Albumin endocytosis in endothelial cells induces TGF-β receptor II signaling

Shahid S. Siddiqui, Zeba K. Siddiqui, and Asrar B. Malik. Albumin endocytosis in endothelial cells induces TGF-β receptor II signaling. Am J Physiol Lung Cell Mol Physiol 286: L1016–L1026, 2004.—Vascular endothelial cells undergo albumin endocytosis using a set of albumin binding proteins. This process is important for maintaining cellular homeostasis. We showed by several criteria that the previously described 73-kDa endothelial cell surface albumin binding protein is the 75-kDa transforming growth factor (TGF)-β receptor type II (TβRII). Albumin coimmunoprecipitated with TβRII from a membrane fraction from rat lung microvascular endothelial cells. Albumin endocytosis-negative COS-7 cells became albumin endocytosis competent when transfected with wild-type TβRII but not when transfected with a domain-negative kinase mutant of TβRII. An antibody specific for TβRII inhibited albumin endocytosis. A mink lung epithelial cell line, which expresses both the TGF-β receptor type I (TβRI) and the TβRII receptor, exhibited albumin binding to the cell surface and endocytosis. In contrast, mutant L-17 and DR-26 cells lacking TβRI or TβRII, respectively, each showed a dramatic reduction in binding and endocytosis. Albumin endocytosis induced Smad2 phosphorylation and Smad4 translocation as well as increased protein expression of the inhibitory Smad, Smad7. We identified regions of significant homology between amino acid sequences of albumin and TGF-β, suggesting a structural basis for the interaction of albumin with the TGF-β receptors and subsequent activation of TβRII signaling. The observed albumin-induced internalization of TβRII signaling may be an important mechanism in the vessel wall for controlling TGF-β responses in endothelial cells.

Eight Smad proteins, identified in mammals, have been classified into the following three subgroups: receptor-regulated Smads (R-Smads); a common-partner Smad (co-Smad); and inhibitory Smads (I-Smads). R-Smads directly interact with the activated TβRII and are themselves activated through phosphorylation of their COOH-terminal SSXS motifs. These activated R-Smads have been shown to form a heteromorphic complex with the co-Smad, Smad4. R-Smads have been shown to move into the nucleus where they regulate gene transcription (3, 5, 14, 17, 21, 22, 30). I-Smads, such as Smad7, have been shown to negatively regulate signaling initiated by TGF-β, activin, and BMP (13, 25). Internalization of TGF-β receptor-activated signaling via endocytosis is required for these discrete functional responses (1, 2, 14, 15, 18, 28, 38, 40–43). The fate of the internalized receptor was distinct whether it is partitioned in caveolae or clathrin-coated pits (where receptor signaling emanates from early endosomes; see Ref. 7).

Albumin has been shown to induce endocytosis in the endothelial cells lining the vessel wall (23, 36). Albumin endocytosis is extensively studied in endothelial cells, and four albumin-binding proteins, including a 73-kDa protein, are described (10, 33–35). In the present study, we addressed the possibility that albumin endocytosis facilitates the internalization of the 75-kDa TGF-β receptor, TβRII, and induces the internalization of TβRII signaling.

EXPERIMENTAL PROCEDURES

Reagents. Drs. Peter ten Dijke and Carl-Henrik Heldin (Ludwig Cancer Institute, Uppsala, Sweden) provided the rabbit polyclonal antibody to Smad2-P. Polyclonal antibodies against Smad2 (sc-6200), Smad4 (sc-7154), TβRII (sc-7004), fluorescein isothiocyanate (FITC)- and Texas red isothiocyanate (TRITC)-conjugated polyclonal goat anti-rabbit IgG, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG, normal mouse IgG, normal rabbit IgG, rTβRII protein (sc-4122), and Smad7 peptide (sc-7040p) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A primary culture of rat lung microvascular endothelial cells (KLMVEC; passage 11) was obtained from American Type Culture Collection (Bethesda, MD). The mink lung epithelial cell line (Mv1.Lu) was a kind gift of Dr. J. Massague (Sloan Kettering Memorial Institute, New York, NY), and mutant lines, L-17 and DR-26, lacking TβRI and TβRII receptors, respectively, were kind gifts of Dr. E. Loeff (Mayo Clinic, Rochester, MN). Antibody to BSA [no. A0433; BSA, fraction V 99% pure, endotoxin-free (BSA, Sigma no. A3059)]; human serum albumin (HSA), A3782; FITC-labeled BSA (FITC-albumin); TRITC-BSA (TRITC-albumin); 4′,6-diamidino-2-phenylindole (DAPI); methyl-β-cyclodextrin; and disuccin-
imidyl substrate were purchased from Sigma (St. Louis, MO). n-Propylgallate was purchased from Molecular Probes. Cell culture reagents were purchased from BioWhittaker (Walkersville, MD). Drs. T. Imamura and K. Miyazono (Cancer Institute, Tokyo University, Tokyo, Japan) provided the wild-type and dominant-negative kinase mutant [K277R (39); cDNA constructs of human TβRII, subcloned into pcDNA3.1]. Protein G-Sepharose and ECL reagents were purchased from Amersham-Pharmacia (Piscataway, NJ). Dr. R. Minshall, University of Illinois at Chicago, provided 125I-albumin (BSA and HSA; see Ref. 23).

