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TGFβ receptor internalization into EEA1-enriched early endosomes: role in signaling to Smad2

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Transforming growth factor (TGF)β is an important physiological regulator of cellular growth and differentiation. It activates a receptor threonine/serine kinase that phosphorylates the transcription factor Smad2, which then translocates into the nucleus to trigger specific transcriptional events. Here we show that activated type I and II TGFβ receptors internalize into endosomes containing the early endosomal protein EEA1. The extent of TGFβ-stimulated Smad2 phosphorylation, Smad2 nuclear translocation, and TGFβ-stimulated transcription correlated closely with the extent of internalization of the receptor. TGFβ signaling also requires SARA (Smad anchor for receptor activation), a 135-kD polypeptide that contains a FYVE Zn^{++} finger motif. Here we show that SARA localizes to endosomes containing EEA1, and that disruption of this localization inhibits TGFβ-induced Smad2 nuclear translocation. These results indicate that traffic of the TGFβ receptor into the endosome enables TGFβ signaling, revealing a novel function for the endosome as a compartment specialized for the amplification of certain extracellular signals.

Introduction

Receptors for hormones and growth factors, as well as protein machineries complexed to their cytoplasmic domains, transit from the plasma membrane into the endosomal compartment. The fate of these signaling complexes is determined in the endosome; the receptor complex can recycle or proceed to downstream compartments in which the receptor generally becomes degraded. One of the factors that determine the fate of proteins within the endosome is the activity of phosphatidylinositol-3-kinase. Inhibition of PI 3-kinase leads to alterations in receptor traffic and degradation rates (Corvera, 2001).

The actions of PI 3-kinases are mediated in part by proteins that interact directly with the products of PI 3-kinase activity. These proteins contain specific domains that bind with high affinity to 3′ phophoinositides. One of these domains, the FYVE domain, is present in ∼40 mammalian proteins, of which several have been implicated in membrane traffic both in yeast and mammalian cells. The FYVE domain binds to PtdIns(3)P with high affinity, and its presence in molecules such as EEA1 (Stenmark et al., 1996), Rabenosyn5 (Nielsen et al., 2000), and Rababtin4 (Cormont et al., 2001), which interact with Rab GTPases in the endocytic pathway, provides a molecular link between PtdIns(3)P and the membrane-trafficking events that occur during early endocytosis and postendocytic sorting of ligands.

FYVE domain-containing proteins have also been found in the context of signal transduction. For example, the mammalian protein Hrs-2, which is rapidly tyrosine phosphorylated in response to polypeptide growth factors such as EGF and HGF, contains a FYVE domain (Komada and Soriano, 1999; Miura et al., 2000). Another FYVE domain containing protein involved in signal transduction is SARA (Smad anchor for receptor activation),* a 135-kD polypeptide that contains a binding domain for the transcription factor Smad2 and a putative binding domain for the TGFβ receptor (Tsukazaki et al., 1998). SARA is thought to be required for the phosphorylation of Smad2 by the activated TGFβ receptor, and thus for nuclear translocation after phosphorylation.

The presence of endosomal localization signals such as the FYVE domain in proteins involved in signal transduction suggests that, in addition to its role in establishing correct traffic patterns of internalized proteins, the endosome might form an essential part of the signal transduction machinery of the cell. The endosome may provide a specialized environment, analogous to those established within the plasma membrane by the localized enrichment of specific lipids (Sedwick and Altman, 2002). Here we have begun to test this hypothesis by analyzing the localization of the TGFβ receptor, and the ef-

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Key words: FYVE; PI 3-kinase; transforming growth factor; SARA; endocytosis

*Abbreviations used in this paper: SARA, Smad anchor for receptor activation; TGF, transforming growth factor.

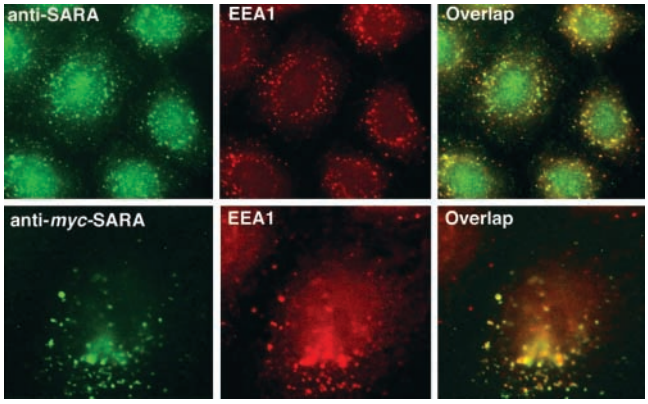


Figure 1. **SARA localizes to EEA1-containing endosomes.** (Top) HeLa cells were stained with antibodies to endogenous SARA (left) and EEA1 (middle). (Bottom) HeLa cells were transfected with *myc*-SARA and stained with a mouse antibody against myc (left) and a monoclonal antibody against EEA1 (middle). The overlap between the signals is displayed in yellow in the far right panel.

fects of inhibitors of endocytosis, on TGFβ-stimulated signaling. Our results suggest that, in Mv1Lu and HeLa cells expressing endogenous wild-type TGFβ receptors, localization of the TGFβ receptor to endosomes containing EEA1 and SARA is an important element in eliciting Smad2 nuclear translocation. These results thereby extend the role of the endosome to that of a compartment specialized for the propagation of certain extracellular signals.

Results

Immunofluorescence analysis of HeLa cells with a polyclonal antiserum raised to SARA revealed a high level of colocalization with EEA1 (Fig. 1, top), a marker previously characterized as specific for a subset of early endosomes (Wilson et al., 2000). The specificity of the immunostaining was verified by analyzing the localization of full-length myc epitope-tagged SARA transfected into HeLa, Mv1Lu, and Cos-7 cells. In all these cell types, myc-SARA was found exclusively in intracellular vesicular structures overlapping substantially with EEA1 (Fig. 1, bottom). Similar results have been recently reported in BHK cells (Itoh et al., 2002).

The localization of SARA on EEA1-enriched endosomes, and its putative requirement for TGFβ-signaling to Smad2, suggested that activated TGFβ receptors would also localize to these endosomes after activation. To directly address this question, as well as to determine the route of internalization of activated full-length type I and II TGFβ receptors, receptors tagged on their extracellular domains with the HA or myc epitopes were cotransfected with wild-type dynamin or with dynamin K44E, a dominant-negative construct that inhibits clathrin-mediated endocytosis (Gilboa et al., 1998;

