Coated Pit-mediated Endocytosis of the Type I Transforming Growth Factor-β (TGF-β) Receptor Depends on a Di-leucine Family Signal and Is Not Required for Signaling

Background: The role of TGF-β receptor endocytosis in signaling is controversial.

Results: Two clathrin-mediated endocytosis signals induce internalization of the type I TGF-β receptor (TβRI). Endocytosis-defective TβRI mutants signal, whereas endocytosis-enhanced TβRI exhibits reduced Smad signaling.

Conclusion: TβRI endocytosis is dispensable for Smad signaling and correlates with reduced response.

Significance: Understanding the role of TGF-β receptor endocytosis is relevant to cell biology and cancer.
reported in some studies but not in others (24–27, 34–36). In addition, the type III TGF-β receptor has been demonstrated to modulate TβRII and TβRI endocytosis via interaction with β-arrestin2 (37, 38). Importantly, the roles of TGF-β receptor endocytosis in signaling are still controversial. Thus, CME was linked to either enhanced (25, 26) or reduced (27, 39) Smad signaling in response to TGF-β, although other reports suggested that Smad signaling is insensitive to inhibition of CME (40, 41). Moreover, opposite effects of caveolar endocytosis on TGF-β non-Smad signaling were reported (41, 42). However, most of these studies have relied on treatments that result in a general inhibition of CME or caveolar endocytosis. Under such conditions, the inhibitory effects are not restricted to the TGF-β receptors and can alter the trafficking and cellular distribution of multiple signaling proteins. Such effects could seriously limit data interpretation.

An alternative approach to assess the role of TGF-β receptor endocytosis in signaling, which circumvents the above problems, is the use of TGF-β receptor mutants with defective or enhanced endocytosis. Here, we applied this approach to TβRI, which directly phosphorylates Smad2/3. We find that TβRI undergoes constitutive internalization via CME, mediated by an internalization signal from the di-leucine family (Leu180-Ile181), which functions in conjunction with an acidic cluster motif. Using TβRI mutants lacking these signals (CME-defective) or containing an additional strong CME targeting motif (YRIL; CME-enhanced), we show that TβRI endocytosis is not required for Smad signaling; rather, it correlates with reduced signaling in response to TGF-β, in line with a role in signal termination. An additional effect of mutating Leu180-Ile181 to alanine is partial constitutive activation of TβRI, which apart from retaining the receptor at the plasma membrane, may be most likely due to the proximity of this sequence (located in the αG1 region) to the binding sites of FKBP12 (43), Smads (44, 45), and additional regulatory proteins (46).

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant TGF-β1 was from PeproTech. Fatty acid-free bovine serum albumin (BSA; fraction V), dyesore hydrate (DYN), and chlorpromazine hydrochloride (CPZ) were from Sigma. Hanks’ balanced salt solution (HBSS) and nystatin suspension were from Biological Industries Beit Haemek. Anti-myc tag (αmyc) 9E10 mouse ascites (47) was purchased from Covance Research Products. Goat anti-mouse (GaM) F(ab’)2, conjugated to Alexa 546, biotin-goat anti-rabbit (GaR) IgG, and Alexa 488-avidin were from Invitrogen. Fluorescent F(ab’)2 were converted to monovalent Fab’ as described (48).

**Plasmids**—Expression vectors encoding human TβRI (in pcDNA3) or TβRII (in pcDNA1) with an EC myc epitope tag were purchased from us earlier (34, 48, 51, 52). Constitutively active myc-TβRII-T204D was generated by inserting the T204D point mutation into myc-TβRII by the QuikChange mutagenesis kit (Stratagene). The TGF-β-responsive luciferase reporter constructs p3TP-Luc (+) in pGL3 (53) and (CAGA)$_{12}$-Luc in pGL3ti (54) were gifts from P. Knaus (Free University of Berlin, Germany). The latter construct is considered to be highly specific for TGF-β-mediated Smad transcriptional activation, due to the specific binding of Smad3 and Smad4 to the CAGA boxes in the promoter (54).