Cell culture. Cells were grown and incubated in phenol red-free DMEM with 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C, 5% CO2 in the presence or absence of FBS as stated.

FITC/TRITC-albumin binding and internalization assays. Albumin internalization by RLMVEC, Mv1Lu, DR-26, L-17, and COS-7 cells was visualized using FITC- or TRITC-BSA as a tracer. Cells were grown in monolayers to 80–90% confluence in six-well culture plates in DMEM made 10% (37°C, 5% CO2). Before the assay, cells were grown for 24 h in serum-free DMEM to starve for albumin. The cells were washed in 4°C DMEM and incubated with DMEM (37°C); the incubation medium concentration was made 100 μg/ml with unlabeled BSA and 10 μg/ml of either FITC-BSA or TRITC-BSA for the times described to determine the albumin uptake. Cells were washed on ice, washed with 100 mM NaAc, pH 2.5, and 150 mM NaCl to remove cell surface albumin, and with 100 mM NaHPO4, pH 7.4 (to restore the pH to neutral). All reagents were used at 4°C. The antifade agent, n-propylgallate in glycerol (5% wt/vol), was added to the mounting medium to reduce photobleaching. Fluorescence and differential interference contrast (DIC) images of cells were visualized using appropriate excitation and barrier filters for fluorescein, rhodamine, and DAPI in an Eclipse E800 microscope (Nikon). The images were captured with a CCD [cooled integrated charging-coupled camera (Hamamatsu Photonics, Hamamatsu, Japan)] and analyzed using Metamorph imaging software (Universal West Imaging, West Chester, PA). The fluorescence measurements were reported as average intensity per unit area of three cell fields, normalized to uptake at 30 min.

Competition of 125I-labeled albumin uptake with unlabeled albumin by RLMVEC. Albumin-starved RLMVEC were incubated with 125I-labeled albumin (50,000 cpm) in the absence or presence of increasing concentrations of unlabeled albumin (0.1–2 mg/ml) for 20 min at 37°C. The cells were placed on ice and washed to remove surf ace BSA, scraped in lysis buffer (50 mM Tris-HCl buffer, pH 7.4, 1% Triton X-100, and 0.5% SDS), and homogenized. The protein concentration of homogenates was determined. Aliquots of cell homogenates were counted using a Beckman gamma counter. The concentration of homogenates was determined.

Inhibition of albumin endocytosis by anti-TβRII antibody. Albumin-starved RLMVEC cells were preincubated with 25–100 μg/ml rabbit anti-TβRII (or normal rabbit IgG) for 1 h. Cells were incubated for 60 min with 100 μg/ml FITC-BSA and 10 μg/ml FITC-BSA, and internal FITC-BSA was visualized as described above.

Vector constructs and transfection of COS-7 cells. cDNA encoding the entire human TβRII sequence (19) or a TβRII dominant-negative (K277R) kinase mutant (gift from K. Miyazono and Dr. T. Imamura, Cancer Institute, Tokyo University) subcloned into pcDNA3.1 [under control of the cytomegalovirus (CMV) promoter] was used to transfect COS-7 cells. Cells were cotransfected with pcDNA3.1 encoding green fluorescent protein (GFP) to assess transfection efficiency. COS-7 cells were transfected with 5 μg/ml DNA, using the Qiagen Superfect reagent, following the manufacturer’s protocol. Cells positive for GFP expression were used in the studies. TRITC-BSA uptake was measured 48 h after transfection. In control experiments, the GFP-encoding vector was transfection alone or with empty vector.

Surface binding and uptake of 125I-albumin. Mv1Lu and two related mutant cell lines that lacked either the TβRII (DR-26) or the TβRI (L-17; see Ref. 39) were grown as described. HSA (1 mg/ml) was added to each well, together with 5 μl 125I-labeled albumin HSA (50,000 cpm). Cells were incubated 10 min at 4°C, when they were placed on ice and washed one time with PBS (pH 7.4). In the internalization assays, cell surface albumin was removed by washing the cells two times with 100 mM NaAc, pH 2.5, and 150 mM NaCl, 0.2 mM PMSF, and 0.5 μg/ml leupeptin, and pepstatin; MIB). The cells were washed in MIB and centrifuged at 500 g for 2 h. The pellet was centrifuged again at 100,000 g for 60 min. The final pellet was dissolved in 1 ml MIB and stored in small aliquots at −70°C. The protein concentration of the whole cell lysate and membrane fraction was determined.

Immunoblotting. Standard procedures were followed. Proteins were subjected to SDS-PAGE and transferred to nitrocellulose. The membrane was blocked in 20 mM Tris-HCl, pH 7.5, and 150 mM NaCl (0.1% nonfat dried milk). The membrane was blocked in PBS at 4°C, then incubated with 100 μg/ml FITC-BSA and 10 μg/ml FITC-BSA. The membrane was washed (TBS-Tr). An HRP-conjugated secondary antibody (1:1,000 in TBS-Tr) was used to detect the primary antibody. The membranes were washed as before and developed using ECL; signals were detected using Kodak X-AR film.

