Smad2 mutant was phosphorylated by the receptors. Surface receptors and that a SARA binding-deficient
finally, we demonstrated that Smad2 interacted with cell
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was unaffected by inhibition of hVPS34 activity with

Let us assume this natural text: "Like many other cell surface receptors, transforming growth factor β (TGF-β) receptors are internalized upon ligand stimulation. Given that the signaling-facilitating molecules Smad anchor for receptor activation (SARA) and Hrs are mainly localized in early endosomes, it was unclear whether receptor internalization is required for Smad2 activation. Using reversible biotin labeling, we directly monitored internalization of the TGF-β type I receptor. Our data indicate that TGF-β type I receptor is endocytosed via a clathrin-dependent mechanism and is effectively blocked by depletion of intracellular potassium or by expression of a mutant dynamin (K44A).

However, blockage of receptor endocytosis by these two means has no effect on TGF-β-mediated Smad2 activation. Furthermore, TGF-β-induced Smad2 activation was unaffected by inhibition of hVPS34 activity with wortmannin or inhibitory anti-hVPS34 antibodies. Finally, we demonstrated that Smad2 interacted with cell surface receptors and that a SARA binding-deficient Smad2 mutant was phosphorylated by the receptors. Thus, our findings suggest that receptor endocytosis is dispensable for TGF-β-mediated activation of Smad2 and that this activation can be mediated by both SARA-dependent and -independent mechanisms.

Receptor endocytosis has long been regarded as an attenuation mechanism to switch off receptor signaling. However, accumulating evidence suggests that endocytosis may facilitate signaling by targeting signaling complexes to specific subcellular localization, either to increase access of activated receptor kinases to their substrates or to compartmentalize signaling complexes (1–3). Consistent with this idea, blocking of clathrin-mediated endocytosis was found to attenuate the activation of the extracellular signal-regulated kinases by receptor tyrosine kinases and G-protein-coupled receptors (4–7). Upon the activation of G-protein-coupled proteinase-activated receptor 2, a multiprotein signaling complex that contains β-arrestin 1, Raf-1, and extracellular signal-regulated kinases is formed on endocytic vesicles as well (8).

TGF-β1 binds to its cell surface receptors, resulting in the formation of type I and type II receptor complexes. In the complex, the TGF-β type II receptor (TβRII) phosphorylates and activates the TGF-β type I receptor (TβRI), which in turn phosphorylates the C-terminal serine residues of Smad proteins Smad2 and Smad3. As a result, Smad proteins accumulate in the nucleus, bind to DNA, and regulate transcription. The ligand-stimulated receptor complexes undergo endocytosis and are eventually degraded in an ubiquitin/lysosome-dependent pathway (9, 10).

The FYVE domain-containing proteins Smad anchor for receptor activation (SARA) and Hrs (the hepatocyte growth factor-regulated tyrosine kinase substrate) have been suggested to facilitate Smad2 phosphorylation by bringing Smad2 to TGF-β or activin receptors (11, 12). Interestingly, immunofluorescence studies revealed that both SARA and Hrs are predominantly localized in early endosomes (11, 13). This finding raises an important question: does Smad2 phosphorylation occur at the plasma membrane, where the receptors are exposed to TGF-β, or in early endosomes, where the signal-facilitating molecules SARA and Hrs are mainly localized? To investigate the role of TGF-β receptor endocytosis in Smad2 activation, we directly followed endocytosis of TβRII using a cell surface biotinylation protocol. Our data reveal that TβRII is rapidly internalized via clathrin-mediated endocytosis but that inhibition of receptor internalization does not block Smad2 activation.

EXPERIMENTAL PROCEDURES

Cell Culture and Materials—L17-TβRII cells and HeLa cells stably expressing wild-type and K44A dynamin were maintained as described previously (14, 15). TGF-β1 was purchased from R&D Systems, wortmannin was purchased from Sigma, anti-phospho-Smad2 antibody was purchased from Upstate Biotechnology, anti-phospho-Thr308-AKT and anti-AKT were purchased from Cell Signaling Technology, and other antibodies were purchased from Santa Cruz Biotechnology. Anti-VPS34 antibodies have been described previously (22). Unless indicated, all chemicals were from Fisher/ICN, SARA(dFYVE) and SARA(665-end) were generated by PCR-based deletion, and the sequences were confirmed by DNA sequencing.

Transfection, reporter assay, immunoprecipitation, Western analysis, and immunofluorescence microscopy were performed as described previously (16). Receptor affinity labeling was carried out as described previously (17), except that 5 mM KCl was not included in KRH buffer for KCl samples.

Depletion of Intracellular Potassium—Potassium depletion was performed for publication, April 11, 2002, and in revised form, May 22, 2002, DOI 10.1074/jbc.M203495200

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The abbreviations used are: TGF-β, transforming growth factor β; TβRII, TGF-β type I receptor; TβRII, TGF-β type II receptor; SARA, Smad anchor for receptor activation; PI(3)P, phosphatidylinositol 3-phosphate; HA, hemagglutinin.