**Mutagenesis**—The truncation, alanine substitution, and endocytosis-enhanced mutants of human myc-TβRI employed in this study are depicted in Fig. 1. The C-terminal truncation mutants (TβRI-V158, -P164, -D179, and -D183) were generated by PCR using QuikChange, with the myc-TβRI plasmid serving as a template, as described by us earlier for TβRII mutagenesis (34). A forward primer recognizing the T7 promoter region of the plasmid was employed, and one of the following primers (containing a stop codon and a NotI restriction site) served as the reverse primer (the consensus stop codon is depicted in boldface letters, and the NotI restriction site is underlined): 1) TβRI-V158, 5’-TTTTCCCTTTTGGCCGGCCTCTACACGCTAGTGGAAAATGACATTGCCG3’; 2) TβRI-P164, 5’-GGAGGCGCCGGCTTAGCTCTCTTCTTCTG3’; 3) TβRI-D179, 5’-TTTTCCCTTTGCGCCGGCTCTAGTCTTTTCTGATGACCTCCCTG3’; and 4) TβRI-D183, 5’-TTTTCTTTTCGTCCGCGCTCTAACTCATAAATGATCTTCCACG3’. The PCR products were digested with HindIII and NotI and inserted into the same mutagenesis kit; the forward primer for each mutant is shown (with Ala codon in boldface letters), and the complementary sequence served as the reverse primer as follows: 1) TβRI-2A, here, myc-TβRI in pcDNA3 served as a template, with the forward mutagenesis primer 5’-TGGAAACACGGCTTCTATGTATGACACGGTACCT3’; 2) TβRI-3A, the template was myc-TβRI in pcDNA3, with the forward mutagenesis primer 5’-AGTGCCCAATATCCAGCGGCCCCTTCATTAGATCGCCCGTTTATTTTCTT3’; and 3) TβRI-3A2, here, TβRI-3A served as a template, employing the same mutagenesis primers used to generate TβRI-2A. For making the endocytosis-enhanced TβRI plasmid, a primer targeting the T7 promoter was used as the forward primer. A reverse primer (as follows) was designed such that the DNA sequence encoding the YRIL peptide (italics) was inserted between the original C-terminal residue of myc-TβRI (Met$^{505}$) and the stop codon (boldface), immediately followed by a HindIII restriction site (underlined): 5’-CAACAGGAGGCATCAATGATTCGGATTATTAAAGCTTTG3’. The PCR product was

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**Cells Lines**—COS7 cells (CRL 1651) and Mv1Lu mink lung epithelial cells (CRL 6584) were from American Type Culture Collection. R1B-L17 (Mv1Lu lacking TβRII) and DR26 (Mv1Lu lacking TβRII) cells (49) were a gift from A. Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden). They were grown as described previously (34, 50). All media and cell culture reagents were from Biological Industries Beit Haemek.
digested with HindIII and inserted into pcDNA3 digested with the same enzyme. The TβRII-2A-YRIL was generated by using TβRI-YRIL as template with the same mutagenesis primers used to generate TβRII-2A. All mutants were verified by sequencing.

**Immunofluorescent Labeling of myc-tagged TGF-β Receptors at the Cell Surface**—COS7 or Mv1Lu-derived cell lines (Mv1Lu, R1B-L17, DR26) grown on glass coverslips in 6-well plates were transfected by TransIT-LT1 (Mirus; COS7 cells) or Lipo-fectamine™ 2000 (Invitrogen; Mv1Lu-derived cells) with 1 μg of DNA of a plasmid encoding one of the myc-TβRI-based constructs or myc-TβRII. After 24 h, cells were incubated (30 min, 37 °C) in serum-free medium, washed with cold HBSS/HEPES/BSA (20 mM HEPES, pH 7.2, 2% BSA), blocked with goat γ-globulin (200 μg/ml, 30 min, 4 °C), and labeled with amyc (20 μg/ml, 45 min, 4 °C) followed by Alexa 546-GaM Fab’ (40 μg/ml, 30 min, 4 °C), all in HBSS/HEPES/BSA. After washing at 4 °C, cells were fixed with 4% paraformaldehyde in PBS (10 min at 4 °C followed by 30 min at 22 °C). Labeled slides were mounted with fluorescence mounting medium (Golden Bridge International). Fluorescent images were recorded with CoolSNAP HQ-M camera (Photometrics) using a ×63/1.4 NA (COS7 cells) or a ×100/1.4 NA (Mv1Lu-derived cell lines) oil immersion objectives mounted on Axioimager D.1 microscope (Carl Zeiss Microimaging).

**Internalization Measurements**—COS7 or R1B-L17 cells growing on glass coverslips were transfected with plasmids encoding one of the myc-TβRI-based constructs. After 24 h, the internalization of the myc-tagged receptors was quantified by the point confocal method employing the fluorescence recovery after photobleaching setup under nonbleaching illumination conditions as described by us earlier (34). Briefly, the cell surface receptors were labeled at 4 °C as described for immunofluorescent labeling of myc-tagged receptors. Labeled cells were either fixed immediately with 4% paraformaldehyde or warmed to 37 °C for the indicated periods to allow endocytosis; they were then transferred back to 4 °C, fixed, and mounted for immunofluorescence as above. Endocytosis was quantified by measuring the reduction in the fluorescence intensity levels at the plasma membrane, focusing the laser beam through the ×63 objective at defined spots (1.86 μm²) in the focal plane of the plasma membrane away from vesicular staining, passing the fluorescence through a pinhole in the image plane to make it a true

![FIGURE 1. Schematic representation of TβRI and its truncation, alanine substitution, and endocytosis-enhanced mutants.](https://example.com/figure1.png)
confocal measurement (34). At each time point, 100–200 cells were measured.