Coimmunoprecipitation of internalized albumin and TβRII. Albumin-starved RLMVEC were incubated with BSA (100 μg/ml) for 30 min. Harvested cells were washed in 100 mM NaAc, pH 2.5, and 150 mM NaCl to remove surface-bound albumin, washed in DMEM to restore pH to neutral, lysed, immunoprecipitated with an anti-albumin antibody or an anti-TβRII antibody, and subjected to immunoblot analysis, as described above. Antialbumin immunoprecipitates were probed with anti-TβRII and anti-TβRII immunoprecipitates with antialbumin.

The cells were homogenized in a glass hand-held homogenizer and then sonicated (30-s pulse 6 times). The cell homogenate was centrifuged at 2,500 g for 10 min to remove cell debris and unbroken cells. The supernatant was centrifuged at 100,000 g for 2 h. The pellet was centrifuged again at 100,000 g for 60 min. The final pellet was dissolved in 1 ml MIB and stored in small aliquots at −70°C. The protein concentration of the whole cell lysate and membrane fraction was determined.

Inhibition of albumin endocytosis by anti-TβRII antibody. Albumin-starved RLMVEC cells were preincubated with 25–100 μg/ml rabbit anti-TβRII (or normal rabbit IgG) for 1 h. Cells were incubated for 60 min with 100 μg/ml FITC-BSA and 10 μg/ml FITC-BSA, and internal FITC-BSA was visualized as described above.
was counted using a Beckman gamma counter. The radioactive counts were normalized to protein concentration. Duplicate samples were counted from three independent experiments.

**TGF-β-induced translocation of Smad2-P.** RLMVEC were grown in serum-free DMEM to 80–90% confluence and stimulated with TGF-β (10 ng/ml) for 16 h. In control cells, no TGF-β was added. Postincubation, the cells were fixed in formaldehyde (2% in PBS), permeabilized with 0.1% Triton X-100 in PBS, and incubated for 1 h with a polyclonal rabbit antibody against Smad2-P (1:200), a gift from Dr. ten Dijke and Dr. C. Heldin. Secondary antibody staining, washing, and cell observations were as described above.

**Data analysis.** All experiments were performed a minimum of three times, and in all cases data from three separate experiments are reported. Results are reported as means ± SE.

**Sequence analysis.** Sequence homology was computed using the Huang and Miller Alignment Algorithm (16).

### RESULTS

**Albumin endocytosis in endothelial cells.** We monitored endocytosis of albumin by RLMVEC by incubating albumin-starved cells with FITC-albumin. Albumin internalization was observed by fluorescence microscopy of cells incubated, for different periods of time, with 100 μg/ml albumin and 10 μg/ml FITC-BSA, used as a tracer. At each of the time points, endocytosis was stopped by placing the cells on ice. Surface-bound albumin was removed by acid wash, followed by washes in DMEM to restore the pH to neutral. Maximum fluorescence units, defined as FITC-albumin uptake at 30 min, were considered as 100%. Albumin uptake was evident in RLMVEC within 5–10 min, with the maximal value attained between 15 and 30 min (Fig. 1A, f–j). Albumin uptake reached saturation at 30 min as it did not increase significantly beyond 30 min (Fig. 1A).

We next tested the ability of unlabeled BSA to compete with 125I-albumin for uptake by RLMVEC (Fig. 1B). Unlabeled albumin was found to successfully compete with 125I-albumin for uptake by RLMVEC, as shown in Fig. 1B. Additionally, to determine whether endocytosis depended on the native conformation of albumin, we carried out uptake assays using albumin that had been denatured by heat or reduction with dithiothreitol. We found that denatured albumin was not internalized by RLMVEC (data not shown). From these data, we conclude that the binding of albumin to RLMVEC is saturable, subject to competition, and dependent on the native conformation of the ligand. These data support the conclusion that albumin binding to RLMVEC occurred via receptor-mediated endocytosis.

**MBC inhibits albumin endocytosis.** Because caveolae have been shown to have a role in albumin endocytosis in endothelial cells (27), we examined the role of caveolae in albumin uptake by RLMVEC. We evaluated the effect of MBC, known to disrupt the integrity of cholesterol-rich caveolae, on albumin uptake in RLMVEC. RLMVEC, preincubated with 3 mM MBC for 2 h, exhibited a marked reduction in uptake of TRITC-albumin compared with untreated controls (Fig. 2A, a and b). We conclude that the effect of cycloexetrin on albumin endocytosis suggested a role for caveolae in albumin endocytosis in RLMVEC.

