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Early Endosomal Regulation of Smad-dependent Signaling in Endothelial Cells*

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Transforming growth factor β (TGFβ) receptors require SARA for phosphorylation of the downstream transducing Smad proteins. SARA, a FYVE finger protein, binds to membrane lipids suggesting that activated receptors may interact with downstream signaling molecules at discrete endocytic locations. In the present study, we reveal a critical role for the early endocytic compartment in regulating Smad-dependent signaling. Not only is SARA localized on early endosomes, but also its minimal FYVE finger sequence is sufficient for early endosomal targeting. Expression of a SARA mutant protein lacking the FYVE finger inhibits downstream activin A signaling in endothelial cells. Moreover, a dominant-negative mutant of Rab5, a crucial protein for early endosome dynamics, causes phosphorylation and nuclear translocation of Smads leading to constitutive (*i.e.* ligand independent) transcriptional activation of a Smad-dependent promoter in endothelial cells. As inhibition of endocytosis using the K44A negative mutant of dynamin and RN-tre did not lead to activation of Smad-dependent transcription, the effects of the dominant-negative Rab5 are likely to be a consequence of altered membrane trafficking of constitutively formed TGFβ/activin type I/II receptor complexes at the level of early endosomes. The results suggest an important interconnection between early endosomal dynamics and TGFβ/activin signal transduction pathways.

The transforming growth factor β (TGFβ)[1] superfamily is a large group of secreted polypeptide growth factors, which include the TGFβs, the activins, and the bone morphogenetic proteins. Members of this family play critical roles during embryogenesis and in maintaining tissue homeostasis in adult life. Deregulated TGFβ family signaling has been implicated in multiple developmental disorders and in various human diseases, including cancer (1). Some of these disorders, such as hereditary hemorrhagic teleangiectasia and primary pulmonary hypertension, involve altered TGFβ family signaling regulating vasculogenic and angiogenic responses of endothelial cells (2–6). Indeed, TGFβ1 is known to influence both endothelial cell proliferation and critical endothelial cell-pericyte interactions occurring during vessel maturation (7). We have recently shown also that activin A affects endothelial cell function leading to inhibition of angiogenesis and decreased vessel wall integrity (8).

The TGFβ/activin family members signal through heteromeric complexes of transmembrane type I and type II serine-threonine kinase receptors. The type II receptor kinase phosphorylates the type I receptor kinase which in turn phosphorylates the downstream transducer proteins, Smad2 and Smad3 (reviewed in Ref. 9). The latter associate with Smad4 and the resulting complex translocates to the nucleus, where they control transcription of target genes. Recent data show that, in the case of the TGFβ receptor and most likely in the case of activin (10), the Smad-binding protein SARA plays an important role in phosphorylation of Smad2 and Smad3 by TGFβRI and ActRIB receptors (10, 11). SARA recruits Smad2 and Smad3 to intracellular membranes that contain the receptor. This targeting requires a FYVE finger, which by analogy to other FYVE fingers (reviewed in Ref. 12) has been speculated to bind specifically to phosphatidylinositol 3-phosphate (PI(3)P). Even though the subcellular localization of SARA (11) is as yet uncharacterized, we hypothesized that this protein may localize to early endosomes, which are known to be enriched for PI(3)P (13).

The concept that activated receptors interact with downstream signaling molecules at discrete endocytic locations has long been suspected (reviewed in Ref. 14). Furthermore, membrane trafficking plays an important role in controlling the location of signaling interactions and in regulating receptor degradation and/or recycling (reviewed in Ref. 15). In the case of the TGFβ/activin receptors, little is known about how endocytosis and receptor trafficking influences the assembly, localization, and activation of ligand-receptor-SARA-Smad complexes. Indeed, the temporal and spatial regulation of these interactions is not fully understood. Since SARA binds to membranes via its FYVE finger providing a potential link between membrane trafficking and TGFβ/activin signaling, we have addressed the intracellular localization of SARA and investigated the requirements for its FYVE finger-membrane lipid

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[1] The abbreviations used are: TGFβ, transforming growth factor β; PI(3)P, phosphatidylinositol 3-phosphate; BBCE, bovine brain capillary endothelial; FCS, fetal calf serum; SBE, Smad-binding element; luc, luciferase; GFP, green fluorescent protein; CMV, cytomegalovirus; β-gal, β-galactosidase; TRITC, tetramethylrhodamine isothiocyanate.

interaction. Motivated by our finding that SARA is localized on early endosomes, we reasoned that experimental perturbation of proteins which regulate endosome function would allow us to address the contribution of the endocytic pathway in the control of TGFβ/activin signaling. Toward this purpose, we have investigated the effects on Smad-dependent signaling of constitutively active and dominant-negative mutant forms of Rab5, a small GTPase that plays a key role in endosome dynamics and receptor signaling (16–20).

EXPERIMENTAL PROCEDURES

Cell Culture—Primary bovine brain capillary endothelial (BBCE) cells were cultured in Dulbecco's modified Eagle's medium, 1 g/liter D-glucose, 10% newborn calf serum. Basic fibroblast growth factor (2.5 ng/ml) was added to the cells every second day until confluent. Cells were maintained at 10% CO_2. Baby hamster kidney-21 cells were cultured in Glasgow minimal essential medium supplemented with 5% heat-inactivated fetal calf serum (FCS) and 10% tryptose phosphate. Human kidney embryonal (293) cells were cultured in RPMI 1640 containing 10% FCS. Human keratinocytes (HaCaT) were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose and 10% FCS. African green monkey kidney (COS-1) cells were cultured in Dulbecco's modified Eagle's medium containing 1 g/liter D-glucose and 10% FCS. Baby hamster kidney-21, 293, HaCaT, and COS-1 cells were maintained at 5% CO_2. All media were supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (4 mM for COS cells). All media and reagents for cell culture were purchased from Invitrogen and were endotoxin-free.