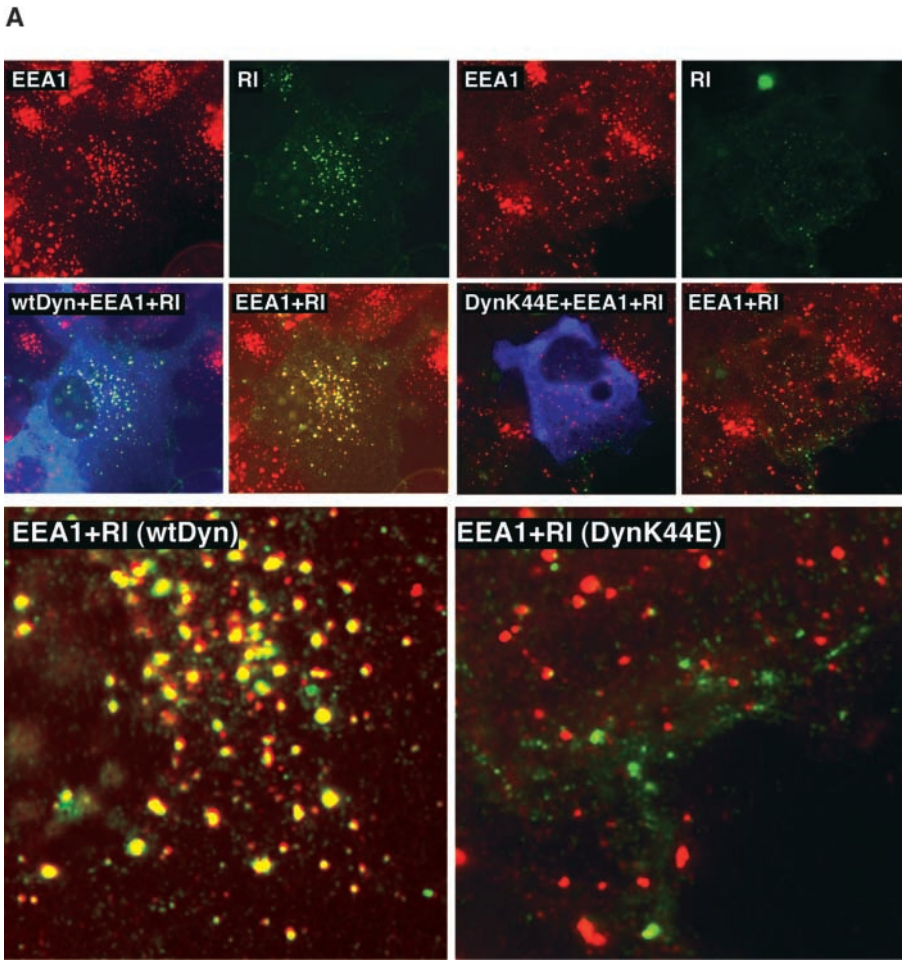


Figure 2. **TGFβ receptor internalization into to EEA1-positive endosomes is blocked by dominant-negative dynamin.** (A) Cos-7 cells cotransfected with HA-tagged type I receptor (RI) and myc-tagged type II TGFβ receptor, and either wild-type (WtDyn) or K44E dynamin (left, WT; right, K44E) were incubated for 1 h at 4°C with 100 pM TGFβ and antibodies against the HA epitope, and then for 60 min at 37°C. Cells were fixed and stained with a human antiserum against EEA1 (indicated as EEA1, red) and a mouse antiserum against dynamin I (blue), and secondary antibodies to the anti-HA antibody (RI, green). The overlap between the three signals is displayed in the panel labeled Dyn + EEA1 + RI. The overlap between EEA1 + RI is shown as indicated, and the large bottom panel represents an enlarged area of this panel (EEA1+RI (wtDyn or DynK44E). (B) Cos-7 cells cotransfected with HA-tagged type I receptor and myc-tagged type II TGFβ receptor (RII), and either wild-type (WtDyn) or K44E dynamin (left, WT; right, K44E) were incubated for 1 h at 4°C with 100 pM TGFβ and antibodies against the myc epitope, and then for 60 min at 37°C. Cells were fixed and stained and panels labeled as described in Fig. 2 A for RI.

Wells et al., 1999). Transfected live, nonpermeabilized cells were incubated at 4°C for 60 min with TGFβ and with antibodies to HA or myc to label expressed type I or II receptors at the cell surface, respectively. Cells were then rapidly warmed to 37°C to allow endocytosis. After 0–60 min, cells were fixed, permeabilized, and stained with antibodies to detect endogenous EEA1, transfected dynamin constructs, and the type I or II receptor bound to respective anti-tag antibodies. Before incubation at 37°C, the receptors were localized to the cell surface, and no colocalization with endogenous EEA1 was seen, as expected (unpublished data). By 15 min at 37°C, a more vesicular pattern appeared which increased progressively with time and colocalized substantially with endogenous EEA1 for up to 60 min after internalization.

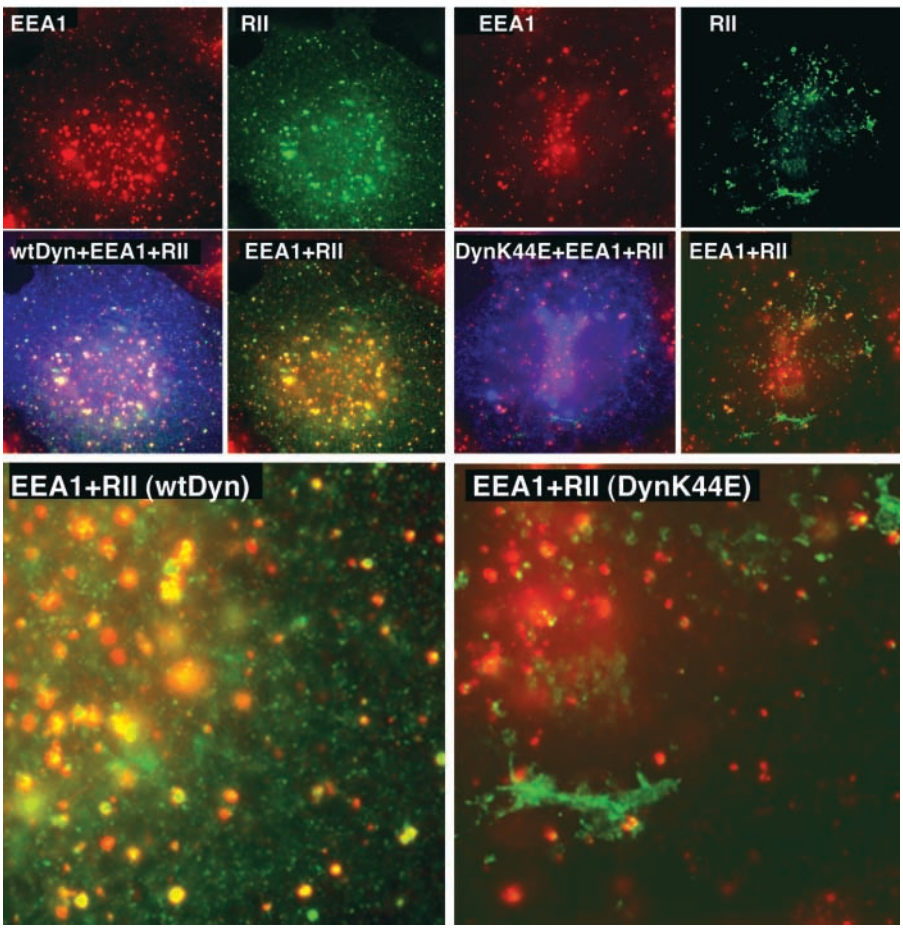
Overexpression of wild-type dynamin did not affect internalization of either the type I or II receptors (Fig. 2, A and B, left). These results indicate that activated wild-type TGFβ receptors internalize and remain localized into SARA and EEA1-enriched endosomes for a substantial amount of time. In contrast, expression of K44E dynamin profoundly blocked receptor internalization. Even after 60 min of incubation at 37°C, only very few EEA1-enriched endosomes were found to contain internalized receptor (Fig. 2, A and B, right). These results indicate that the activated type I and II TGFβ receptors internalize into EEA1-enriched endosomes via a dynamin-dependent pathway.

The rate of internalization of the TGFβ receptors correlates with the rate of nuclear translocation of Smad2 (unpublished data). To directly test if internalization of TGFβ receptors into endosomes plays a role in signaling to Smad2, the effects of dynaminK44E on Smad2 nuclear translocation were examined. Nontransfected cells responded to TGFβ with a significant increase in nuclear/cytoplasmic intensity of the Smad2/3 signal (Fig. 3). Such an increase in nuclear/cytoplasmic staining of Smad2 was blocked in cells expressing dynaminK44E (Fig. 3).