**Expression of TβRII in endothelial cells.** Because TβRII has a reported molecular mass, 75 kDa, similar to that of one of the described albumin binding proteins (10), we determined if albumin bound TβRII on the surface of RLMVECs. We first assessed the subcellular distribution of TβRII in RLMVEC by

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**Fig. 1.** A: albumin binds to endothelial cells in a saturable manner. Albumin-starved rat lung microvascular endothelial cells (RLMVEC) were induced with albumin: 100 μg/ml unlabeled BSA with 10 μg/ml fluorescein isothiocyanate (FITC)-albumin, used as a tracer. At specific time points (0–30 min), plates were removed to ice and washed at 4°C. To visualize the internalized albumin selectively, the external FITC-albumin was removed by washing the cells first with 100 mM NaOAc, pH 2.5, 150 mM NaCl, and then with 100 mM NaHPO4, pH 7.4. Cells accumulated albumin within 5–10 min and reached a peak over 15–30 min. f–i show the punctate staining of internalized FITC-albumin, and a–e show 4',6-diamidine-2-phenylindole (DAPI) staining of the same fields. Results are representative of 3 experiments. Bar represents 10 μm. B: albumin competes with 125I-labeled albumin for uptake by endothelial cells. Albumin-starved RLMVEC were incubated with 125I-albumin (50,000 cpm), in the absence or presence of increasing concentrations (0.1–2 mg/ml) of nonradio-labeled albumin. After 20 min, the cells were placed on ice, and cell surface 125I-albumin was removed as described in A. The washed cells were lysed (50 mM Tris-HCl, pH 7.4, with 1% Triton X-100 and 0.5% SDS) and homogenized. The aliquots of the lysates were transferred to tubes for counting (Beckman gamma counter). The quantitative data show that unlabeled albumin successfully competed with 125I-albumin for uptake by endothelial cells.
To determine whether albumin binds to the 75-kDa TβRII, we immunoprecipitated TβRII from albumin-starved RLMVEC incubated with albumin for 30 min. The cells were harvested, washed in low-pH buffer (pH 2.5) to remove surface-bound albumin, washed in DMEM to restore pH to neutral (pH 7.4), lysed, and immunoprecipitated with an anti-TβRII antibody, and the eluate was subjected to immunoblot analysis as previously described in EXPERIMENTAL PROCEDURES, but probing with an antialbumin antibody. Immunoblot results showed that the internalized albumin specifically coprecipitated with TβRII (Fig. 4A, lane 2). This ~66-kDa band was not seen when the membrane was probed with normal rabbit IgG (Fig. 4A, lane 1) or when the anti-albumin antibody was preblocked with albumin (Fig. 4A, lane 3). The reverse experiment was also performed. An anti-albumin immunoprecipitate probed with an anti-TβRII antibody demonstrated an ~75-kDa TβRII-specific immunoreactivity

Fig. 2. Cyclodextrin impairs albumin endocytosis: albumin-starved RLMVEC were incubated with or without the caveola-disrupting agent cyclodextrin (3 mM) for 2 h, washed, and incubated with DMEM containing albumin (1 mg/ml) trace labeled with Texas red isothiocyanate (TRITC)-albumin (0.1 mg/ml). Cells incubated with 3 mM cyclodextrin showed a reduction in TRITC-albumin uptake compared with untreated control cells. Bar is 2 µm. Results are representative of 3 experiments.

Fig. 3. A: intracellular and cell surface transforming growth factor (TGF)-β receptor type II (TβRII) in RLMVEC. RLMVEC monolayers were grown on coverslips in DMEM with 10% FBS. Cells were washed with PBS, fixed in chilled paraformaldehyde (2%) for 1 h, washed with PBS-Triton X-100 (0.1%), incubated with an FITC-labeled goat anti-rabbit IgG secondary antibody for 2 h at 4°C. In control experiments, normal rabbit IgG replaced the anti-TβRII IgG, and no staining was seen (data not shown). Results are representative of 3 experiments. B: TβRII is present as a 75-kDa protein in RLMVEC. Proteins (50 µg) from a whole cell lysate of RLMVEC were subjected to SDS-PAGE and transferred to nitrocellulose. Lane 1, staining of total protein with India ink (LI). The remainder of the membrane was blocked in 5% nonfat dry milk in TBS, probed with anti-TβRII antibody (lane 3) or with anti-TβRII antibody preincubated with the cognate peptide against which the serum was raised and affinity purified (lane 4). 

Carrying out indirect immunofluorescence on permeabilized cells. Incubation with an anti-TβRII antibody resulted in punctate staining of RLMVEC (Fig. 3A). Analysis of different focal planes with confocal microscopy demonstrated that the immunostaining was present both within the cell and cell surface. Immunoblot analysis of RLMVEC whole cell lysates and of a membrane fraction (obtained by differential high-speed centrifugation) with an anti-TβRII antibody revealed an ~75-kDa (range 70–80) species (Fig. 3B, lanes 3 and 5) that corresponded to the reported size of TβRII, 75 kDa.

To assess the specificity of the anti-TβRII antibody binding, we preincubated the antibody with the cognate full-length TβRII recombinant protein against which the antibody had been made and affinity purified. As shown in Fig. 3B, lane 2, no band was seen, indicating that the antibody is specific for TβRII. Additionally, normal rabbit IgG did not recognize this band (Fig. 3B, lane 4). These data show that RLMVEC expresses a 75-kDa protein that was found on the endothelial cell surface and that is recognized by an affinity-purified anti-TβRII antibody. The cytosolic fraction also showed the presence of TβRII (data not shown); however, the membrane fraction consistently showed a somewhat greater amount of TβRII than the cytosolic fraction (i.e., densitometry analysis showed ~45% in the cytosolic pool and ~55% in the membrane pool). From these data, we conclude that the 75-kDa TβRII was expressed on the cell surface of RLMVEC and therefore available for binding.