Cell Proliferation Assays—BBCE cells were infected with recombinant adenoviruses expressing GFP alone, or GFP and Rab5S34N, or GFP and Rab5Q79L at an multiplicity of infection of 25 (70 for the GFP-Rab5Q79L set), for 2 h (medium replacement). The next day, cells were split into 12-well dishes at 5,000 cells per well, and 24 h later, control wells were counted to ensure equal cell numbers. Experimental wells were then stimulated with basic fibroblast growth factor (2.5 ng/ml) and counted 2 days later in triplicate. HaCaT cells were first split into 12-well dishes, at 10,000 cells per well, and then infected, for 2 h, at an multiplicity of infection of 100, with adenoviruses expressing either GFP alone, or GFP and Rab5S34N. Cells in triplicate wells were counted every day, for three consecutive days, starting the day following infection.

Intracellular Localization of SARA—BBCE cells were trypsinized 24 h prior to transfection and were seeded onto 11-mm glass coverslips. Cells were infected with T7 RNA (16) or modified Ankara T7 RNA polymerase recombinant vaccinia viruses (21) and transfected with pGEM-T7-FLAG-SARA and T7-human transferrin receptor (T7-hTR) or pGEM-T7-FLAG-SARA and pGEM-T7-GFP-Rab5Q79L, using LipofectAMINE or LipofectAMINE Plus (Invitrogen). The cell number (100,000 cells), DNA concentration, and lipid were constant. Following transfection, the cells were incubated in OptiMEM medium (Invitrogen) for 4 h at 37 °C in 5% CO_2. Alexa transferrin (50 μg/ml) (Molecular Probes) was internalized for 20 min in the cells transfected with pGEM-T7-FLAG-SARA and T7-hTR. Lysotracker (Molecular Probes) was internalized for 30 min at 50 nM concentration in cells expressing pGEM-T7-FLAG-SARA alone. Hydroxyurea was present at all times to prevent late viral gene expression (16).

Cells were fixed either with methanol for 20 min at −20 °C or with 3.7% paraformaldehyde, quenched with 50 mM ammonium chloride for 15 min, permeabilized with 0.1% Triton X-100 for 4 min, and nonspecific sites were blocked with 10% FCS. Primary and secondary antibodies were diluted in 5% FCS. Fluorescein isothiocyanate or TRITC-conjugated donkey anti-mouse and anti-rabbit IgG secondary antibodies were purchased from Dianova and used at 1:200 dilution. Coverslips were mounted in Mowiol containing 100 mg/ml diazabicyclo(2.2.2)octane (Sigma), and viewed using a Leica TCS-SP scanning confocal microscope, equipped with an Argon/Krypton laser and Leica TCS software. The 488 and 568 wavelengths were used to excite fluorescein isothiocyanate (or Alexa transferrin) and TRITC (or Lysotracker), respectively.

Surface Plasmon Resonance—Surface plasmon resonance was recorded at 25 °C on a BiaCore X (BiaCore, Sweden) as described (22). The liposomes used contained 63% phosphatidylcholine, 20% phosphatidylserine, 15% phosphatidylethanolamine, and 2% PI(3)P (Echelon). Liposomes (0.35 mg/ml) were loaded onto a Biacore L1 chip by three successive injections of 80 μl of liposomes at a flow rate of 5 μl/min. The reference cell was loaded with similar liposomes lacking PI(3)P. Sensograms were recorded upon the injection of 0.1–2 μg of protein at a flow rate of 20 μl/min. The lipid surface was regenerated using 10 mM NaOH.

Constructs—The reporter construct used to monitor activin A transcriptional activity consisted of multiple copies of the Smad-binding element fused to luciferase (SBE-luc) (23), and was kindly provided by Peter ten Dijke (The Netherlands Cancer Institute, Amsterdam). NF-κB-luciferase (κB-TK5-luc) was kindly provided by Christoph Esslinger (Ludwig Institute for Cancer Research, University of Lausanne, Switzerland) and E-selectin-luciferase (E-selectin-luc) by Shosaku Narumi (Department of Preventive Medicine, University of Tokyo, Japan) (24). An SV40-luciferase construct in pGL3 (SV40-luc) was purchased from Promega.

FLAG-tagged SARA wild type (wt) and SARAΔ1–664 mutant constructs have been previously described (11). FLAG-tagged SARA wt was cloned into pGEM-1 to generate pGEM-T7-FLAG-SARA. SARA FYVE finger residues 574–660 were amplified by PCR and cloned into pGEM-T7-myc to generate the pGEM-T7-mycSARA574–660 construct. The pGEX-SARA498–660 construct was generated by cloning a PCR fragment of SARA into pGEX (Amersham Biosciences). The SARA498-660-glutathione *S*-transferase fusion protein was purified using glutathione-Sepharose 4B (Amersham Biosciences). For expression using the T7 RNA polymerase recombinant vaccinia system, full-length or truncated SARA constructs were amplified by PCR and cloned downstream of a Myc epitope in pcDNA3 (Invitrogen). Kinase-dead ALK2, ALK4, and ALK5 constructs and HA-ALK4 wt and ActRIIB were kindly provided by Peter ten Dijke. Myc-tagged SmadΔ3c and -Δ4c were kindly provided by Rik Derynck (University of California, San Francisco, CA).

PGEM-1-green fluorescent protein (GFP)-tagged Rab5Q79L construct (GFP-Rab5Q79L) was generated by cloning, in-frame, the coding region of Rab5Q79L in pEGFP-C3 (CLONTECH) and ligating into pGEM-1. Rab5S34N-myc-tagged and Rab5Q79L-myc-tagged were cloned into a CMV expression cassette p163/7 (25) where the H2 promoter was exchanged with the CMV promoter. The RN-tre construct was kindly provided by Pier Paolo Di Fiore (European Institute of Oncology, Milan, Italy), and wt and K44A Dynamin constructs by Sandra Schmid, Dept. of Cell Biology, The Scripps Research Institute, La Jolla, CA. All DNA constructs generated by PCR were sequenced and purified using the Endo-Free kit from Qiagen to avoid toxicity from LPS on endothelial cells.