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RESULTS

Rapid Clathrin-dependent Internalization of TβRI—To study the relationship between receptor signaling and endocytosis, we chose to directly monitor TβRI internalization in the stable cell line L17-TβRI that is derived from the TβRI-deficient mink lung epithelial L17 cells and expresses HA-tagged TβRI under the control of tetracycline (15). Cell surface proteins were labeled at 4 °C using a reducible biotinylation method (19). After additional incubation of labeled cells for various times at 37 °C, biotin remaining at the cell surface was removed by incubation with impermeable reducing agents at 4 °C. Internalized receptors, which are protected from reduction, were then precipitated with streptavidin beads and analyzed by immunoblotting.

First, we examined the ligand dependence of TβRI internalization. The cell surface TβRI was well labeled, and treatment of the cells with reducing agents efficiently removed biotin from the cell surface (Fig. 1A, lanes 1 and 2). After the cells were shifted to 37 °C, TβRI was rapidly internalized, and its internalization was further increased by TGF-β treatment (Fig. 1A, lanes 3–8; Fig. 1B).

To examine whether TβRI endocytosis is a clathrin-mediated process, we used two protocols that have been previously demonstrated to disrupt clathrin-mediated vesicle formation. Depletion of intracellular potassium decreases clathrin-coated pit formation and thus inhibits clathrin-dependent endocytosis (18). Clathrin-mediated endocytosis is also regulated by the GTPase dynamin, and expression of dynamin mutants defective in GTP binding or hydrolysis blocks the endocytosis of transferrin and epidermal growth factor receptors (20).

L17-TβRI cells were treated with hypotonic medium followed by isotonic potassium-free buffer to deplete intracellular potassium. Potassium depletion significantly inhibited TβRI internalization (Fig. 2A, lane 4). Moreover, ligand-mediated TβRI endocytosis was restored when 10 mM KCl was included in the buffer (Fig. 2A, lane 5). Therefore, depletion of intracellular potassium potently blocked TβRI internalization.

To confirm the clathrin dependence of TβRI internalization, we employed stable HeLa cell lines that express wild-type dynamin or the K44A mutant under the control of tetracycline.
FIG. 3. Smad2 activation is independent of receptor endocytosis. A, L17-TßRI cells were subjected to potassium depletion (-KCl) or control treatment (+KCl) for 30 min, followed by TGF-ß1 treatment for another 30 min. Smad2 phosphorylation (top panel) and protein expression (bottom panel) were analyzed by immunoblotting with anti-phospho-Smad2 and anti-Smad2 antibodies, respectively. B, after tetracycline withdrawal for 16 h, parental and dynamin-expressing HeLa cells were treated with or without 100 pt TGF-ß1 for 30 min. Smad2 phosphorylation was examined by anti-phospho-Smad2 immunoblotting (top panel). Smad2 protein expression was confirmed by anti-Smad2 (middle panel), and dynamin expression was confirmed by anti-HA immunoblotting (bottom panel). C, HeLa cells were continuously maintained in the medium containing tetracycline or subjected to tetracycline withdrawal for 16 h, and then they were treated with or without 100 pt TGF-ß1 for 30 min. Intracellular localization of Smad2 was examined by immunofluorescence. Similar results were obtained from three different experiments. D, transcriptional activity of TGF-ß does not require clathrin-mediated endocytosis. HeLa cells were transfected with the 3TP-luciferase construct (left panel), and Hep3B cells were transfected with ARE-luciferase and FAST2 as well as various forms of dynamin constructs (right panel). After TGF-ß1 treatment for 20 h, luciferase activity was determined. Relative luciferase activity (RLU) is expressed as the mean ± S.D. from triplicates. Similar results were obtained from three different experiments.
The cells were transfected with HA-tagged T/H9252RI and maintained in the growth medium in the absence of tetracycline, and T/H9252RI endocytosis was measured. As shown in Fig. 2B, T/H9252RI is efficiently endocytosed in HeLa cells expressing wild-type dynamin (lanes 3 and 4); a high level of ligand-independent T/H9252RI internalization was observed, which could be due to the high level of receptor expression. Nonetheless, expression of K44A dynamin completely blocked T/H9252RI internalization (Fig. 2B, lanes 7 and 8).