To quantify the effect of dominant-negative dynamin, cells were incubated without or with TGFβ and with Alexa595-labeled transferrin for 30 min. Cells were then fixed and stained for K44E dynamin and Smad2/3 (Fig. 4 A). The intensity of Smad2 fluorescence in the cytoplasm and nucleus of these cells was measured in overlapped images as described in Materials and methods. Regions within the cytoplasmic space, detected by the presence of endosomes containing Alexa 594 transferrin in nondynamin K44E-transfected cells, or by presence of dynamin K44E in transfected cells were recorded. Identical size regions in the center of the nucleus, identified by exclusion of cytoplasmic signal of transferrin or dynamin, were also recorded. The ratio of nuclear to cytoplasmic fluorescence for each of the cells displayed in Fig. 4 C.

The ratio of nuclear/cytoplasmic fluorescence from many more cells is shown in Fig. 5 A. In nontransfected cells

Figure 2 continued



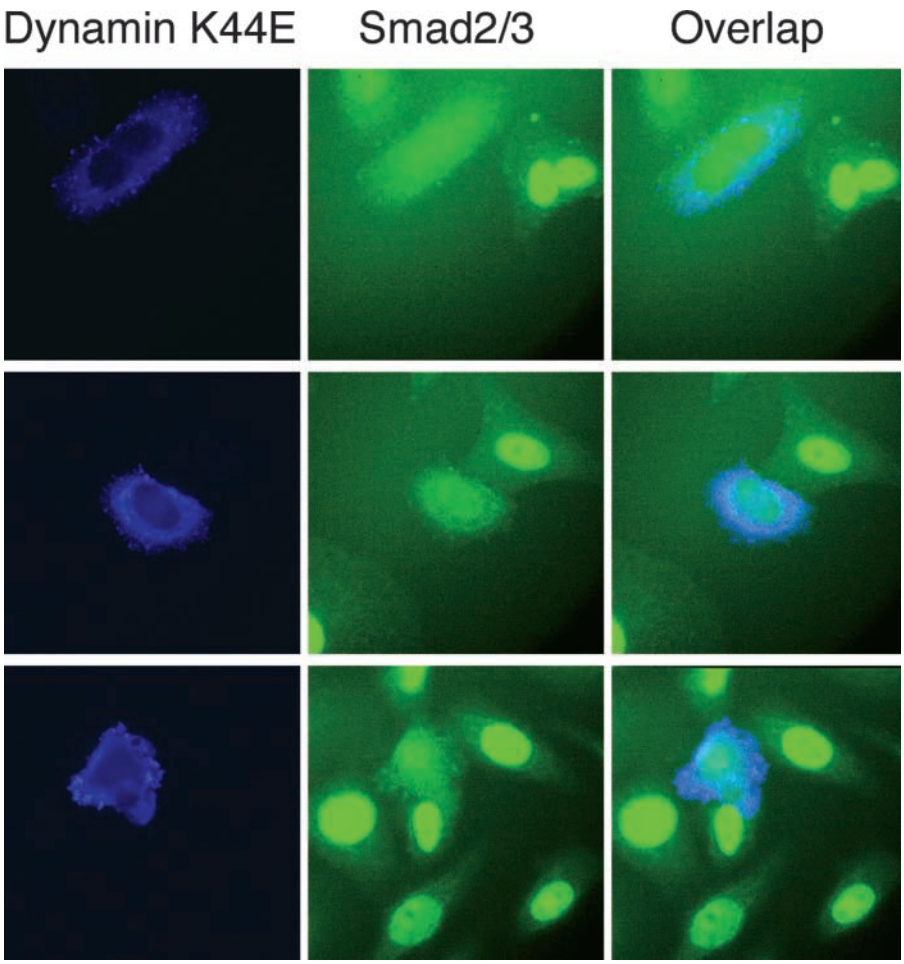


Figure 3. **Dominant-negative dynamin inhibits Smad2 translocation.** HeLa cells transfected with myc-tagged dynamin K44E were incubated with 100 pM TGFβ for 30 min. Cells were fixed and stained with rabbit antibodies against myc (left) and mouse antibodies against endogenous Smad2/3 (right). Overlap is shown in far right column.

treated with TGFβ this ratio was consistently >2, whereas the value for dynamin K44E-expressing cells was lower and similar to that measured in cells that were not stimulated with TGFβ (Fig. 5 A). These results indicate that dynamin K44E impairs the nuclear translocation of Smad2/3 in response to TGFβ. Consistent with this finding, TGFβ-stimulated transcription of the luciferase gene driven by 3Tp-Lux, a Smad2-responsive promoter, was significantly impaired in cells overexpressing dominant-negative, but not wild-type dynamin (Fig. 5 B).

Clathrin-mediated endocytosis can be rapidly blocked by depletion of cellular potassium (Larkin et al., 1985, 1986). This procedure is acute, thus decreasing the probability of inducing compensatory responses that could occur in cells expressing dynamin mutants. Receptor internalization was measured as described above, but the final brief acid wash was omitted to allow observation of plasma membrane receptor under conditions of potassium depletion. In cells incubated in the presence of potassium, the signal was found both on the plasma membrane and extensively colocalized with EEA1 (Fig. 6). In contrast, receptor staining in the potassium-depleted cells was detected on the cell surface with some concentration in membrane ruffles. Virtually no colocalization of the receptor and EEA1was detected.

To determine the effects of potassium depletion on TGFβ signaling, the effect on TGFβ-stimulated Smad2 phosphorylation was measured. Control or potassium-depleted cells were incubated with TGFβ and Alexa-594–labeled transferrin for 0–30 min, solubilized in SDS sample buffer, and extracts analyzed by sequential immunoblotting with a phospho-specific antibody against Smad2 and an antibody to Smad2 protein (Fig. 7 A). The effectiveness of potassium depletion on endocytosis was reflected directly by the concentration of Alexa595-transferrin, which could be measured by scanning the immunoblot with the appropriate laser line of the Storm 860 phosphorimager (Fig. 7 A). In control cells, transferrin uptake and TGFβ-induced Smad2 phosphorylation both reached maximal levels within 15–30 min of incubation (Fig. 7, A and B). In potassium-depleted cells, transferrin uptake and Smad2 phosphorylation were both impaired, reaching submaximal uptake/phosphorylation after 30 min (Fig. 7, A and C). The correspondence between the extent of Smad2 phosphorylation and transferrin uptake suggests that endocytosis enhances TGFβ-stimulated Smad2 phosphorylation.

Interestingly, whereas the effects of potassium depletion on Smad2 phosphorylation were attenuated with time of incubation with TGFβ, the inhibitory effects of potassium depletion on Smad2 nuclear translocation were very pronounced for up to 30 min of stimulation (Fig. 8 A). This inhibition was readily reversed upon addition of KCl, indicating that the inhibitory effects of potassium depletion were not due to irreversible mistargeting of receptors into nonfunctional compartments (unpublished data).