Coimmunoprecipitation of albumin and TβRII. To determine whether albumin binds to the 75-kDa TβRII, we immunoprecipitated TβRII from albumin-starved RLMVEC incubated with albumin for 30 min. The cells were harvested, washed in low-pH buffer (pH 2.5) to remove surface-bound albumin, washed in DMEM to restore pH to neutral (pH 7.4), lysed, and immunoprecipitated with an anti-TβRII antibody, and the eluate was subjected to immunoblot analysis as previously described in EXPERIMENTAL PROCEDURES, but probing with an antialbumin antibody. Immunoblot results showed that the internalized albumin specifically coprecipitated with TβRII (Fig. 4A, lane 2). This ~66-kDa band was not seen when the membrane was probed with normal rabbit IgG (Fig. 4A, lane 1) or when the anti-albumin antibody was preblocked with albumin (Fig. 4A, lane 3). The reverse experiment was also performed. An anti-albumin immunoprecipitate probed with an anti-TβRII antibody demonstrated an ~75-kDa TβRII-specific immunoreactivity.
Ectopic expression of TβRII in COS-7 cells results in albumin endocytosis and TβRII signaling. To address whether the TβRII was sufficient to induce endocytosis of albumin, we evaluated the effects of transient, ectopic expression of a vector containing human TβRII cDNA in COS-7 cells and cells that lack both TβRI and TβRII. A human cDNA clone encoding the full-length transcript of TβRII (19), subcloned in the pcDNA 3.1 vector under the CMV promoter, was cotransfected into COS-7 cells together with gfp subcloned in pcDNA 3.1. COS-7 cells normally do not exhibit albumin endocytosis. However, transient expression of TβRII cDNA in COS-7 cells resulted in significant uptake of TRITC-BSA (Fig. 6C). In a control experiment, there was no increase in albumin endocytosis in COS-7 cells transfected with pcDNA 3.1, which expressed only GFP (Fig. 6A). We further tested the role of TβRII by transfecting COS-7 cells with a dominant-negative kinase mutant (K277R) of TβRII. As shown in Fig. 6E, this TβRII kinase mutant markedly reduced albumin endocytosis seen with transfected wild-type TβRII, suggesting that albumin endocytosis is greatly facilitated by the functional kinase domain of TβRII. GFP marker shows the transfected cells with the wild-type and kinase mutant transfected cells (Fig. 6, D and E). From these data, we conclude that albumin endocytosis is mediated by TβRII in a TβRI transfected cell line that is otherwise incompetent in the endocytosis of albumin.

Fig. 4. Coimmunoprecipitation of TβRII and albumin. RLMVEC were grown in monolayers in DMEM and 10% FBS. After 24-h albumin starvation, endocytosis was induced by incubation with 100 µg/ml albumin for 30 min. Cells were washed with PBS, and with 100 mM NaOAc, pH 2.5, and 150 mM NaCl, followed by washes in PBS. All reagents were at 4°C. The cells were lysed and incubated with anti-TβRII antibody (Fig. 4A, lane 2). The antibodies were concentrated with protein G-Sepharose and immunoblotted, as described above. The nitrocellulose membranes containing proteins immunoprecipitated with anti-TβRII were probed with antialbumin (A, lane 2) and show a diffused band corresponding to 63 kDa (marked as Alb). Alternatively, membranes containing proteins immunoprecipitated (IP) with antialbumin were probed with anti-TβRII. The membranes were incubated with normal rabbit IgG (lane 1 in A and B) or immunoprecipitated with anti-TβRII antibodies that identified a 75-kDa band corresponding to TβRII (B, lane 2). When anti-TβRII is preblocked with cognate TβRII protein (B, lane 3) no staining is observed.

Fig. 5. Preincubation of RLMVEC with anti-TβRII antibody results in marked reduction of TRITC-albumin uptake. Cells were preincubated with the rabbit polyclonal anti-TβRII antibody. RLMVEC show TRITC-albumin endocytosis of cells preincubated with normal rabbit IgG (a). b shows TRITC-albumin uptake after preincubation (1 h) with anti-TβRII antibody. The anti-TβRII antibody resulted in a marked reduction of albumin endocytosis by RLMVEC. Preincubation of the anti-TβRII antibody with its cognate protein (rTβRII) prevented the block in endocytosis seen with the unblocked antibody (data not shown). Bar represents 2 µm. Results are representative of 3 experiments.
However, as shown in Fig. 7, the role for TβRII is greater than that of TβRI (Fig. 7C). These results indicate that these two TGF-β receptors play a crucial role both in the binding and endocytosis of albumin.