**Smad Nuclear Translocation Assay**—Mv1Lu cells were transfected with a myc-TBR1 construct or empty vector as described above. After 24 h, cells were starved in serum-free medium (30 min, 37 °C) and stimulated (or not) with 200 pm TGF-β1 at 37 °C for the times indicated in the figures. To identify transfected cells, the surface myc-tagged receptors were labeled for immunofluorescence detection as above. The cells were fixed with 4% paraformaldehyde, permeabilized with Triton X-100 (0.2%; 5 min), blocked with goat γ-globulin (200 μg/ml, 30 min, 22 °C) in HBSS/HEPES/BSA, and labeled successively (each incubation for 45 min at 22 °C in the same buffer) by the following: (i) rabbit IgG reactive with Smad2/3 (3 μg/ml); (ii) biotin GaR IgG (2 μg/ml); and (iii) Alexa 488-avidin (2 μg/ml). Cells were mounted and imaged as described for “immunofluorescent labeling.”

**Smad Phosphorylation Assay**—R1B-L17 cells in 6-well plates were transfected as described under “Immunofluorescent Labeling of myc-tagged TGF-β Receptors at the Cell Surface” with one of the myc-TBR1 constructs or empty vector. At 24 h post-transfection, the cells were starved (2 h) in serum-free medium and stimulated (or not) with 200 pm TGF-β1 (30 min). Cells were lysed and subjected to SDS-PAGE (10% polyacrylamide; 100 μg of protein/lane) followed by immunoblotting as described (55). The blots were probed by anti-phospho-Smad2/3 (1:1000), αmyc (0.6 μg/ml), or anti-β-actin (1:20,000), followed by peroxidase-GaR or -GoM IgG (1:5000). After stripping (56), they were reprobed for total Smad2/3 using rabbit anti-Smad2/3 (1:1000). Bands were visualized by ECL (Western Bright, Advansta) and quantified by densitometry (EZQuant Gel 2.2, EZQuant Ltd.).

**Down-regulation of Cell-surface myc-TBR1**—Down-regulation of TBR1 was assayed by measuring the time dependence of the reduction in the level of biotinylated cell-surface receptors (57). Mv1Lu cells in 10-cm plates were transfected by Lipofectamine™ 2000 with 4 μg of DNA of an expression plasmid for myc-TBR1 or empty vector. After 24 h, cells were rinsed three times with ice-cold HBSS/HEPES and incubated for 45 min with 0.5 mg/ml membrane-impermeable sulfo-NHS-LC-Biotin (Pierce) in borate buffer (10 mM boric acid, 150 mM NaCl, pH 8). Cells were rinsed twice with cold HBSS/HEPES containing 15 mM glycine, followed by incubation at 37 °C in complete growth medium for the indicated times (see figure legends). Cells were lysed on ice with RIPA buffer as described (38). After removal of cell debris, 6% (v/v) of each lysate was taken for SDS-PAGE and Western blotting to probe total myc-TBR1 (0.6 μg/ml αmyc) and β-actin (1:20,000) followed by peroxidase-GaM (1:5000) as above. The remainder of each lysate was subjected to immunoprecipitation by 2 μg of αmyc IgG and protein G-Sepharose (30 μl) as described (58). Immunoprecipitates were rinsed and subjected to SDS-PAGE (10% gel) and blotting as described under “Smad Phosphorylation Assay,” using peroxidase-streptavidin (Invitrogen; 1:2500) and ECL to detect biotinylated myc-TBR1. Densitometry was as described under “Smad Phosphorylation Assay.”

**Transcriptional Activation Assays**—Luciferase reporter transcriptional activation assays were performed on Mv1Lu-derived cell lines using two protocols, adapted for unperturbed and endocytosis-inhibited cells. Unperturbed cells in 6-well plates were co-transfected with the following: (i) 1 μg of luciferase reporter construct, p3TP-Luc(+)(或(CAGA)12-Luc; (ii) 0.3 μg of pRL-TK (Renilla luciferase, Promega); and (iii) 1 μg of empty vector or a vector encoding a myc-TBR1 construct or myc-TBR1. At 12 h post-transfection, the cells were split in parallel onto glass coverslips in 6-well plates (to assess the level of the myc-TGF-β receptor at the cell surface) and onto 96-well plates (for the luciferase assays). After another 24 h, the cells in the 96-well plates were serum-starved (2 h), stimulated (or not) with 300 pm TGF-β1 (18 h in starvation medium), lysed, and analyzed by the DLR assay system. The results were normalized for transfection efficiency using the Renilla luminescence. To calibrate for the expression levels of the myc-TGF-β receptors at the cell surface, the latter were determined on the coverslip-plated cells after fluorescent labeling of the cell-surface receptors, using the point-confocal fluorescence intensity measurement as described under “Internalization Measurements.” Transcriptional activation assays on cells subjected to treatments that inhibit endocytosis were conducted similarly, except that the stimulation with TGF-β1 (300 pm) was for a shorter period (6 h), during which the cells were subjected (or not) to the inhibition treatment (for treatment description, see “Treatments Affecting Internalization” below).