Construction of Recombinant Adenoviruses—Recombinant adenoviruses were made according to He *et al.* (26), as fully described (www.coloncancer.org/adeasy.htm). All vectors and bacteria for the generation of adenoviruses were kindly provided by Bert Vogelstein (The John Hopkins Medical Institutions, Baltimore, MD). Briefly, FLAG-tagged SARAΔ1–664 (11) was cloned as a *Sna*BI-*Sma*I fragment into the expression vector pADTrack-CMV in the *Eco*RV site. In the case of Rab5S34N, the Myc-tagged coding sequence was cloned into the *Bgl*II site of pADTrack-CMV. Rab5Q79L was also cloned into the *Bgl*II site of pADTrack-CMV. Homologous recombination was carried out in BJ5183 bacteria, recombinants were characterized by restriction analysis and transformed into DH10B cells. The *Pac*I-digested recombinants were transfected into 293 cells and the adenovirus produced was amplified exactly as described (www.coloncancer.org/adeasy.htm). Viral titers were determined in 293 cells by counting GFP positive cells. A control adenovirus expressing GFP alone was also generated. 293 cell lysates of amplified viruses were used for these experiments.

Antibodies and Recombinant Proteins—A polyclonal antibody recognizing EEA1 was previously described (27). Anti-FLAG M2 antibody (F3165) was purchased from Sigma. Anti-phospho-Smad2 polyclonal antibody was a gift from Aris Moustakas (Ludwig Institute, Sweden). 9E10 (anti-Myc) and 12Ca5 (anti-HA) monoclonal antibodies were purified from the corresponding hybridomas using standard techniques. A rabbit polyclonal antibody raised against Rab5 was used following affinity purification. A rabbit polyclonal antibody recognizing SARA was purchased from Santa Cruz. Recombinant activin A was purified from WAC2 human neuroblastoma cells overexpressing the protein.[2] The activity of the recombinant activin A was tested for its ability to inhibit BBCE cell proliferation (8).

Reporter Assays for Smad-dependent Transcription—BBCE cells were trypsinized and plated into 6-well plates at 500,000 cells per well. Transfection was carried out the next day using either LipofectAMINE or LipofectAMINE Plus in OptiMEM medium (all from Invitrogen). OptiMEM medium was removed and full medium was added to the cells 4 h after transfection and 20 h later the cells were placed into reduced

[2] E. Panopoulou *et al.*, manuscript in preparation.

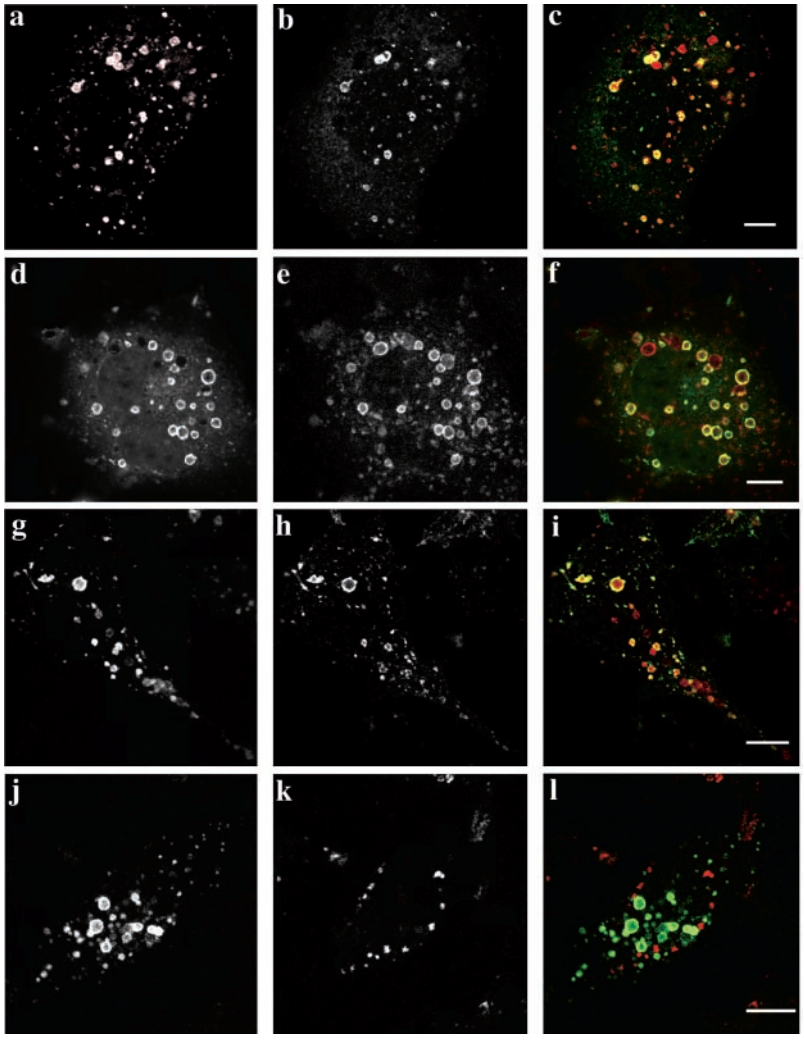

FIG. 1. **Intracellular localization of SARA in endothelial cells.** BBCE cells transfected with FLAG-tagged SARA were processed for immunofluorescence using anti-FLAG antibody (*a*, *e*, *g*, and *j*). EEA1 was stained by a specific antibody/fluorescein isothiocyanate-labeled secondary antibody complex (*b*), GFP-Rab5Q79L was detected by visualizing GFP directly (*d*), human transferrin receptors were labeled by fluorescent Alexa-transferrin uptake (*h*), and late endosomes/lysosomes were localized by Lysotracker (*k*). Overlays are shown in *c*, *f*, *i*, and *l*. Size bars are 10 μm.

serum medium (0.2% newborn calf serum) for 8 h. Then, cells were treated or not with 50 ng/ml activin A and incubated for an additional 16 h. Finally, cells were processed for luciferase as described in the Promega E4030 luciferase kit, and β-galactosidase (β-gal) was measured using a standard protocol. Relative light units were measured using a Berthold luminometer and standardized for transfection efficiency using the β-gal values. Assays were repeated 3 times.