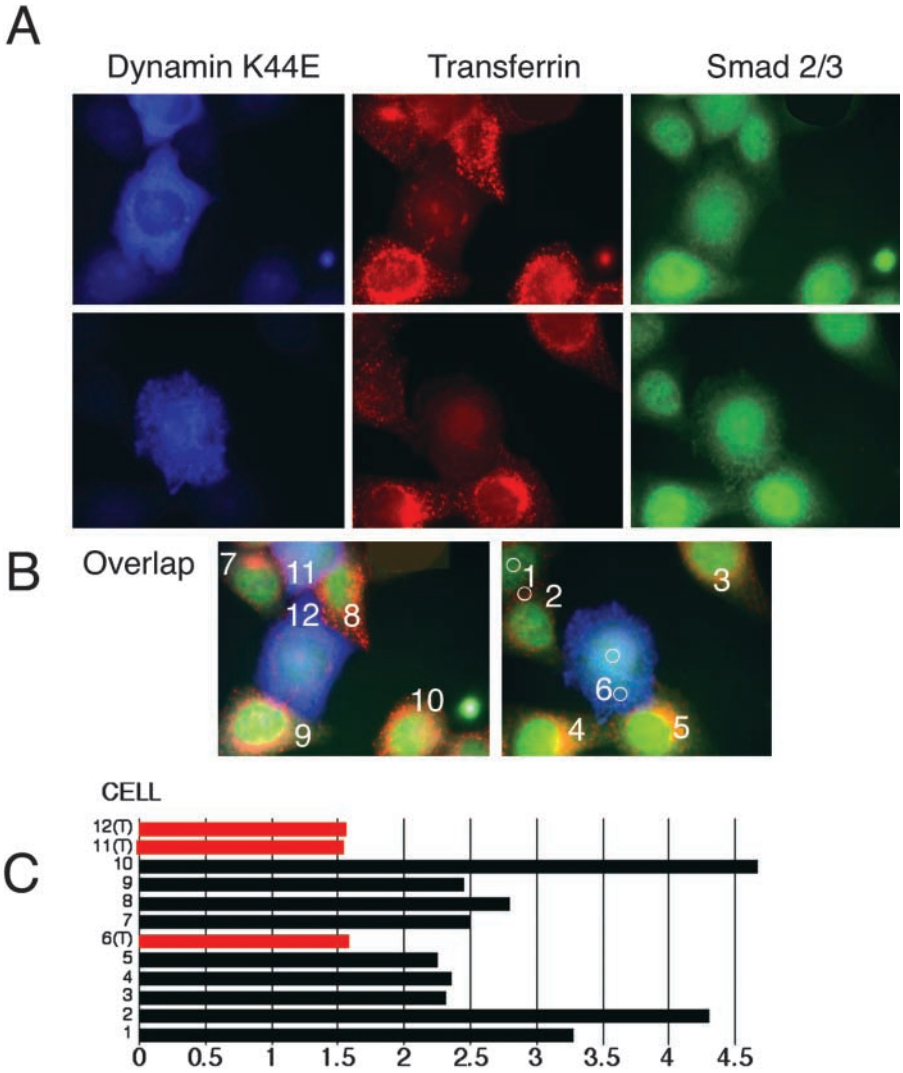


Figure 4. **Quantification of the effects of dominant-negative dynamin on Smad2 translocation and transferrin uptake.** (A) HeLa cells transfected with myc-tagged dynamin K44E were incubated with Alexa 594–labeled transferrin and 100 pM TGFβ for 30 min. Cells were fixed and stained to detect transfected Dynamin (left) and endogenous Smad2/3 (right). Internalized transferrin is visualized in the middle panels. Top and bottom panels represent two representative fields. (B) The three images shown in A were merged. The overlap for the top row is shown on the left; the overlap for the bottom row is shown on the right. Regions within the cytoplasm and nucleus selected as exemplified by the circles in cells 1 and 6. All the cells are numbered. (C) The green fluorescence intensity was recorded for the cytoplasmic and nuclear regions and the nuclear/cytoplasmic ratio was calculated for each cell indicated by number in B. The red bars indicate the transfected cells; the black bars indicate the nontransfected cells.

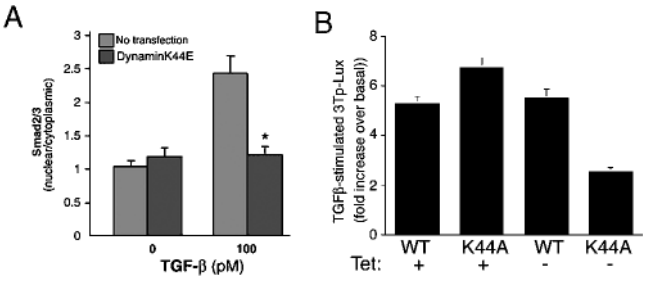


Figure 5. **Dominant-negative dynamin inhibits Smad2 signaling.** (A) The nuclear/cytoplasmic ratio of Smad2/3 was measured in 24 independent fields derived from two separate experiments. Plotted are the means ± SEM, and asterisk indicates a statistically significant ($P < .01$) difference from the nontransfected cohort. (B) TGFβ-induced transcription of 3Tp-Lux was measured in HeLa cells stably expressing wild-type (WT) or K44A dynamin under a tetracycline repressible promoter. Cells were transfected with reporter plasmids and grown in the continued presence or absence of tetracycline (Tet) as indicated. Bars represent the fold-stimulation over basal luciferase activity induced by a 14–16 h incubation with 100 pM TGFβ. Bars are means and lines represent standard errors of the mean of three experiments performed in triplicate.

To determine whether potassium depletion affects more proximal aspects of TGFβ function, the phosphorylation of TGFβ receptors was measured after incubation of transfected cells with ^{32}P-orthophosphate. Cotransfection of type I and II receptors resulted in a high basal phosphorylation of the type I receptor (Fig. 8 B), similar to what has been reported for the type I and II activin receptors (Lebrun and Vale, 1997). Nevertheless, a small enhancement of phosphorylation of the type I receptor was observed upon addition of TGFβ.

Potassium depletion did not detectably impair phosphorylation of the type I receptor, nor did it interfere with constitutive phosphorylation of the type II receptor (Fig. 8 B). The lack of an inhibitory effect of potassium depletion on type I receptor activation is also consistent with the finding shown above, in which some Smad2 phosphorylation can be observed in potassium-depleted cells after prolonged incubation with TGFβ (Fig. 7). This phosphorylation can be attributed to either the induction of compensatory endocytic mechanisms, or to internalization-independent Smad2 phosphorylation. The finding of detectable transferrin uptake after prolonged incubation in potassium-depleted cells