Endocytosis activates TGF-β receptor signaling. To ascertain the involvement of albumin endocytosis in TGF-β receptor-specific signaling in endothelial cells, we investigated the downstream signaling pathways of this receptor. On ligand binding, phosphorylation of TβRI by the constitutively phosphorylated TβRII has been shown to phosphorylate Smad-2 and Smad-3, thereby promoting their association with Smad-4 (14, 17, 21, 22, 30). We observed a basal level of cell surface expression of TβRII in RLMVEC grown in albumin-free medium (Fig. 8Aa). However, after albumin endocytosis, TβRII expression was induced, as evidenced by a marked increase in the cell surface-immunoreactive TβRII punctate staining seen using confocal microscopy (Fig. 8Ab). This analysis is based on the visualization of specific cellular focal planes, determined by deconvolution microscope settings that allow one to distinguish the cell surface from the cytosolic and perinuclear focal planes. We also examined levels of Smad2 phosphorylation (Smad2-P; using a Smad2-P-specific antibody) in RLMVEC. We observed an increase in the amount of Smad2-P after albumin endocytosis compared with the basal level of Smad2-P found in cells grown under albumin-free conditions. We also observed an albumin-induced increase in Smad2-P-specific staining in the membrane, cytosolic, and perinuclear regions (Fig. 8Ad). Albumin endocytosis also increased the phosphorylation of Smad2, as detected by immunoblotting (Fig. 8Ai). An increase in the level of Smad2-P was observed at a low concentration of albumin (1 mg/ml) and was found to increase up to 3 mg/ml albumin. Interestingly, at albumin concentrations >3 mg/ml, we observed inhibition of Smad2 activation, as measured by immunoblotting of Smad2-P (data not shown). Under similar conditions when TGF-β1 (10 ng/ml) was used to stimulate RLMVEC, we observed that Smad2-P moved primarily into the nucleus (Fig. 8Bb), as previously reported (11, 12).

The TGF-β family members are known to activate discrete regulatory Smads (e.g., Smad1, Smad2, Smad3, and Smad5), but all identified regulatory Smads share Smad4 as a key partner (4, 5, 17, 30). As a result of stimulation by TGF-β, the coactivator Smad4 forms a complex with activated Smad1-P or Smad2-P, and together the heteromeric complexes have been shown to move into the nucleus. In light of this, we investigated the distribution of Smad4 after albumin endocytosis. Smad4 was predominantly found in the perinuclear region of the cells grown in albumin-free medium (Fig. 8 Ae) with a distribution similar to that of Smad2-P. However, after albumin endocytosis for 30 min to 2 h, Smad4 was distributed throughout the cell, in the cytosol, perinuclear region, and nucleus (Fig. 8Af). TGF-β1 also induced the translocation of Smad4 from the cytosol to the nucleus in RLMVEC (data not shown), as reported in Mv1.Lu cells (26). This finding is in contrast to the translocation of Smad2-P to the perinuclear region observed after albumin endocytosis (Fig. 8Ad). Although both TGF-β and albumin induced translocation of Smad2-P from the cytosol to the nuclear region of the cell, TGF-β stimulation resulted in Smad2-P movement in the nucleus, whereas TGF-β1 resulted in Smad4 movement to the nucleus.

Inhibition of albumin binding and endocytosis in cells expressing mutant TGF-β receptors. To see whether both TGF-β receptors are required for albumin endocytosis, we took advantage of two mutant mink lung epithelial cell lines, L-17 and DR-26, defective in TβRI and TβRII, respectively. These mutant cells were derived from the Mv1.Lu cell line, which expresses both wild-type TβRI and TβRII and thereby provides a positive control for albumin endocytosis. Figure 7Aa shows that, although the wild-type Mv1.Lu cells accumulated TRITC-albumin, DR-26 (Fig. 7Ab), L-17 (Fig. 7Ac) and cells that lacked functional TGF-β receptors each showed a marked inhibition in albumin endocytosis. These data strongly implicate a role for both TGF-β receptors in albumin endocytosis. However, as shown in Fig. 7B, the role for TβRII is greater than that of TβRI. We further investigated the binding of 125I-albumin tracer to the surface of Mv1.Lu, L-17, and DR-26 cells by incubating the cells at 0°C for 1 h with radiolabeled albumin.

Figure 7B shows the relative binding of 125I-albumin to the cell surface of the three cell lines. We observed a reduction in the surface binding of 125I-albumin to cells lacking either the TβRI (~12%) or TβRII (~20%) when compared with wild-type Mv1.Lu cells. A similar inhibition of endocytosis was found in the TβRI and TβRII mutant cell lines, although the requirement of TβRII appears to be higher than that of the TβRI (Fig. 7C). These results indicate that these two TGF-β receptors play a crucial role both in the binding and endocytosis of albumin.
albumin endocytosis resulted in Smad2-P accumulation in the perinuclear area. Thus the two stimuli, TGF-β and albumin, resulted in different subcellular localizations of this R-Smad.

We assessed the time course of Smad2-P in RLMVEC after albumin endocytosis by densitometric analysis of immunoblots. Phosphorylation of Smad2, in response to albumin endocytosis (0.5 mg/ml), peaked at 1 h (Fig. 8C). From 1 h through 5 h the level of Smad2-P dropped, returning to baseline by 5 h.