RESULTS

SARA Localizes to an Early Endocytic Compartment—To determine the intracellular localization of SARA, we expressed the FLAG-tagged protein in BBCE cells and checked the co-localization of SARA with markers of cellular compartments. As has been previously shown (11), SARA was localized to vesicular structures (Fig. 1*a*). There was a high degree of co-localization of SARA and the early endosome marker EEA1 on these vesicular structures, indicating that these vesicles represented early endosomes (Fig. 1, *a–c*). However, some SARA positive vesicles were negative for EEA1. At high expression levels, SARA increased the size of early endosomes thus suggesting a possible role of SARA in endosome fusion, a possibility that merits further investigation.

Further experiments supported the early endosomal localization of SARA, as it co-localized with two other early endosome markers, Rab5Q79L, a GTPase-deficient mutant of Rab5, and internalized transferrin (Fig. 1, *d–f* and *g–i*). In neither case, however, was the co-localization complete, some vesicular structures which were positive for SARA being negative for Rab5 and transferrin. Localization of SARA to the late endocytic and lysosomal compartment was excluded as it did not co-localize with Lysotracker, a marker of late endosomes and lysosomes (Fig. 1, *j–l*) (28).

The FYVE Finger of SARA Interacts Strongly with the Membrane Lipid PI(3)P and Is Sufficient for Endosomal Targeting—Two FYVE finger-containing proteins, EEA1 and Hrs, require additional amino acid sequences to the FYVE finger for their endosomal targeting (29, 30). To study if the same occurs also with SARA, we expressed its FYVE finger alone (residues 574–660) fused to the c-Myc epitope tag. Surprisingly, this protein localized to early endosomes where it colocalized with EEA1 (Fig. 2, *d–f*), in a manner identical to that of full-length SARA (Fig. 2, *a–c*). This suggested that the SARA FYVE finger may have higher affinity for PI(3)P than the FYVE fingers of Hrs and EEA1. We therefore measured its binding to PI(3)P using surface plasmon resonance. A lipid mixture containing 2% PI(3)P was immobilized onto a sensor surface and sensograms recorded upon injection of SARA498–660-glutathione *S*-transferase protein at the indicated concentrations (Fig. 3*A*). The SARA FYVE finger was indeed found to bind strongly to PI(3)P, with an estimated K_D value of 30 nM (Fig. 3*A*). This is a lower value than those measured previously for Hrs and EEA1 (38 and 45 nM, respectively) (13, 22). This small difference, albeit significant, may account for the more efficient targeting of the SARA FYVE finger to early endosomes, compared with the Hrs and EEA1 FYVE fingers. To investigate whether the membrane association of SARA *in vivo* requires PI 3-kinase activity to generate PI(3)P, we treated cells expressing SARA with the PI 3-kinase inhibitor wortmannin (100 nM).

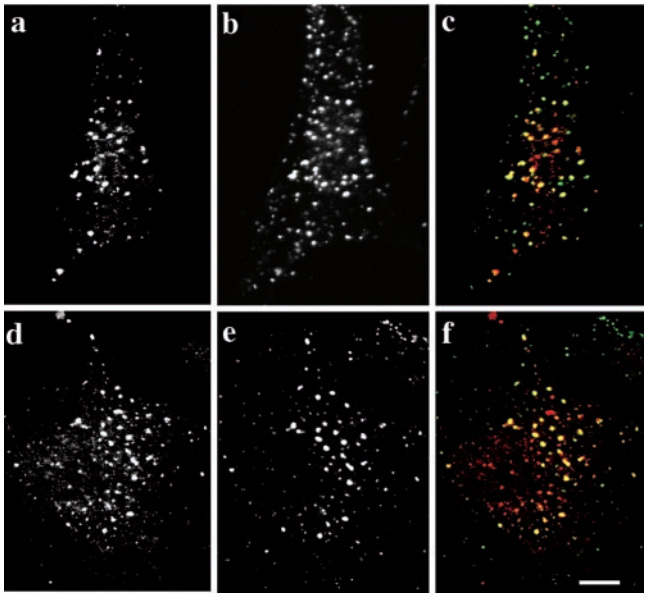

FIG. 2. **A minimal FYVE finger is sufficient to localize SARA to early endosomal membranes**. Baby hamster kidney cells were transfected with Myc-tagged full-length SARA (*a*) or a minimal FYVE finger of SARA consisting of amino acids 574 to 660 (SARA574–660) (*d*) and processed for immunofluorescence using an anti-Myc antibody. Endogenous EEA1 was detected in the same cells (*b* and *e*) using an anti-EEA1 antibody. Overlays are shown in *c* and *f*. Size bars are 10 μm.