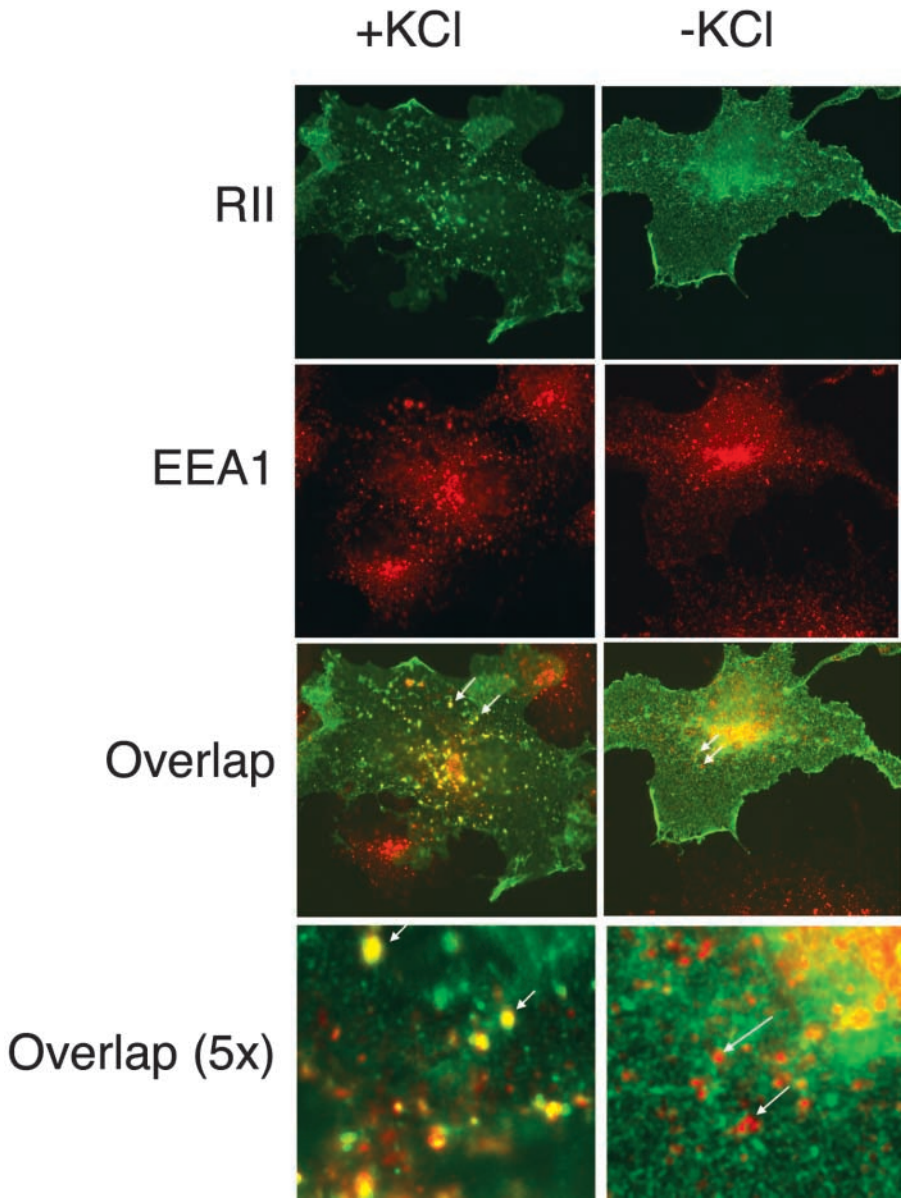


Figure 6. **Potassium depletion inhibits TGFβ receptor endocytosis.** (A) Cells expressing myc-tagged type II receptors (RII) were subjected to the potassium-depletion protocol in the absence (−KCl) or presence (+KCl) of 10 mM KCl, and incubated with 100 pM TGFβ and anti-myc antibodies for 60 min at 4°C. After incubation at 37°C for 20 min cells were fixed, permeabilized, and stained to detect RII (top row) and endogenous EEA1 (second row). The overlap is shown in the third row and a region from this panel magnified five times in the fourth row. Arrows indicate endosomes stained for both EEA1 and RII in the control cells; none were detected in potassium depleted cells.

(Fig. 7 C) supports the former possibility, although the latter cannot be ruled out. Nevertheless, even after prolonged incubation with TGFβ and detectable Smad2 phosphorylation, Smad2 nuclear translocation is greatly impaired in potassium-depleted cells.

The greater sensitivity to endocytosis inhibition displayed by Smad2 nuclear translocation compared with Smad2 phosphorylation suggest that factors additional to phosphorylation may be involved in TGFβ-induced Smad2 nuclear translocation, and that these may require endosomal localization. One possible explanation for the inhibitory actions of potassium depletion could be the disruption of the interaction between Smad2 and SARA. To test this possibility, the localization of endogenous Smad2 in cells overexpressing myc-tagged full-length SARA was analyzed. Potassium depletion did not impair the interaction between SARA and Smad2 (Fig. 8 C), nor the localization of EEA1 (Fig. 6) to early endosomes. The inhibition of Smad2 nuclear translocation by two independent complementary techniques that block clathrin-mediated endocytosis suggests that traffic of the receptor into the endosome is required for productive signaling by TGFβ.

To explore the hypothesis that this endocytosis requirement is due to the presence of SARA on the endosome we sought to measure TGFβ signaling under conditions that disrupt the interaction of SARA with the endosomal membrane. Because the isolated FYVE domain of SARA binds to endosomal membranes, overexpression of this domain might be expected to interfere with the binding of endogenous SARA to the endosome. To directly test this hypothesis, the localization of endogenous SARA was analyzed in cells overexpressing GFP-SARA-FYVE at different levels. At relatively low levels of expression, endogenous SARA colocalized with expressed GFP-SARA-FYVE on distinct endosomal structures (Fig. 9 A, top). However, at higher levels of overexpression endogenous SARA displayed a more diffuse appearance, and colocalized poorly with intracellular structures that contained a large GFP signal (Fig. 9 A, bottom).

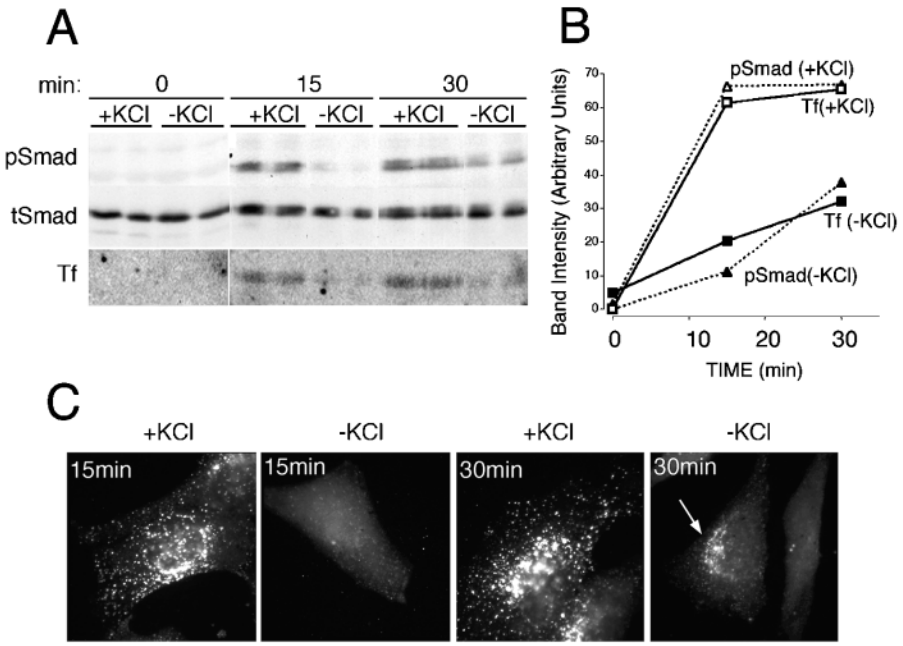


Figure 7. **Potassium depletion impairs Smad2 phosphorylation and transferrin endocytosis.** (A) Control (+KCl) or potassium-depleted (−KCl) HeLa cells were treated with 100 pM TGFβ for the indicated time. Cell lysates were analyzed by immunoblotting with antibody against phospho-Smad2 (pSmad2), and reprobed with anti–Smad2/3 (tSmad). Fluorescent transferrin on the nitrocellulose blot was visualized using the red fluorescence option the Strom 860 phosphoimager (Tf). (B) The intensity of the pSmad and Smad2/3 bands was measured and the ratio is graphed and compared with the intensity of the transferrin band. (C) Control (+KCl) or potassium-depleted (−KCl) Mv1Lu cells were incubated with 50 μg/ml Alexa-594 labeled mouse transferrin. The arrow indicates transferrin internalized after 30 min of incubation in KCl depleted cells, which represents 10–20% of that measured in the perinuclear region of control cells.