Smad2 Activation Is Unaffected by Inhibition of T/H9252RI Endocytosis—To test whether internalization of TGF-β receptors is required for Smad activation, L17-T/H9252RI cells were subjected to depletion of intracellular potassium. The cells were then stimulated with TGF-β1 at a concentration as low as 10 pM and were also observed in HeLa and Hep3B cells (data not shown). Smad2 is localized in the cytoplasm at the basal state, and TGF-β treatment resulted in its translocation into the nucleus (Fig. 3C). Expression of either wild-type or K44A dynamin had no effect on TGF-β-induced nuclear accumulation of Smad2 (Fig. 3C). To examine whether T/H9252RI endocytosis is necessary for the transcriptional activity of TGF-β, we examined the expression of two TGF-β-responsive reporters (3TP-luciferase and ARE-luciferase), whose expression is Smad-mediated. The 3TP-luciferase construct with or without various forms of SARA constructs. After TGF-β1 treatment for 20 h, luciferase activity was determined. Relative luciferase activities are expressed as the mean ± S.D. from triplicates. Similar results were obtained from three different experiments.
Receptor Endocytosis Is Unessential for Smad2 Activation

These data suggest that production of PI(3)P, presumably in the early endosome, is important for TgRI signaling. To test this hypothesis, we treated L17-TgRI cells with 100 nM wortmannin, which inhibits all phosphatidylinositol 3-kinases except the class II phosphatidylinositol 3-kinase C2δ isoform. As shown in Fig. 4A, wortmannin had no effect on TGF-β-stimulated Smad2 phosphorylation (lane 3), although it inhibited insulin-mediated Akt/protein kinase B phosphorylation at threonine 308 (lane 10). Similar results were obtained in Hep3B cells (data not shown). In addition, treatment of HeLa cells with wortmannin did not affect TGF-β-induced Smad2 nuclear localization (data not shown).

To specifically examine the role of PI(3)P in TGF-β signaling, we microinjected HepG2 cells with specific inhibitory antibodies against the class III phosphatidylinositol 3-kinase, hVPS34. These antibodies disrupt the trafficking of internalized platelet-derived growth factor receptors and the endosomal localization of EEA1 (22). Moreover, they completely disrupt the localization of the intracellular PI(3)P marker 2X-FYVE-GFP fusion protein in Chinese hamster ovary (23) and HepG2 cells (data not shown). However, microinjection of inhibitory anti-VPS34 antibodies had no effect on TGF-β-stimulated nuclear accumulation of Smad2 in HepG2 cells (Fig. 4B).

To directly address whether the FYVE domain is required for SARA function, we examined TGF-β-induced expression of 3TP-luciferase in the presence of SARA(dFYVE), a mutant that lacks the FYVE domain. This mutant was shown to lose a punctate subcellular localization but still interact with Smad2 (11). We reasoned that this mutant should have a dominant negative effect on TGF-β signaling by sequestering Smad2 and Smad3 away from the membrane. However, like the wild-type SARA, SARA(dFYVE) has no effect on TGF-β activity in mediating the expression of 3TP-luciferase. This is in contrast to SARA(665-end), a mutant lacking both the N terminus and the FYVE domain, which does inhibit TGF-β function (Fig. 5) (11). These results suggest that interactions between SARA and intracellular PI(3)P are not required for Smad2 activation.

Smad2 Is Recruited to the Receptor Complexes on the Plasma Membrane—To address whether Smad2 is activated on the plasma membrane, the receptor-Smad2 complexes wereexamined when receptor endocytosis was blocked. COS1 cells transfected with FLAG-tagged Smad1 or Smad2, TgRI, and TgRII were subjected to potassium depletion and then incubated with 125I-TGF-β1 to label cell surface receptors. After treatment with a cross-linking reagent, receptor-Smad2 complexes were examined by anti-FLAG immunoprecipitation and visualized by SDS-PAGE and autoradiography. Because the interaction between TgRI and Smad2 is transient, Smad2 stably associated with the receptors only when the kinase-deficient TgRII mutant was expressed (Fig. 6A, lane 3). Importantly, Smad2 still interacted with TGF-β receptor complex even when receptor endocytosis was inhibited by potassium depletion (lane 5), strongly indicating that Smad2 is recruited into receptor complexes on the plasma membrane.

To further investigate the role of SARA in Smad2 activation, we examined phosphorylation of Smad2(N381S), a mutant deficient in SARA-binding (24), in dynamin(K44A)-expressing HeLa cells. FLAG-tagged Smad2(N381S) was transfected into dynamin(K44A)-expressing HeLa cells together with TgRI and TgRII constructs. After induction of dynamin K44A expression for 16 h, the cells were treated with TGF-β1, and Smad2(N381S) phosphorylation was revealed by anti-phospho-Smad2 immunoblotting after anti-FLAG immunoprecipitation. As shown in Fig. 6B, Smad2(N381S) was phosphorylated when coexpressed with TGF-β receptors, and this phosphorylation was further stimulated by TGF-β. Phosphorylation of