**Treatments Affecting Internalization**—Experiments employing internalization-inhibitory treatments were conducted on COS7 or R1B-L17 cells using two protocols, one for testing the effects of a given treatment on endocytosis and the other for studying the effects on transcriptional activation. All endocytosis assays were conducted in HBSS/HEPES/BSA, and all treatments were initiated by a 15-min preincubation (37 °C) with the inhibitory drug/medium. The cells were retained under the inhibitory condition throughout the fluorescent labeling and endocytosis measurement. For the luciferase assays, which were conducted in starvation medium, the endocytosis-disrupting treatment was started upon addition of TGF-β1, and the cells were kept under the same conditions until the lysis step (6 h). The treatments employed were as follows: (i) hypertonic treatment to disrupt the structure of clathrin-coated pits (59) was conducted in HBSS/HEPES/BSA (for endocytosis studies) or starvation medium (for transcriptional activation assays) supplemented with 0.45 m sucrose (34); (ii) treatment with CPZ, which redistributes AP2 from the plasma membrane to endosomes (60), was done using 80 μM CPZ, which redistributes AP2 from the plasma membrane to endosomes (60), was done using 80 μM CPZ, and (iv) nystatin treatment to inhibit caveolar endocytosis (26, 27, 62) employed 25 μg/ml of the drug.

**RESULTS**

**TBR1 Is Endocytosed Mainly through Clathrin-coated Pits Irrespective of Its Activation**—The major aim of this study was to explore the interdependence between the endocytosis of TBR1, which is the receptor responsible for Smad activation, and its signaling output. First, we set to define the relative contribution of CME and caveolar endocytosis to TBR1 internalization. TBR1 carrying an EC myc epitope tag (myc-TBR1) was
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expressed in COS7 cells; the cell surface receptors were labeled at 4 °C as described under “Experimental Procedures,” followed by shifting the cells to 37 °C for defined periods, and measuring TβRI endocytosis by the point-confocal assay (34). The initial surface distribution of myc-TβRI prior to warming to 37 °C was homogeneous, typical of cell-surface labeling in the absence of endocytosis (Fig. 2A). Upon incubation at 37 °C, the fluorescence shifted in a time-dependent manner to a vesicular pattern characteristic of endocytic vesicles (Fig. 2, B–D). Quantitative measurement by the point-confocal method (34) of fluorescently labeled myc-TβRI remaining at the cell surface as a function of the incubation time at 37 °C yielded an internalization half-time (t1/2) of ∼13 min (Fig. 2G), close to that found by us earlier for TβRII (34). Notably, myc-TβRI internalization was fully blocked by various treatments that inhibit CME, including incubation with sucrose-containing hypertonic medium, CPZ, or DYN (which inhibits both CME and caveolar endocytosis) (Fig. 2, E and G). Conversely, treatment with nystatin (an inhibitor of caveolar endocytosis) had no effect (Fig. 2, F and G). We next extended the experiments on myc-TβRI endocytosis to R1B-L17 cells, which are a TβRII-deficient cell line derived from the TGF-β-responsive Mv1Lu epithelial cells (63), providing a highly suitable system for TGF-β signaling studies (50). The sensitivity of R1B-L17 cells to the various endocytosis-inhibiting treatments were similar to those of COS7 cells (supplemental Fig. 1A), with a somewhat faster endocytosis rate in untreated R1B-L17 cells (t1/2 ∼9 min). To explore whether TβRI endocytosis depends on its activation, the experiments were repeated with constitutively active myc-TβRI-T204D; the endocytosis of this mutant and its sensitivity to various inhibitors were identical to myc-TβRI (Fig. 2H), demonstrating that TβRI endocytosis does not depend on its activation state. In line with these results, the internalization rate of TβRI was not affected by ligand (200 pm TGF-β1) in either COS7 or R1B-L17 cells (data not shown). Although it is still possible that a subpopulation of TβRI can associate with lipid raft domains and/or caveolae (26, 64), these results indicate that the major pathway for TβRI endocytosis is via clathrin-coated pits, in accord with several former reports on the endocytosis of TGF-β receptors (24, 25, 27, 34, 35, 39, 40).