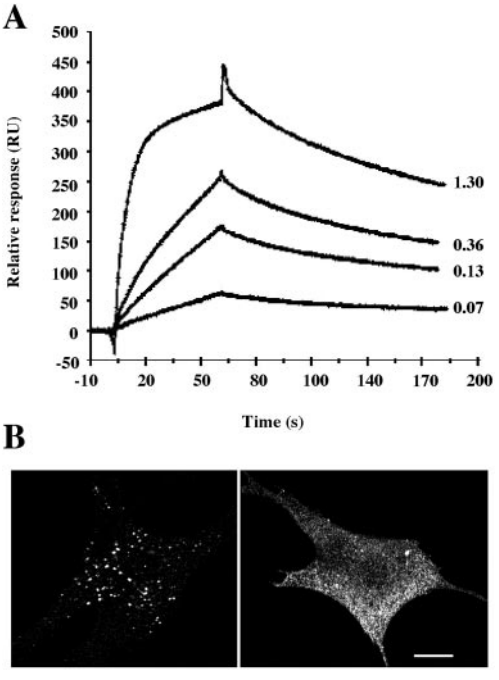

FIG. 3. **Association and dissociation kinetics of SARA FYVE finger with PI(3)P and removal of SARA from membranes by wortmannin.** *A*, plasmon resonance sensograms obtained by injecting the indicated quantities (μg) of SARA FYVE finger. A K_D of 30 nM for the association of SARA FYVE finger with PI(3)P was calculated. *B*, baby hamster kidney cells transfected with Myc-SARA were either left untreated (*left panel*) or were treated (*right panel*) with wortmannin. The cells were then fixed and incubated with the 9E10 antibody recognizing Myc-tagged SARA. Size bar is 10 μm.

SARA was removed from the endosomal membrane upon wortmannin treatment (Fig. 3*B*), indicating that the membrane localization of SARA, like that of EEA1 and Hrs, requires PI 3-kinase. Taken together, our results show that SARA is targeted to early endosomes through the binding of its FYVE finger to PI(3)P.

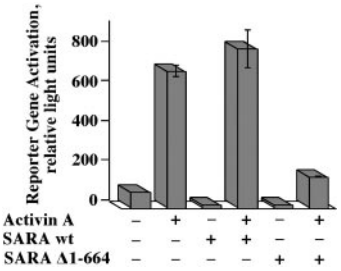

FIG. 4. **SARA mutants lacking the FYVE finger inhibit activin A signaling in endothelial cells**. BBCE cells were transfected with SBE-luc, CMV-β-gal, and either control vector or SARAwt or SARAΔ1–664 constructs and 24 h later were placed in 0.2% serum for an additional 8 h. Cell were then treated or not with 50 ng/ml activin A and incubated for 16 h. Relative light units were measured and standardized for transfection efficiency using β-gal values. Data are mean ± S.D. of triplicate determinations. The inhibition by SARAΔ1–664 is statistically significant ($p < 0.001$).

SARA Participates in Activin A Signaling in Endothelial Cells—Alhough SARA is known to participate in TGFβ signaling (11), it is not clear whether it is also a component of the signaling cascade of activin A in endothelial cells. For this purpose, we transfected BBCE cells with a construct consisting of multiple copies of Smad-binding element fused to the luciferase gene (SBE-luc) in the presence of wild type SARA or a mutant SARA lacking the FYVE finger, SARAΔ1–664, which cannot associate with endosomal membranes (Ref. 11 and data not shown). Whereas wild type SARA did not alter the response of the SBE-luc to activin A, SARAΔ1–664 clearly blocked the activin A-dependent transcription of SBE reporter construct (Fig. 4). Thus, it appears that, in endothelial cells, SARA recruits Smad proteins also to activin A receptor complexes (10).

Dominant-negative Rab5 Activates Transcription of Smad-dependent Promoters in a Ligand-independent Manner—Our findings show that SARA is an early endosomal protein involved in TGFβ/Smad signaling. The localization of SARA on early endosomes raises the question regarding the role of endocytosis and endosome dynamics on this signaling pathway. To investigate the possible influence of endocytic dynamics in the regulation of TGFβ/activin signaling, we investigated the effect of Rab5 on Smad-dependent transcriptional activation. Rab5 is a key regulator of early endocytic trafficking, and several mutants of this protein have been well characterized with regard to their activity on endosome function. A constitutively active, GTPase-deficient mutant of Rab5, Rab5Q79L, enhances endocytosis and early endosome fusion causing the formation of enlarged endosomes (31), whereas a GDP-binding dominant-negative mutant, Rab5S34N, inhibits endocytosis and early endosome fusion. We transfected endothelial cells with a GTPase-deficient (Rab5Q79L) or a dominant-negative mutant of Rab5 (Rab5S34N) and tested the effect on SBE-luc transcription. Remarkably, in the absence of ligand, Rab5S34N caused a large increase of Smad-dependent transcription (Fig. 5*A*). In the presence of activin A, the constitutively active Rab5Q79L caused a 50% inhibition, whereas the dominant-negative mutant Rab5S34N appeared to slightly stimulate activin A-induced SBE-luc transcription (Fig. 5*B*). To rule out the possibility that Rab5S34N causes TGFβ or activin A release from BBCE cells with potential autocrine stimulation of SBE-luc, we collected the medium from BBCE cells expressing Rab5S34N and tested it on cells expressing the SBE-luc construct. We found no stimulation of SBE-luc transcription, indicating that the stimulatory effect of Rab5S34N is not due to secreted molecules (data not shown). Since experiments in the absence of ligands, in this and other studies, are carried out in 0.2% serum which do not exclude the presence of minute quan-