The TGFβ-mediated increase in nuclear/cytoplasmic intensity of Smad2/3 was diminished in cells overexpressing GFP-SARA-FYVE (Fig. 9, B and C). These results suggest that disruption of endosome function and/or SARA association by overexpression of the isolated FYVE domain can impair Smad2 nuclear translocation.

Discussion

In this manuscript we have examined the route of internalization of wild-type TGFβ receptors. Both type I and II receptors appear to rapidly internalize and accumulate in endosomes enriched in EEA1. The route by which the receptors reach EEA1-containing endosomes is likely to be clathrin-dependent, given that internalization is inhibited both by expression of dominant-negative dynamin and by potassium depletion, two methods that disrupt this endocytic pathway by distinctly different mechanisms. We also demonstrate that SARA, a soluble cytoplasmic protein required for TGFβ function (Tsukazaki et al., 1998), is localized virtually exclusively to EEA1-enriched endosomes (Fig. 1; Panopoulou et al., 2002).

The finding that activated TGFβ receptors internalize into EEA1/SARA-enriched endosomes is of interest given the reported relationship between TGFβ receptor function and SARA (Tsukazaki et al., 1998). SARA directly binds to Smad2, and is required for TGFβ-stimulated Smad2-dependent transcriptional activation. In our experiments, all detectable SARA localized to early endosomes, and thus the accumulation of receptor in this compartment is likely to play an important role in its ability to stimulate sustained Smad2 phosphorylation and Smad2-dependent transcriptional activation. Indeed, inhibition of receptor internalization by two methods that disrupt this endocytic pathway by distinctly different mechanisms greatly impairs the stimulation of Smad2 nuclear translocation and diminishes transcriptional activation in cells highly responsive to TGFβ though activation of their endogenous receptors (HeLa and Mv1Lu cells). Although differing results may be obtained upon receptor overexpression, our data as well as that of Penheiter et al. (2002) support the hypothesis of a crucial role for internalization in TGFβ function.

Interestingly, genetic evidence currently in the literature indeed supports a crucial role for endocytosis in TGFβ function. The *Drosophila* TGFβ homologue Dpp functions through its receptor to activate its target gene Spalt and regulate wing development. Entchev et al. (2000) have shown that the propagation of a Dpp gradient and the range of activation of Spalt require endocytosis, as both are severely compromised by cells that express a temperature sensitive dynamin mutant (Shibire) or a dominant negative form of the small GTPase Rab5, which is essential for endosomal function. These genetic experiments indicate that the range of Dpp signaling is controlled by endocytic trafficking, which is involved in either establishing the proper distribution of the ligand, or for its signaling function at the single cell level, or both. Our results strongly suggest that endocytosis positively regulates signaling of TGFβ at the single cell level.

Studies on the role of internalization on other ligand-activated receptor systems has, in general, supported the concept that endocytosis is a mechanism to attenuate signaling. Thus, inhibition of internalization leads to either no effect or to enhanced proximal signaling events (Kao et al., 1998). Attenuating effects of dominant negative dynamin on the MAP kinase pathway have been reported, but these only partially affect MAP kinase activation (Ceresa and Schmid, 2000; Johannessen et al., 2000). Thus, the marked impairment of TGFβ-stimulated Smad2 phosphorylation, and apparent competitive inhibition of Smad2 nuclear translocation upon inhibition of endocytosis is unprecedented. Moreover, the requirement for membrane trafficking to achieve a productive association between two components of a signaling pathway (e.g., receptor and adapter) is also unprecedented.

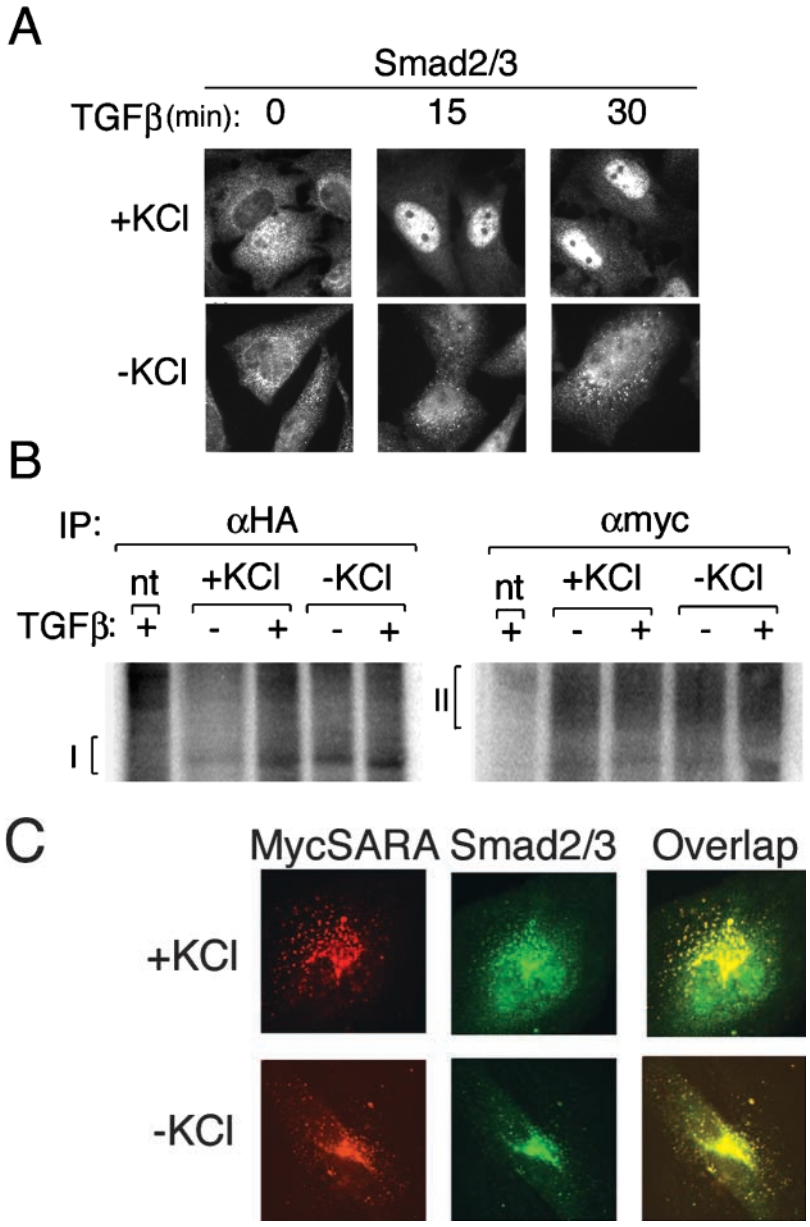


Figure 8. **Potassium depletion impairs TGFβ-induced Smad2/3 translocation, but not TGFβ receptor activation or the interaction between SARA and Smad2.** (A) Control (+KCl) or potassium-depleted (−KCl) Mv1Lu cells were incubated for the indicated time with 100 pM TGFβ. Cells were stained for Smad2/3. (B) Cos-7 cells were transfected with myc-tagged type II TGFβ receptor (RII) and HA-tagged type I receptor (RI). Cells were labeled with ^{32}P-labeled inorganic phosphate as described in Materials and methods. Cells were subjected to the potassium depletion protocol in the absence (−KCl) or presence (+KCl) of 10 mM KCl and the incubated with (+) or without (−) TGFβ for 15 min. Receptors were immunoprecipitated using antibodies against the epitope. (C) Control (+KCl) or potassium-depleted (−KCl) Mv1Lu cells expressing *myc*-SARA were fixed and stained with a monoclonal antibody against Smad2/3 (left) and polyclonal antibody against *myc* (middle). The overlap between the signals is displayed in yellow (right).