To determine the effects of the treatments that inhibit TβRI endocytosis on TGF-β-mediated Smad signaling, we employed the DLR assay using TGF-β-responsive luciferase reporter constructs. The experiments were conducted in R1B-L17 cells, where the mock-transfected cells lacking TβRI provide a direct control for potential TβRI-independent activation by a given treatment. The various CME-inhibiting treatments employed (CPZ, DYN, or hypertonic treatment with sucrose) did not inhibit Smad-responsive transcriptional activation. Rather, they induced variable degrees of constitutive activation (supplemental Fig. 1, B and C), in line with reports on continued or enhanced TGF-β signaling in cells subjected to CME-blocking treatments (39–41). The hypertonic treatment (sucrose) induced some transcriptional activation even in the absence of TβRI, most likely due to effects involving reduction of the cellular and nuclear volumes by partial dehydration. These variable effects suggest that although endocytosis-blocking treatments are useful for establishing the relative contribution of
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Identification of the Motifs Required for TβRI Endocytosis—
To generate endocytosis-defective TβRI mutants, it was first necessary to identify the endocytosis motif(s) responsible for its internalization. Because CME-targeting motifs are cytoplasmic, and TβRI internalization proceeds mainly via CME (Fig. 2), we initially generated a series of myc-TβRI mutants with progressive truncations of the cytoplasmic domain (generated by introducing stop codons at the desired positions). These mutants are depicted in Fig. 1 (first four mutants). The first truncation mutant, TβRI-D183, was chosen to retain the Leu\(^{180}\)-Ile\(^{181}\) sequence, based on our previous studies that identified an LI di-leucine family motif on TβRII as its major CME-targeting signal (34). TβRI-D183 was internalized in COS7 cells as fast as the wild type TβRI (Fig. 3), suggesting that no internalization signals are located beyond Asp\(^{183}\). Notably, further truncation at Asp\(^{179}\) (eliminating Leu\(^{180}\).Ile\(^{181}\)) reduced the endocytosis rate ~2-fold (Fig. 3), indicating that Leu\(^{180}\)-Ile\(^{181}\) may play a role in TβRI internalization. To validate this assumption, we have replaced Leu\(^{180}\)-Ile\(^{181}\) by alanines in the context of the full-length TβRI to generate the TβRI-2A alanine-replacement mutant (depicted in Fig. 1). The internalization rate of TβRI-2A was compromised to the same extent as TβRI-D179 (Fig. 3).

The partial inhibition of the endocytosis of TβRI-2A and TβRI-D179 suggests that TβRI may contain additional endocytosis signals. One possibility is that TβRI may also undergo caveola-like endocytosis. Yet the failure of nystatin, a known blocker of caveolar endocytosis, to inhibit the internalization of TβRI(WT) (Fig. 2, F and G) argues against this possibility. This notion is strongly supported by the demonstration that the residual endocytosis of TβRI-2A is insensitive to nystatin but is fully blocked by CME-blocking treatments (see Fig. 3). This suggests that in addition to Leu\(^{180}\)-Ile\(^{181}\), there is another CME-targeting signal in the membrane-proximal region of TβRI. To identify this signal, we further truncated TβRI to generate TβRI-P164 and TβRI-V158 (Fig. 1), and we measured their endocytosis in COS7 cells. Although the internalization of TβRI-P164 was similar to that of TβRI-2A, the endocytosis of TβRI-V158 was nearly eliminated (Fig. 3). This suggests that

either nystatin, CPZ, or sucrose (hypertonic medium) as described under “Experimental Procedures.” The surface receptors were then labeled at 4 °C as above, followed by a 20-min incubation at 37 or 4 °C (time 0) in media containing inhibitors where indicated (see under “Experimental Procedures”). Endocytosis of the myc-TβRI mutants was quantified by the point confocal microscopy (see “Experimental Procedures” and Fig. 2). Results are mean ± S.E. of 150–200 cells in each time point. Intensity at time 0 for each sample was taken as 100%. G. endocytosis of TβRI mutants is sensitive to CME inhibitors but not to an inhibitor of caveolar endocytosis. At 24 h post-transfection, cells were left untreated or treated with specific endocytosis pathways to the internalization of a given receptor, the interpretation of their effects on signaling is confounded by the alteration of the trafficking and cellular distribution of multiple signaling proteins by the same treatment. To bypass this problem, we turned to an alternative approach, based on measuring the signaling responses of endocytosis-defective or endocytosis-enhanced TβRI mutants.