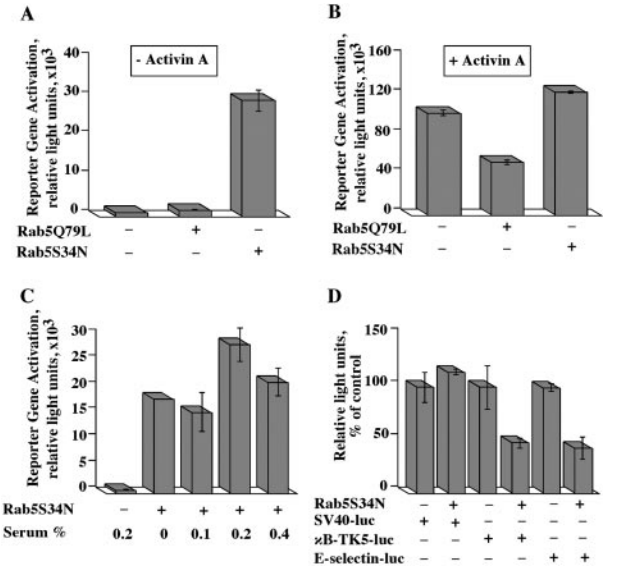

FIG. 5. **Rab5S34N stimulates Smad-dependent transcription specifically and in a ligand-independent manner.** BBCE cells were transfected with SBE-luc, CMV-β-gal, and either control vector or Rab5Q79L or Rab5S34N constructs and 24 h later were placed in 0.2% serum for an additional 8 h. Cells were then left untreated (*A*) or treated with 50 ng/ml activin A (*B*) for 16 h before measuring relative light units as in Fig. 4. The stimulation by Rab5S34N (*A*) and the inhibition by Rab5Q79L (*B*) are both statistically significant ($p < 0.001$). *C*, BBCE cells were transfected with SBE-luc, CMV-β-gal, and either control vector or the Rab5S34N construct and 24 h later were placed in 0, 0.1, 0.2, and 0.4% serum for an additional 24 h before measuring relative light units. *D*, BBCE cells were transfected with Rab5S34N, CMV-β-gal, and SV40-luc, κB-TK5-luc, or E-selectin-luc constructs and 24 h later were placed in 0.2% serum for an additional 24 h before measuring relative light units. The latter were expressed as percentage of the values obtained from the experimental wells in which the reporter construct was transfected alone with CMV-β-gal (100%). All data in *A-D* are mean ± S.D. of triplicate determinations.

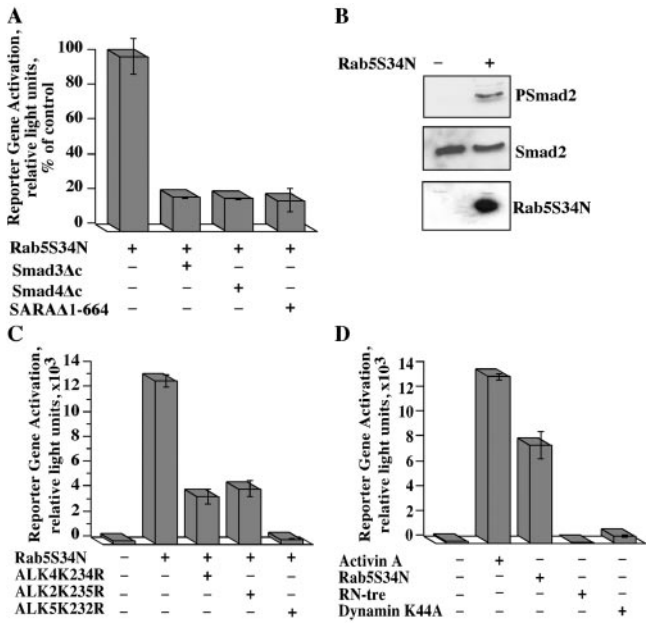

FIG. 6. **Rab5S34N effect on ligand-independent activation of Smad transcription is mediated by phosphorylation of Smads; inhibitors of endocytosis do not mimic these effects.** *A*, BBCE cells were transfected with SBE-luc, CMV-β-gal, Rab5S34N, and either control vector or Smad3Δc, Smad4Δc, or SARAΔ1–664 constructs and 24 h later were placed in 0.2% serum for an additional 24 h before measuring relative light units. The inhibition by the mutant Smads and SARA were all statistically significant ($p < 0.001$). *B*, COS cells were transfected with Myc-tagged Smad2 alone or in combination with Myc-tagged Rab5S34N. Lysates were Western blotted and probed with 9E10 anti-Myc and an anti-phospho-Smad2 antibody. *C*, BBCE cells were transfected with SBE-luc, CMV-β-gal, and either control vector or Rab5S34N or Rab5S34N plus ALK4K234R, Rab5S34N plus ALK2K235R, or Rab5S34N plus ALK5K232R constructs and 24 h later were placed in 0.2% serum for an additional 24 h before measuring relative light units. The inhibition by the mutant ALK receptors were all statistically significant ($p < 0.001$). *D*, BBCE cells were transfected with SBE-luc, CMV-β-gal, and either control vector or Rab5S34N or RN-tre or K44A dynamin constructs and 24 h later were placed in 0.2% serum for an additional 24 h before measuring relative light units. As a control (*columns 1* and *2*), 24 h after SBE-luc, CMV-β-gal, and control vector transfections, the BBCE cells were placed in 0.2% serum for an additional 8 h. Cells were then treated with 50 ng/ml activin A for 16 h before measurement of relative light units. The differences between the effect of RN-tre and dynamin K44A *versus* Rab5S34N were both statistically significant ($p < 0.001$). All data in *A*, *C*, and *D* are mean ± S.D. of triplicate determinations.

tities of TGFβ/activins, we carried out the reporter assays in serum-free conditions and in the presence of increasing quantities of serum. The effect of Rab5S34N was similar in serum-free and 0.4% serum conditions, exhibiting no dose response dependence (Fig. 5*C*). Moreover, to determine whether Rab5S34N stimulates transcription nonspecifically, we also tested the effect of Rab5S34N on E-selectin, SV40, and NF-κB promoter constructs. Rab5S34N did not stimulate the transcription of these promoters, exhibiting either no effect or slight inhibition (Fig. 5*D*).

