSNAP ES N&B; Roper Scientific) and a 63× Hcx Pl Apo NA 1.32 oil objective (Leica). Z steps were submitted to deconvolution (nearest neighbor method) by using MetaMorph software (Universal Imaging Corp.).

Real-time PCR

Total RNA were extracted by the guanidinium thiocianate/cesium chloride method, and real-time quantitative PCR was performed by the fluorescent dye SYBR green methodology as previously described (Le Pabic et al., 2003). Primer pairs for target genes were as follows: PAI-1, sense (5′-GTCTTTCCGACCAAGAGCAG-3′) and antisense (5′-CGATCCTGACCTTTTGCAGT-3′); ADAM12, sense (5′-GTTTGGCTTTGGAGGAAGCACAG-3′) and antisense (5′-TGCAGGCAGAGGCTTCTGAGG-3′); COL1A2, sense (5′-GGTGGTGGTTATGACTTTG-3′) and antisense (5′-ATACAGGTTTCGCCGGTAG-3′); and 18S, sense (5′-CGCCGCTAGAGGTGAAATTC-3′) and antisense (5′-TTGGCAAATGCTTTCGCTC-3′).

Online supplemental material

Fig. S1 A shows the effect of increasing amounts of ADAM12 on expression of the TGF-β–responsive gene PAI-1. Fig. S1 B shows the TGF-β–dependent expression of endogenous ADAM12 or COL1A2 in cells treated with ADAM12 antisense or control oligonucleotides. Fig. S2 shows the colocalization of ADAM12 with EEA1 or TβRII in Mv1Lu cells. Fig. S3 shows the association of TβRII with several transmembrane proteins as indicated by labeling with a membrane-impermeable biotinylation reagent. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200612046/DC1.

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