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the additional endocytosis signal resides in the short segment between Val^{158} and Pro^{164}. Notably, this sequence contains an acidic amino acid cluster (EED, at positions 161–163); such a cluster was reported to act as a CME-targeting signal (65–67). Mutation of Glu^{161}, Glu^{162}, Asp^{163} to alanines generated the TβRI-3A mutant (Fig. 1), whose endocytosis rate was reduced to the same extent as TβRI-2A (Fig. 3). Moreover, similar to TβRI-2A, the residual endocytosis of TβRI-3A was blocked by CME-inhibiting treatments but not by nystatin (Fig. 3G), in line with the CME-targeting character of both signals. Importantly, the endocytosis of the double TβRI mutant containing both the 3A and 2A alanine replacement mutations (TβRI-3A2A; for schematics, see Fig. 1) was drastically inhibited (Fig. 3). We conclude that the internalization of TβRI in COS7 cells is mediated by a di-leucine and an acidic cluster CME signals, reminiscent of the situation encountered for insulin-regulated amino peptidase (67).

The endocytosis-defective TβRI mutants enable studies on the impact of specific inhibition of TβRI endocytosis on signaling. Such studies can be complemented by the use of a TβRI mutant that undergoes enhanced CME, revealing the effects of augmented TβRI internalization on the signaling outcome. To this end, we grafted the strong CME signal YRIL, which belongs to the YXXZ (X indicates any amino acid; Z indicates hydrophobic amino acid) family of CME signals (68, 69), onto the C terminus of TβRI (TβRI-YRIL; Fig. 1). In line with the CME targeting capability of the YRIL peptide, the endocytosis rate of TβRI-YRIL was markedly enhanced relative to TβRI(WT) (Fig. 4, A–D). The enhanced endocytosis is via the CME pathway, as shown by the ability of CME inhibitory treatments (but not nystatin) to block it (Fig. 4E).

Early studies reported some differences between the endocytosis of chimeric TGF-β/GM-CSF receptors in different cell types (32, 33), suggesting that TGF-β receptor endocytosis may depend on the cellular context. Thus, after the initial characterization of TβRI endocytosis signals in the COS7 cell system, we turned to validate the results in Mv1Lu-derived cells, which are better suited for signaling studies. To this end, we measured the internalization of the full-length endocytosis-defective and endocytosis-enhanced TβRI mutants in R1B-L17 cells. As in the case of COS7 cells, the Leu^{180}-Ile^{181} motif was required for TβRI endocytosis, and addition of the strong CME-targeting YRIL signal to TβRI markedly enhanced its endocytosis (Fig. 5). However, the role of the Leu^{180}-Ile^{181} signal in TβRI internalization in the R1B-L17 cells is much more prominent than in COS7 cells, as shown by the near-complete loss of endocytosis in the TβRI-2A mutant (Fig. 5). The notion that the Leu^{180}-Ile^{181} signal is sufficient for TβRI endocytosis in R1B-L17 cells is supported by the very minor effect of the 3A mutation (eliminating the acidic cluster motif) on TβRI endocytosis in these cells and by the similar and very low internalization rates of TβRI-2A and TβRI-3A2A (Fig. 5). The strong YRIL signal is predominant in TβRI-YRIL endocytosis, as demonstrated by the similar endocytosis rates of TβRI-YRIL and the TβRI-2A-YRIL double mutant lacking the Leu^{180}-Ile^{181} signal (Fig. 5); the endocytosis rates of these two mutants were also identical in COS7 cells (data not shown). The partial difference between COS7 cells and Mv1Lu-derived cells in the usage of specific endocytosis signals may reflect cellular context differences in the repertoire of clathrin adaptor proteins.