Mechanistically, the requirement of endocytosis for TGFβ signaling can be explained by two different models. In the first model, internalization of the TGFβ receptor is required for its functional interaction with SARA/Smad2 complexes, which are restricted to the endosome by virtue of the interaction of the SARA FYVE domain with PI(3)P. In the second model, complexes between SARA, Smad2, and the TGFβ receptor may form at the plasma membrane, but their productive interaction requires internalization into the endosome. In this case, the endosome may provide a more favorable biochemical environment, for example by being less enriched in phosphatases, or a more favorable cellular environment being positioned more closely to the nuclear membrane. Although further experiments are necessary to distinguish among these mechanisms, the important role for endosomal localization for TGFβ signaling extends our insights on the biological role of the endosome as a compartment specialized for the assembly and propagation of specific extracellular signals.

Materials and methods

Constructs

The plasmid encoding myc-tagged hSARA was provided by Dr. Jeffrey Wrana (University of Toronto, Toronto, Ontario). A plasmid encoding the dominant-negative dynamin K44E was provided by Dr. Richard Vallee (Columbia University, New York, NY). The plasmid encoding the 3TP-Lux reporter gene was provided by Dr. Malcom Whitman (Harvard Medical School, Boston, MA). Plasmids encoding TβRI with the HA epitope inserted between amino acids 27 and 28 and TβRII with the c-myc epitope between amino acids 26 and 27 were provided by Dr. Rebecca Wells (Yale University, New Haven, CT). Plasmids were recloned into pcDNA3.1+ (Invitrogen) using HindIII/NotI restriction sites for the type I receptor and BamHI/EcoRI for the type II receptor, and confirmed by sequencing. The pRL CMV plasmid was purchased from Promega. The pGreenLantern plasmid encoding GFP was purchased from GIBCO BRL. GFP-SARA-FYVE was generated by inserting a 204-bp fragment (coding for amino acids 589–656) into a modified pGreen Lantern vector (having an XbaI site after GFP) at the Xba1 site.

Cell culture and transfection

Cos, HeLa, and Mv1Lu cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Life Technologies, Inc.). HeLa cells stably transfected with HA wild-type or dynamin K44A under a

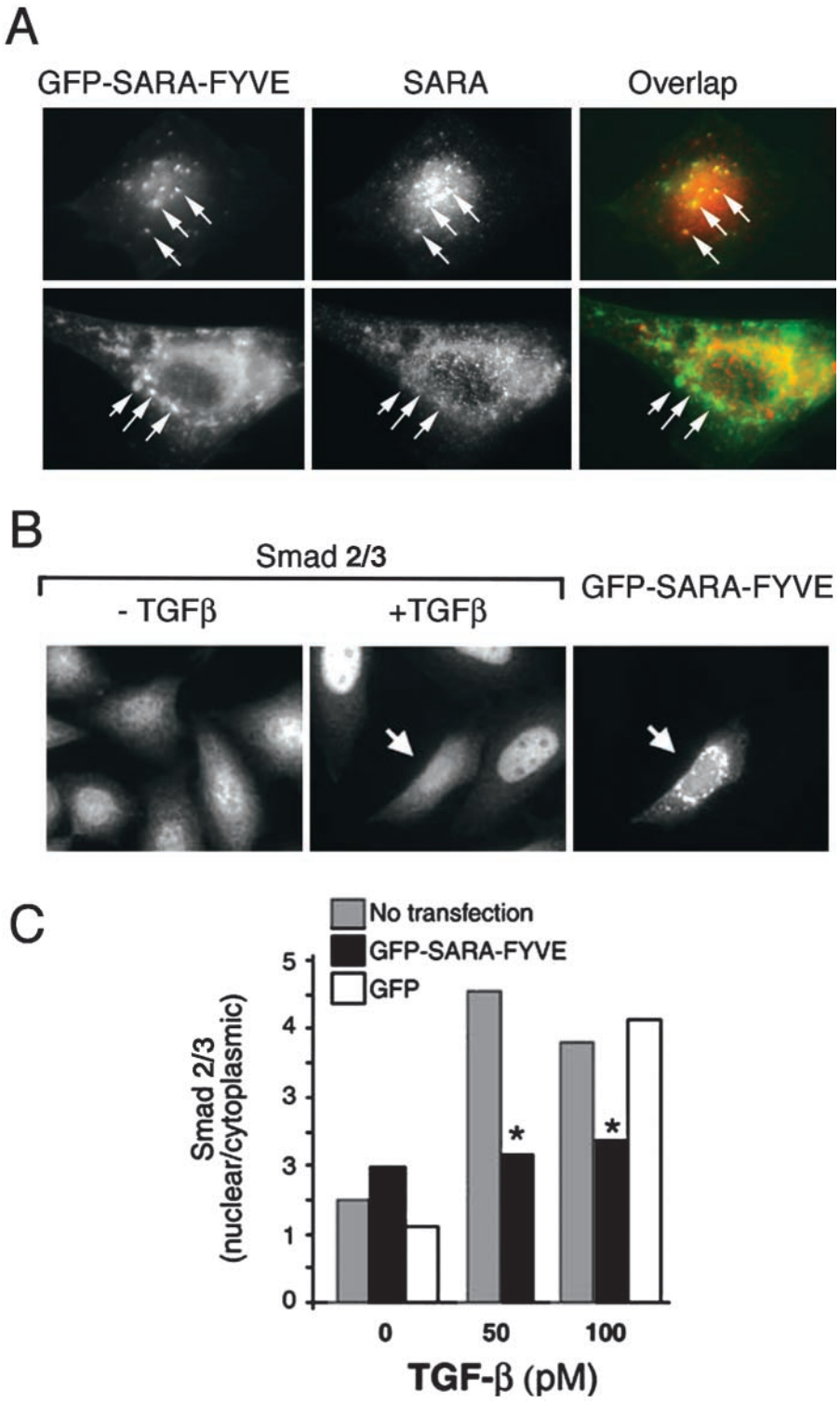


Figure 9. **Effect of GFP-SARA-FYVE overexpression on SARA Localization and TGFβ induced Smad2/3 nuclear translocation.** (A) Mv1Lu cells were transfected with GFP-SARA FYVE. Cells were fixed and stained for endogenous SARA (middle). The overlap of the two signals is displayed in the right-most panels. The localization of several endosomes containing GFP-SARA-FYVE is indicated by the arrows; note the colocalization with endogenous SARA at low levels of expression (top right, yellow signal), and its absence at higher levels of overexpression (bottom right, green signal). (B) HeLa cells were transfected with GFP-SARA-FYVE and treated without (left) or with 100 pM TGFβ for 30 min (middle and right). The cells were stained with antibodies against endogenous Smad2/3 (left and middle). GFP SARA FYVE (left) was viewed using a GFP filter set. The arrow indicates the transfected cell. (C) The nuclear/cytoplasmic ratio of Smad2/3 in nontransfected cells (gray bars), cells expressing GFP-SARA-FYVE (black bars) or cells expressing GFP alone (white bars) was measured in HeLa cells treated with 0, 50, or 100 pM TGFβ. Plotted are the averages of 20 cells per condition, taken from two independent experiments. Statistically significant ($P < .01$) differences between nontransfected and transfected cells are indicated by the asterisk.

tetracycline repressible promoter (Damke et al., 1995) were provided by Dr. Sandra L. Schmid (The Scripps Research Institute, La Jolla, CA). Transfections were performed by the calcium phosphate precipitation method or by using FuGENE 6 Transfection reagent (Roche).