**Albumin endocytosis induces Smad7 expression.** Because albumin is the major protein in plasma (29), we addressed the possibility that TGF-β receptor signaling was subject to negative feedback regulation in response to a sustained stimulus. It has been shown that Smad7 forms a stable association with the ligand-stimulated TGF-β receptor complex, thereby inhibiting phosphorylation of Smad2 and Smad3, precluding their association with Smad4, translocation into the nucleus, and subsequent transcriptional activation of target genes (13, 26). Indirect immunostaining of RLMVEC with anti-Smad7 antibodies after 4-h incubation in the absence (Fig. 9Aa) or in the presence (Fig. 9Ab) of albumin demonstrated that albumin endocytosis induced Smad7 protein expression. In control experiments, we tested the specificity of anti-Smad7 antibody by preincubating it with a Smad7 blocking peptide. Preincubation with the blocking peptide prevented Smad7 immunostaining (data not shown). We observed by immunoblotting that protein expression of Smad7 was increased in 4 h after starved RLMVEC exposure to albumin (Fig. 9B) at the point when Smad2-P levels were close to the baseline. We conclude that Smad7 expression was induced by albumin in a time-dependent manner at a time consistent with downregulation of albumin-induced signaling.

**Structural homology between albumin and TGF-β.** Because albumin endocytosis activated TGF-β receptor signaling (as measured by an increase in the level of Smad2-P and induction of Smad7 protein expression), we examined the possibility of structural homology between human albumin and human TGF-β precursor and mature protein using a protein alignment algorithm (16). We observed that the mature TGF-β 112-residue protein has marked homology with the human albumin amino acid sequence (Fig. 10A). The first region, one of 53 amino acids from residue 59–112 in the mature TGF-β1, is 26.4% identical to a region (residues 367–420) in HSA (Fig. 10A). The second region of homology is 12 amino acids (nos. 420) in HSA (Fig. 10A).
10–21) in TGF-β1 that share 50% identity with residues 297–309 of albumin (Fig. 10A). Although in most of the experiments described above we have used BSA, we confirmed the results with HSA. Additionally, the regions of homology described here are highly conserved (90%) between HSA and BSA.

We further identified a highly conserved region in the human TGF-β1 finger II region (residues 88–101) known to bind the TβRII, based on X-ray crystal structure of the ligand and the receptor (12), and HSA (Fig. 10B), which has 38.4% identical amino acids (residues 555–567) and 65% sequence similarity with HSA. These observations point to a structural basis for the interaction of albumin with the TβRII.

**DISCUSSION**

Endocytosis of cell surface receptors is a means of internalizing and compartmentalizing cell signaling and acts, additionally, as a mechanism for receptor degradation and desensitization (1, 2, 7, 28, 32, 40, 42, 43). We studied endothelial cells...
because these vessel wall lining cells actively internalize albumin by endocytosis of caveolae in an Src-dependent manner (23, 36, 37). In this report, we focused specifically on the previously identified ~73-kDa albumin binding protein present in the endothelial cell membrane (10). On the basis of several criteria, we identified this protein as TβRII. Analysis by cross-linking showed that native albumin binds to a ~75-kDa protein, which is immunoprecipitated by an anti-TβRII antibody. We also showed that the membrane fraction of endothelial cells was enriched for an ~75-kDa TβRII immunoreactive protein. Uptake of 125I-labeled albumin by RLMVEC appears to be receptor-mediated, since the uptake is competed by unlabeled albumin and reaches saturation at ~2 mg/ml albumin. In addition, an anti-TβRII antibody specifically inhibited albumin endocytosis, thus linking this endocytic event with TβRII. Importantly, transient expression in COS-7 cells of a vector containing a cDNA encoding human TβRII was sufficient to induce albumin endocytosis in these cells. In contrast, expression of a TβRII dominant-negative kinase mutant failed to support endocytosis. We demonstrated that a mink lung epithelial cell line, Mv1.Lu, endogenously expressing both TβRI and TβRII, was capable of albumin endocytosis, whereas two mutant-related cell lines, L-17 and DR-26, lacking functional TβRI or TβRII, respectively, showed inhibition of cell surface albumin binding, as well as albumin endocytosis. DR-26 cells show higher inhibition compared with the L-17 cells in albumin binding to the surface and in albumin uptake. Thus these data indicate an important role for the TGF-β receptors in the mechanism of albumin endocytosis.

A number of putative albumin binding proteins in addition to p73 (i.e., gp60, p30, and p16; see Ref. 10) have been partially characterized, but the true molecular identity of the albumin receptor proteins has remained elusive. Here, for the first time, we have demonstrated a novel role for TβRII in regulating albumin endocytosis both in RLMVEC and mink lung epithelial cells.

We also demonstrated that induction of albumin endocytosis was accompanied by activation of TβRII-mediated signaling. We observed increased TβRII expression on the cell surface, increased Smad2-P and translocation to the perinuclear region, and translocation of Smad4 from the cytosol to the perinuclear region. Taken together, these data show that the interaction of albumin with TβRII induces endocytosis and intracellular TGF-β receptor signaling in endothelial cells.