**Smad Signaling by Endocytosis-defective and Endocytosis-enhanced TβRI Mutants**—The endocytosis-defective and endocytosis-enhanced TβRI mutants described above enable studies that directly address the role of TβRI endocytosis in signaling. To this end, we evaluated TGF-β-mediated Smad signaling by transcriptional activation studies using the TGF-β-responsive luciferase reporter constructs p3TP-Luc(+) (53) and (CAGA)_{12}-Luc (54). These studies employed R1B-L17 cells, which express TβRII but lack functional TβRI, and therefore do not respond to TGF-β unless they are transfected with TβRI (63). The cells were co-transfected by one of the myc-TβRI constructs (or empty vector; control), together with a luciferase reporter construct and pRL-TK (Renilla luciferase, serving as a transfection calibration control). Because the response to TGF-β is initiated by binding to the cell-surface receptors and the TβRI endocytosis mutants could display different levels of expression at the cell surface, we measured in parallel the surface levels of the myc-tagged receptors by the point confocal method (see “Experimental Procedures”). Although these levels were rather similar (the differences were less than 15%), we have also calibrated the luciferase results accordingly (see “Experimental Procedures”). As shown in Fig. 6, the CME-defective mutations in TβRI had a pronounced effect on Smad signaling in the absence of ligand; transfection with CME-defective mutants (TβRI-2A and TβRI-3A2A)
induced marked transcriptional activation already without ligand, an effect not shown by either TβRI(WT) or the endocytosis-enhanced TβRI-YRIL mutant. The constitutive nature of this activity is further validated by the lack of basal signaling in Mv1Lu cells in complete growth medium, as shown by the similarly low signaling levels in the absence and presence of the TβRI kinase inhibitor SB431542 (Fig. 6, C and D). This notion is reinforced by the constitutive signaling of TβRI-2A and -3A2A in DR26 cells (supplemental Fig. 2), which lack functional TβRII and therefore do not respond to ligand. The ability of the endocytosis-defective mutants to activate the luciferase reporters without TGF-β stimulation was even somewhat higher than that of the constitutively active TβRI-T204D mutant. However, in contrast to TβRI-T204D, whose activity was insensitive to TGF-β, the endocytosis-defective mutants retained a degree of TGF-β responsiveness (Fig. 6). Examination of the fold-increase in the luciferase activity following stimulation with TGF-β shows that this parameter is significantly lower for the endocytosis-defective mutants (~2-fold increase; compare the black and white bar pairs for each receptor in Fig. 6). This effect is not due to low activation in the presence of ligand, but rather it reflects a smaller incremental increase in the presence of ligand due to high basal activity. This suggests that the CME-defective mutants possess some degree of constitutive activity. Notably, the endocytosis-defective TβRI-2A mutant expressed in R1B-L17 cells induced a moderate phosphorylation of Smad2/3 already in the absence of ligand, increasing significantly to at least the same level mediated by TβRI(WT) upon stimulation with TGF-β1 (Fig. 6E). To explore whether this constitutive activation of the 2A-containing TβRI mutants stems from their defective endocytosis or from an independent effect induced by the 2A mutation, we employed the myc-TβRI-2A-YRIL compound mutant, which bears the 2A mutation but undergoes efficient endocytosis by virtue of the YRIL signal. This mutant displayed constitutive activation as well as ligand-mediated stimulation, albeit at lower levels than the endocytosis-defective 2A mutants (Fig. 6). This suggests that the high constitutive activity levels of the latter mutants involve contributions from both mechanisms (reduced endocytosis and activation by the 2A mutation). A negative control is supplied by the TβRI-V158 truncation mutant, which is inactive (lacks the kinase domain) but is also endocytosis-defective; indeed, the kinase-deficient TβRI-V158 failed to activate the luciferase reporter constructs either in the absence or presence of TGF-β.

To validate that the above effects are not specific to the R1B-L17 mutant cell line, we conducted the same experiments in the parental Mv1Lu cells. It is more difficult to detect the effects of the specific TβRI mutants in these cells, because they respond to TGF-β via their endogenous receptors (see the vector-only transfected cells in Fig. 6). For this reason, the increase in signaling upon transfection with TβRI(WT) in Mv1Lu cells was below the detection limit. However, this background issue does not extend to signaling in the absence of ligand, which is very low for the endogenous receptors. Fig. 6 demonstrates that expression of the endocytosis-defective TβRI-2A in Mv1Lu cells also results in constitutive transcriptional activation of the Smad pathway already in the absence of ligand. A similar phenomenon is observed with the constitutively active TβRI-T204D mutant (Fig. 6). Notably, although the TβRI-2A-YRIL mutant exhibited partial constitutive activation, it was significantly lower (p < 0.05) than that of TβRI-2A, in line with the results obtained in R1B-L17 cells.

The translocation of Smad2/3 to the nucleus is a key early step in TGF-β-mediated Smad signaling, which could be utilized to validate the constitutively active character of the endocytosis-defective TβRI mutants. To this end, we expressed myc-TβRI (WT or endocytosis mutants) in Mv1Lu cells, and we investigated the effects on Smad2/3 nuclear translocation. The transfected cells were stimulated for 30 min with TGF-β1 or left untreated, and fluorescence microscopy was employed to score cytoplasmic versus nuclear localization of Smad2/3 in the transfected cells (identified by immunofluorescence using omyc to label myc-TβRI). Typical results are shown in Fig. 7A, and data derived from several independent experiments integrating data from 300 cells are depicted in Fig. 7B. These studies demonstrate that although Smad2/3 is mainly cytoplasmic in unstimulated control (mock-transfected) cells, the vast majority of these cells (which express endogenous TGF-β receptors) display TGF-β1-induced nuclear accumu-
lation of Smad2/3. In sharp contrast, the CME-defective mutants (TβRI-2A and TβRI-3A2A), but not TβRI(WT) or endocytosis-enhanced TβRI-YRIL, induced Smad2/3 nuclear translocation even prior to stimulation with TGF-β (Fig. 7). The TβRI-2A-YRIL double mutant also exhibited constitutive Smad nuclear translocation, but to a lower extent than the endocytosis-defective 2A mutants (Fig. 7), in accord with the results in the luciferase transcriptional activation assays (Fig. 6). As expected, constitutively active TβRI-T204D mutant also induced Smad2/3 nuclear translocation in the absence of ligand (Fig. 7).