Since the SBE element binds to Smad3 and because SBE-luc transcriptional activation implied release and transport of Smad3 to the nucleus presumably in a complex with Smad4, we investigated the effect of Rab5S34N by simultaneous transfection of constructs harboring dominant-negative mutants of Smad3 (Smad3Δc) and Smad4 (Smad4Δc) proteins (32). Indeed, the effect of Rab5S34N on SBE-luc transcriptional activation was dramatically reduced in the presence of Smad3Δc and Smad4Δc (Fig. 6*A*). The same effect was observed (Fig. 6*A*) by a SARA mutant lacking the FYVE finger, but retaining the Smad-binding domain (SARAΔ1–664) (11), suggesting that Rab5S34N released Smads from SARA complexes. It has been suggested that a fraction of Smad proteins are microtubule-associated (33). Confocal microscopy analysis indicated that the microtubule network in endothelial cells overexpressing Rab5S34N seemed normal (data not shown), ruling out the possibility that Rab5S34N could exert its effects by altering the microtubule network and dissociating Smad proteins from it. The observation that transcriptional activation of a Smad-dependent promoter by Rab5S34N was abolished by Smad3 and Smad4 dominant-negative mutants clearly suggests that the stimulatory effect of Rab5S34N requires the activity of Smad proteins. One possibility is that Rab5S34N causes release of Smad3 from early endosomes and translocation to the nucleus following heterodimerization with Smad4. Since such release of receptor-Smads occurs only following phosphorylation from activated TGFβ/activin receptors, we sought evidence regarding the phosphorylation state of Smad proteins following Rab5S34N transfection. Indeed, Rab5S34N overexpression in COS cells caused a dramatic increase in the phosphorylation level of the Smad2 protein (Fig. 6*B*). Furthermore, co-transfection of endothelial cells with kinase-dead, dominant-negative ALK2K235R, ALK4K234R, and ALK5K232R type I receptors inhibited considerably the effect of Rab5S34N on Smad-dependent transcription (Fig. 6*C*), strongly suggesting that phosphorylation of Smads by Rab5S34N is compatible with amplification of ligand-independent, low-level constitutive activation of TGFβ/activin receptors.

Because Rab5 mutants interfere with endocytosis and early endosome fusion, the above results suggested an important role of these processes in Smad-dependent signaling. To further

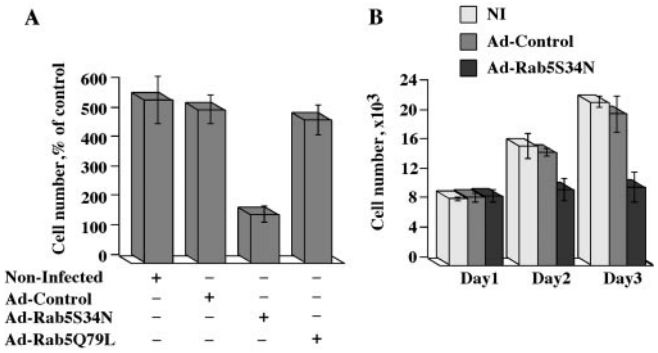

FIG. 7. **Rab5S34N inhibits cell proliferation.** *A*, BBCE cells were infected with recombinant adenovirus expressing either GFP alone or GFP plus Rab5S34N or GFP plus Rab5Q79L for 2 h. The next day, cells were seeded at 5,000 cells per well and 24 h later were stimulated with basic fibroblast growth factor (2.5 ng/ml). Triplicate wells were counted by Coulter counter 2 days later. The inhibition by Ad-Rab5S34N compared with Ad-Control was statistically significant ($p < 0.001$). *B*, HaCaT cells were seeded at 10,000 cells per well, and then infected, for 2 h, with adenoviruses expressing either GFP alone, or GFP and Rab5S34N. Cells in triplicate wells were counted every day, for three consecutive days, starting the day following infection. The inhibition by Ad-Rab5S34N compared with Ad-Control was statistically significant on day 2 ($p = 0.005$) and day 3 ($p = 0.006$). *NI*, non-infected. Data in *A* and *B* are mean ± S.D. of triplicate determinations.

discriminate between plasma membrane and early endosomal events, we transfected endothelial cells with RN-tre, a Rab5 GAP, which inhibits endocytosis by inactivating Rab5 on the plasma membrane (20). Transfection by RN-tre did not increase Smad-dependent transcription (Fig. 6*D*) pointing to a key role in early endosomal events. Similarly, inhibition of endocytosis by clathrin-coated pits and caveolae using the K44A dominant-negative mutant form of dynamin (34, 35) did not increase Smad-dependent transcription (Fig. 6*D*). This suggests that phosphorylation of Smads induced by Rab5S34N may occur due to amplification of low levels of constitutively active TGFβ/activin receptors that may assemble in the early endosome in the absence of exogenous ligand.

Rab5S34N Inhibits the Proliferation of Endothelial Cells and Keratinocytes—Because the anti-mitotic effects exerted by TGFβ/activins are mediated to a great extent by Smad2/3 proteins, we investigated whether Rab5S34N overexpression, parallel to the activation of a Smad-dependent promoter, could mimic the effects of TGFβ/activins on more complex cellular functions. Indeed, BBCE cells infected with Rab5S34N-expressing adenoviruses were substantially less responsive to basic fibroblast growth factor-induced proliferation compared with noninfected cells or cells infected with a control adenovirus (Fig. 7*A*), while cells infected with Rab5Q79L proliferated normally. Similarly, infection of HaCaT cells with Rab5S34N adenoviruses rendered them unresponsive to serum-induced proliferation (Fig. 7*B*). It is, therefore, possible that Rab5S34N-induced phosphorylation and transcriptional activation by Smad2/3 may mimic the effect of TGFβ/activin receptor-induced phosphorylation of Smad2/3 proteins.

DISCUSSION

Internalization of ligand activated receptors has been considered merely as a signal attenuation mechanism (reviewed in Ref. 14). However, there is increasing evidence that endocytosis of ligand-receptor complexes not only leads to signal attenuation (36), but may be also necessary to co-localize activated receptors with downstream effectors (37). Thus, the idea of signaling from the endocytic compartment is gaining momentum. In the case of the TGFβ/activin family receptors, it is unclear whether receptor internalization is required to reach the SARA-bound Smad substrate or to what extent endosome dynamics affect TGFβ family signaling.