Receptor internalization

Experiments were performed 24–36 h posttransfection. Live, nonpermeabilized Cos-7 cells were incubated at 4°C for 1 h mouse anti-Myc (Neo Markers), rabbit anti-myc (Upstate Biotechnology), or rabbit anti-HA (Upstate Biotechnology) and 100 pM TGFβ. The antibodies were dialyzed before use in 50 mM Hepes, 100 mM NaCl, pH 7.4. Antibody- and ligand-bound receptors were allowed to internalize by incubating the cells at 37°C for the times indicated. In experiments involving dynamin function cells were then rapidly washed three times with citrate buffer (150 mM NaCl, 20 mM sodium citrate, pH 2.5), once with ice-cold PBS, fixed in 4% formaldehyde in PBS, and permeabilized with 0.05% digitonin or 0.5% Triton X-100. The localization of the anti-myc or anti-HA antibodies was detected with appropriate fluorescent secondary antibodies.

Immunofluorescence

Cells were routinely fixed in 4% formaldehyde in PBS and permeabilized with 0.05% digitonin or 0.5% Triton X-100 in PBS for 5 min at room tem-

perature. Smad2/3 was detected using mouse anti–Smad2/3 (Transduction Laboratories). *Myc* was detected using rabbit anti-*Myc* (Upstate Biotechnology) or mouse anti-*Myc* (Neo Markers). EEA1 was detected using a mouse monoclonal to EEA1 (Transduction Laboratories) or human autoimmune antiserum to EEA1 (Monash Clinical Immunology Laboratory). HA tag was detected using rabbit anti-HA (Upstate Biotechnology). SARA was detected using a rabbit polyclonal antibody to SARA (H-300) (Santa Cruz Biotechnology, Inc). Dynamin was detected using a monoclonal antibody to dynamin I (Transduction Laboratories). Rhodamine-conjugated donkey anti–rabbit antibody or donkey anti–mouse antibody, fluorescein isothiocyanate-conjugated donkey anti–mouse antibody or donkey anti–rabbit antibody (Jackson ImmunoResearch Laboratories), Alexa 594 or 488 goat anti–human and Alexa-Flour 350, 488, and 594 conjugated goat anti–rabbit or goat anti–mouse secondary antibodies (Molecular Probes) were used to visualize primary antibodies.

Transferrin uptake

Cells were incubated with 5 μg/ml Alexa-594-labeled human transferrin (Molecular Probes) or 25–50 μg/ml mouse transferrin (Jackson ImmunoResearch Laboratories), which was labeled using an Alexa-594 Protein Labeling Kit (Molecular Probes). Cells were incubated for the indicated time at 37°C. Surface-bound transferrin was removed by three washes on ice in citrate buffer (150 mM NaCl, 20 mM sodium citrate, pH 5.0). Cells were either fixed for fluorescence microscopy, or lysed in SDS sample buffer. Lysates were analyzed by SDS-PAGE. Alexa-594 transferrin was visualized on nitrocellulose blots of these gels using the red fluorescence scan option on the Storm 860 phosphoimager (Molecular Dynamics).

Potassium depletion

Cells were depleted of potassium as described before (Larkin, 1986). Briefly, cells were incubated at 37°C for 5 min in DME/H2O (1:1) followed by 10 min in 50 mM Hepes, 100 mM NaCl, pH 7.4 and 30 min in 50 mM Hepes, 100 mM NaCl, 1 mM $CaCl_2$, 2.5% BSA, pH 7.4. Control cells were treated as above but buffers contained 10 mM KCl. In experiments in which TGFβ-receptor internalization was monitored, cells were then cooled to 4°C and incubated for 60 min with TGFβ anti-myc or anti-HA antibodies. In all other experiments, the cells were then incubated for the times indicated at 37°C, in the presence or absence of TGFβ, as indicated.

Smad2 phosphorylation

Cells were grown in 12-well multiwell dishes, and treated as indicated in each experiment. At the end of the experiment, cells were washed three times in ice-cold PBS, and scraped into 100 μl of SDS-sample buffer. Aliquots of the lysate were separated by PAGE, blotted onto nitrocellulose, and probed with a rabbit antibody raised to phosphoSmad2 (Upstate Biotechnology). Blots were acid-washed and reprobed with a monoclonal antibody raised to Smad2/3 (Transduction Labs). Band intensities were measured using the selection tool and histogram functions in Adobe Photoshop (v. 7.0).

Receptor phosphorylation

Cos-7 cells in 100-mm dishes were cotransfected with HA-tagged type I and myc-tagged type II receptors. After 36 h, cells were incubated in phosphate-free DME containing 1% fetal bovine serum for 2 h. Cells were subsequently labeled with 0.5 mCi/ml of ^{32}P-labeled inorganic phosphate (New England Nuclear) for 2 h. Cells were incubated at 37°C for 5 min in DME/H2O (1:1) followed by 10 min in 50 mM Hepes, 100 mM NaCl, pH 7.4, and then for 30 min in 50 mM Hepes, 100 mM NaCl, 1 mM $CaCl_2$, 2.5% BSA, pH 7.4 with 0.5 mCi/ml of ^{32}P-labeled inorganic phosphate. Control cells were treated the same but all buffers contained 10 mM KCl. Cells were then washed three times on ice in 50 mM Hepes, 100 mM NaCl, pH 7.4, and lysed in lysis buffer (50 mM Tris, pH 8.0, 1% Triton X-100, 1% 2-deoxycholate, 0.1% SDS, 50 mM NaF, 0.1 mM sodium vanadate, 1 mM DTT, Tame, 4 μg/ml leupeptin, 0.2 mM PMSF, 1 mM 1,10 phenanthroline, 1 mM benzamidine). Cell lysates were spun at 14,000 rpm for 10 min. Supernatants were incubated sequentially with protein A Sepharose only (30 min), with polyclonal anti-HA antibody (UBI) prebound to protein A Sepharose beads (120 min) and with polyclonal anti-myc antibody (UBI) prebound to Sepharose beads (120 min). After each incubation beads were collected by centrifugation and washed three times for 5 min with lysis buffer. Immunoisolates were separated by SDS-PAGE and transferred to nitrocellulose blots; phosphorylated bands were visualized using a Storm 860 phosphoimager (Molecular Dynamics).

Transcriptional response assay

HeLa cells stably transfected with tetracycline-repressible wild-type or dominant-negative dynamin K44A were plated into 24-well multiwell dishes in tetracycline-free media, and cotransfected with pRL-CMV and 3TP Lux using calcium phosphate. After 48 h cells were treated with 100 pM TGFβ for 14–16 h. Luciferase activity was measured using Dual Luciferase Reporter System (Promega).

Quantification and statistical analysis

Regional fluorescence intensities were quantified using Adobe Photoshop (v. 7.0) software. Black and white images were converted to RGB format, and colorized using the fill command with the multiply option. Images were overlayed using the apply-image command with the screen option. To measure regional intensities, small circles within the cytoplasmic or nuclear regions of each cell were selected using the elliptical marquee tool. The intensity within each circle was obtained using the histogram function for each color channel, which was selected using the layers/channels palette. The values were recorded, and the ratio of the nuclear to cytoplasmic intensity was recorded for at least 20 cells per experiment. The statistical significance of the analyses was evaluated using the paired Student's *t* test.

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