We identified the ~73-kDa albumin binding protein present in the endothelial cell membrane (10) as TβRII. We showed that albumin was capable of increasing the cell surface pool of TGF-β1 that share 50% identity with residues 297–309 of albumin.

The sequence homology between human TGF-β1 and HSA was 26.4% identity across 53 amino acids.

![Diagram](https://example.com/diagram.png)
TBRII, as evidenced by the increase in punctate TBRII, detected by immunofluorescence after exposure to albumin. Because this response was seen within minutes, an explanation is that a population of preexisting TBRII may redistribute from the cytosol to the cell surface. Another explanation is that, since the cells (RLMVEC) were serum starved for >24 h, albumin exposure may produce a membrane topology change that could make TBRII more accessible to immunostaining. It is also possible that the antibody used for detecting the TBRII staining in RLMVEC may work better in the presence of albumin in recognizing the epitope on the cell surface than serum-starved cells.

We examined the profile of effector Smads to address whether albumin endocytosis is capable of activating the TBRII signaling pathway. The three classes of Smad proteins in mammals include the R-Smads, co-Smad (i.e., Smad4), and I-Smad (e.g., Smad6 and Smad7; see Refs. 5, 14, 17, 24, 25). We showed an increase in Smad2 (an R-Smad) phosphorylation and its translocation to the perinuclear region after albumin endocytosis. Thus albumin endocytosis is capable of inducing the internalization of TBRII and activating the canonical TBRII signaling pathway in endothelial cells. However, albumin endocytosis did not mimic the translocation of Smad2-P to the nucleus as in the case of the canonical ligand TGF-β. This disparity suggests a role for albumin endocytosis that is distinct from the TGF-β-induced signal transduction (21, 23). We also observed that Smad7, which serves to negatively regulate TGF-β signaling by blocking the phosphorylation of regulatory Smads (e.g., Smad2) by TBRI kinase (13, 26), was upregulated by albumin.

It has been shown recently that TGF-β receptors can be internalized by two different endocytic mechanisms (26). One mode of internalization is mediated through the clathrin pathway, and the other operates via caveolin-positive vesicles (7). In the present study, TBRII internalization is likely the result of association with caveolae since endothelial cell vesicles are mostly derived from caveolae (20). Also, we observed that endocytosis was inhibited by pretreating the cells with cyclodextrin, a cholesterol binding agent that disrupts caveolae (27, 31). Additionally, we showed that the internalized albumin associated with caveolin-1 (unpublished data), lending further support to the dependence of TBRII internalization on caveolae. The fate of the TGF-β receptors is determined by their association with either clathrin-coated vesicles or caveolae and their subsequent internalization pathways (7). Receptors that partition into caveolae interact with Smad7 and its partner Smurf2 and are destined for degradation (7). Thus it is possible that the albumin-induced internalization of TGF-β receptor signaling via caveolae is a mechanism for downmodulating the TGF-β responses.

A possible explanation for our finding that albumin can activate TBRII is the homology between albumin and the TGF-β precursor protein sequences [Kolmogorov-Simirnov statistics (16)]. Two regions show significant homology between albumin and TGF-β1 (Fig. 10). Further examination of the sequence homology between the mature, processed 112-residue TGF-β1 produced by protease cleavage (6, 8) and HSA also reveals regions of identity (Fig. 10A). The first region, one of 53 amino acids from residue 59–112 in the mature TGF-β1, is 26.4% identical to a region (residues 367–420) in HSA (Fig. 10A). The second region of homology is 12 amino acids (nos. 10–21) in TGF-β1 that share 50% identity with residues 297–309 of albumin (Fig. 10A). Thus both the precursor peptide and protease-cleaved active peptide of TGF-β1 share at least three regions with significant sequence homology with albumin. Significant homology also exists between the finger II region of the human TGF-β1 amino acid sequence; that finger II motif has been previously shown to be critical for binding of the ligand TGF-β1 with the receptor TBRII based on the X-ray crystal structure of the TGF-β1 and TBRII ligand-receptor complex (12). HSA shares 38.4% identity and 65% similarity with the finger II region of the human TGF-β1 (Fig. 10B), suggesting a region of albumin that may interact with the TBRII receptor. On the basis of these homology data, we propose a mechanism in which albumin binds to TBRII, activates endocytosis in endothelial cells, and provides a means for internalizing TBRII signaling. Internalization of TBRII signaling activates Smad2 by phosphorylation, and Smad2-P associates with Smad4 to form a complex. Because of the sequence homology between albumin and TGF-β, both ligands may compete for TBRII, and plasma albumin may continuously modulate and fine-tune TGF-β-activated signaling by shutting it to a caveolar degradation pathway (26). Because albumin-induced activation of TBRII also activates Smad7, this suggests another mechanism of downregulation of TGF-β signaling. Thus the competition between albumin and TGF-β for TBRII may provide an exquisite mechanism for the regulation of TGF-β-activated signaling in endothelial cells.

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