We have addressed the intracellular localization of SARA and found that SARA is present on the early endocytic compartment, suggesting that the Smad pathway signals from the early endosome. Furthermore, SARA significantly increases endosome size, suggestive of a role in endosome dynamics. We have observed by immunofluorescence that the FYVE finger of SARA is sufficient for its endosomal targeting. So far, the identified FYVE finger proteins have been shown to require additional binding partners for targeting to endosomal membranes. Thus, EEA1 and Rabenosyn 5 require the binding of an adjacent domain to endosomal Rab5GTP (29, 38). Surface plasmon resonance experiments indicated that the SARA FYVE finger has a higher affinity (K_D of 30 nM) than those of Hrs (K_D of 38 nM) and EEA1 (K_D of 45 nM) for PI(3)P. This difference in K_D may partially account for the fact that the SARA FYVE finger is efficiently targeted to early endosomes, whereas the EEA1 and Hrs FYVE fingers are mainly cytosolic when expressed as such (13, 22). Since the dimerization of FYVE domains increases their avidity for PI(3)P-containing membranes (13, 39), it is also possible that the isolated FYVE domain of SARA has a higher propensity to dimerize than those of Hrs and EEA1. Although we cannot rule out the possibility that the surface plasmon resonance experiments underestimate the differences in ligand affinities, we favor the view that endosomal targeting of SARA may rely solely on binding to endosome-located PI(3)P (13). For instance, we did not find any direct interactions between SARA and Rab5 by the two-hybrid system (data not shown). However, we cannot fully rule out the possibility that our minimal FYVE finger construct may contain other binding elements which participate in dimerization or endosomal targeting.

In the light of our initial results showing that SARA is located on early endosomes, we sought further evidence regarding the inter-relation between endosome dynamics and signaling. We reasoned that a certain level of regulation may exist at the level of this organelle and proteins, such as Rab5, regulating early endosome function may indeed alter signaling. Moreover, Rab5 has been implicated in EGFR signaling (20) and is activated by EGF stimulation (19). A striking finding of the present study was that Rab5S34N, a dominant-negative Rab5 mutant, stimulated transcription of a Smad-dependent promoter, in the absence of TGFβ/activins, in serum-free conditions. This activation was associated with phosphorylation and nuclear translocation of Smad proteins. Phosphorylation of Smads by Rab5S34N was independent of indirect effects such as establishment of a TGFβ/activin autocrine loop or depolymerization of microtubules. Indeed, microtubules sequester unphosphorylated Smads, and depolymerization of the microtubular network releases active, phosphorylated Smads by an uncharacterized mechanism (33). Because phosphorylation of Smads by Rab5S34N occurred in serum-free conditions and since it has been previously shown that the cytoplasmic domains of type II and type I TGFβ receptors interact physically and functionally with each other in a ligand-independent manner (40), it was reasonable to assume that Rab5S34N might be able to amplify such low-level constitutive TGFβ/activin receptor activation. Indeed, we have observed a considerable inhibition of the transcriptional activation of the Smad-dependent promoter by Rab5S34N when co-transfecting dominant-negative ALK2, ALK4, and ALK5 receptor constructs.

Since Rab5S34N inhibits endocytosis, recycling, and early endosome fusion (31), the data suggested a regulatory role of membrane trafficking on the intensity of signaling cascades. A negligible effect of constitutively formed TGFβ-activin receptor

complexes, on Smad-dependent transcription could be grossly amplified by Rab5S34N. Such amplification was unlikely to be derived from a decreased rate of endocytosis as blocking of plasma membrane endocytosis by expression of RN-tre, a specific Rab5 GAP (20), did not augment Smad-dependent transcription. Similarly, there was no increase in Smad-dependent transcription following inhibition of clathrin-coated pit- and caveolin-dependent plasma membrane endocytosis (41–43) by the dominant-negative K44A dynamin construct (34, 35). These results implied that the Rab5S34N effect was rather a consequence of decreased degradative or recycling trafficking leading to accumulation of constitutively formed TGFβ-activin type I/II receptor complexes on early endosomal membranes, where SARA-Smad complexes reside. Such accumulation of TGFβ-activin type I/II receptor complexes, and increased residence time thereof, presumably accounts for the increase in signaling observed upon RabS34N expression. Likewise, the observed reduction of activin A-induced Smad promoter transcription by the constitutively active Rab5Q79L is likely to be a consequence of enhanced receptor trafficking leading to decreased residence in the early endosomal compartment. It appears that cycling of Rab5 between GTP and GDP forms may influence the length and intensity of TGFβ/activin signaling cascades by regulating TGFβ-activin type I/II receptor trafficking via the early endocytic compartment. Indeed, it has been shown that Rab5S34N reduces epidermal growth factor receptor degradation by influencing membrane trafficking (44). Alternatively, Rab5 could exert its effects by directly binding to components of the TGFβ/activin pathway or affecting TGFβ/activin receptor kinase activity, for instance, by modulating receptor-associated kinases or phosphatases (45). Toward this end, we did not observe any direct interactions between Rab5 and SARA or Smad2/3 proteins using the yeast 2-hybrid system (data not shown).

In conclusion, we have revealed a critical role of early endosomes in regulating Smad-dependent signaling. Not only is SARA localized in the early endocytic compartment but also a dominant-negative Rab5 mutant causes phosphorylation and nuclear translocation of Smads leading to transcriptional activation of a Smad-dependent promoter. Rab5S34N not only stimulated Smad-dependent transcriptional activation, but also inhibited the proliferation of endothelial cells and keratinocytes mimicking the effects of TGFβ/activins. The results suggest an interconnection between events in early endosomes with signal transduction pathways and may have important implications in understanding how cells co-ordinate their cellular functions when responding to extracellular stimuli.

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