**Fig. 6.** A, interaction of Smad2 with cell surface receptors. COS1 cells were transfected with the constructs expressing FLAG-tagged Smad, wild-type TgRII, and wild-type (WT) or kinase-deficient (KR) TgRI, as indicated. Forty h later, cells were subjected to potassium depletion (−KCl) or control treatment (+KCl). Subsequently, cells were incubated with 200 pM 125I-TGF-β1 and treated with the cross-linking reagent DSS. Smad-receptor complexes were isolated with anti-FLAG immunoprecipitation and visualized by SDS-PAGE and autoradiography (top panel). Total cell surface receptors were verified by analysis of total labeled receptors, and Smad proteins were verified by anti-FLAG immunoblotting. B, SARA-Smad2 interaction is not essential for Smad2 phosphorylation. Dynamin-expressing HeLa cells were transfected with the constructs expressing FLAG-tagged Smad, wild-type dynamin, or K44A mutant (Fig. 3B, lane 6), anti-p-Smad2, anti-F-Smad2, and anti-F-Smad2 protein expression was confirmed by anti-FLAG immunoblotting (bottom panel).
Smad2(N381S) in the absence of TGF-β could be due to protein overexpression. Nonetheless, these results demonstrate that SARA binding activity is not essential for receptor-mediated phosphorylation of Smad2. This phosphorylation is presumably mediated by the cell surface receptors because it is not abolished by inhibition of receptor endocytosis.

**DISCUSSION**

Our finding that TβRII is internalized via clathrin-mediated endocytosis is in agreement with the earlier studies, which utilized granulocyte macrophage colony-stimulating factor-TGF-β chimeric receptors (25). The internalization of these chimeric receptors was abolished by potassium depletion. Furthermore, recent studies also suggest that internalization of TβRII is clathrin-mediated (26). However, uptake of T125I-TGF-β1 was not blocked by chloroquine or transient expression of K44A dynamin (27). The mechanism of TGF-β uptake may differ from that of TβRII internalization because TGF-β uptake can be mediated by different binding proteins, such as β-glycan. Thus, TGF-β uptake reflects the sum of internalized TGF-β-bound proteins, some of which may utilize clathrin-independent internalization mechanisms.

SARA has been shown to play a role in Smad-mediated signaling. Given the endosomal localization of this protein, a model has emerged in which the internalization of TβRI receptors into early endosomes facilitates interactions with SARA and enhances TGF-β signaling. In this study, we have tested several aspects of this model. We demonstrate that TβRII internalizes via coated vesicles. However, inhibition of this process using two different methods has no effect on Smad2 phosphorylation and nuclear translocation and has no effect on TGF-β-stimulated transcription. Furthermore, we find that disruption of the interaction between PI(3)P and the SARA FYVE domain, either by inhibiting cellular phosphatidylinositol-3-kinases with wortmannin, by specifically inhibiting hVPS34 with antibodies, or by deleting the FYVE domain, has no effect on TGF-β-mediated signaling. In addition, we provide evidence that Smad2 can be recruited directly to cell surface TGF-β receptors. Therefore, we conclude that TβRII endocytosis is qualitatively dispensable for Smad2 activation and that interactions between the FYVE domain of SARA and PI(3)P are not necessary for Smad2-mediated TGF-β signaling. We further demonstrate that the SARA binding-deficient mutant Smad2(N381S) is still phosphorylated by activated TGF-β receptors. This is consistent with our previous results showing that both wild-type Smad2 and Smad2(N381S) can stimulate ARE-luciferase expression, but only the activity of wild-type Smad2 is enhanced by SARA (24). Thus, receptor-mediated Smad2 activation is mediated by both SARA-dependent and -independent mechanisms.

Our results do not rule out the possibility that receptor endocytosis may contribute to the maximal activation of Smad2. Although not required for TGF-β signaling, interactions with endosomal SARA could modulate the intensity or lifetime of TGF-β signaling in the presence of submaximal levels of ligand. This is suggested by recent experiments in which a dominant negative Rab5 increases TGF-β signaling presumably by influencing early endosomal dynamics (28). In fact, we observed that overexpression of wild-type dynamin significantly enhanced Smad2 phosphorylation. This suggests that overexpression of wild-type dynamin may accelerate receptor endocytosis and thereby facilitate Smad2 activation. Alternatively, enhanced Smad2 phosphorylation may be due to endocytosis-independent effects of dynamin on other signaling pathways, as has been suggested for mitogenic and apoptotic signal transduction (29, 30).

Receptor endocytosis has been shown to be required for certain signaling pathways but not for others. For instance, insulin-like growth factor receptor internalization interferes with the Shc/mitogen-activated protein kinase pathway, but not with the insulin receptor substrate-1 pathway (6). In addition to the Smad pathway, TGF-β has also been implicated to signal through other pathways, such as mitogen-activated protein kinases (reviewed in Ref. 31). It remains to be determined whether these signaling pathways are dependent on TGF-β receptor endocytosis.

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