These results demonstrate that TβRI endocytosis is dispensable for signaling (Figs. 6 and 7 and supplemental Fig. 1). The endocytosis half-time measured for TβRI(WT) in Mv1Lu cells is 9 min. Therefore, faster signaling output kinetics would support the notion that it is independent of TβRI endocytosis. To that end, we measured the kinetics of TGF-β-induced Smad2/3 nuclear translocation (an early TGF-β signaling event that can
FIGURE 7. Smad nuclear translocation by TβRII endocytosis mutants in Mv1Lu cells. Mv1Lu cells were transfected with the indicated myc-TβRII mutants or with pcDNA3 (0.8 μg) supplemented with 0.2 μg of tdTomato (Tomato) in pEBS (a gift from D. Kolakofsky, University of Geneva, Switzerland) to identify the mock-transfected cells. At 24 h post-transfection, the cells were serum-starved (30 min) and incubated with or without TGF-β1 (200 pM, 30 min) in serum-free medium. After labeling the myc-tagged cell surface receptors at 4 °C using myc IgG followed by Alexa 546-G aFab' (red fluorescence), the cells were fixed/permeabilized and the endogenous Smad2/3 proteins were labeled as described under “Experimental Procedures” (green fluorescence). Bar, 15 μm. A, typical images showing cytoplasmic or nuclear localization of Smad2/3. The white arrows in the Smad2/3 images indicate typical images showing cytoplasmic or nuclear localization of Smad2/3. The white arrows in the Smad2/3 images indicate

be measured with high sensitivity) in Mv1Lu cells. The results (Fig. 8, A and B) yield a rate at least 2-fold faster (t1⁄2 ~4 min). In addition, TGF-β receptor endocytosis was also linked earlier to receptor degradation (26, 27); we therefore measured the degradation of cell-surface TβRI in Mv1Lu cells, using a cell-surface biotinylation protocol (see “Experimental Procedures”). The results (Fig. 8, C and D) yield a degradation half-time of ~2 h both in the absence or presence of TGF-β1, in accord with former reports (26, 27). Thus, the degradation rate of TβRI is much slower than its endocytosis rate in the same cells, in line with a role for TβRI endocytosis in signal termination.

DISCUSSION
Numerous studies on the endocytosis of TβRII and TβRI have suggested CME as the major internalization pathway, although a potential contribution by caveolar endocytosis has been contentious (24–27, 34–36). Attempts to define the roles of TGF-β receptor endocytosis (especially TβRI, which is the receptor that directly mediates downstream signaling to the Smad pathway) have led to conflicting results (25–27, 39–41), largely due to reliance on treatments that inhibit altogether CME and/or caveolar endocytosis. In this study, we identified the previously unknown CME-targeting signals on TβRII, generated endocytosis-defective and endocytosis-enhanced TβRII mutants, and we employed them to directly investigate the relationship between TβRII endocytosis and signaling. Our findings indicate that TβRII endocytosis is dispensable for TGF-β signaling and may even play a negative role (enhanced endocytosis may reduce signaling). Notably, mutating the Leu180-Ile181 endocytosis signal to alanines perturbed TβRII internalization and endowed it with partial constitutive activity. Although retention of TβRII at the plasma membrane contributes to this constitutive activity, an additional contributing factor may be the proximity of the mutated residues to TβRI regions involved in regulatory interactions.

Former studies have measured the endocytosis rate of GM-CSF/TβRII chimeric receptors dimerized by GM-CSF (32), but a direct quantitative measurement of TβRII endocytosis was not reported. Using the point-confocal endocytosis assay (34) to measure the internalization of EC-tagged myc-TβRII, we found that it undergoes constitutive endocytosis independent of its activation state (Fig. 2 and supplemental Fig. S1A), in line with former reports that TGF-β receptor internalization does not depend on activation (26, 27). The initial endocytosis rate (the fraction internalized per min, derived from the linear part of the internalization curve) (70) was 0.025 min−1 in COS7 cells, and 0.05 min−1 in R1B-L17 cells, in the same range reported for GM-CSF/TβRII homodimers (32). These rates are also close to those of TβRII internalization (32, 34).

To facilitate the search for TβRII endocytosis signals, it was desired to determine initially the pathways that contribute to its internalization. In both COS7 and Mv1Lu-derived cells, CME
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FIGURE 8. Kinetics of Smad nuclear translocation and degradation of cell-surface myc-TβRI. A and B, immunofluorescence of Smad2/3. Mv1Lu cells were incubated without or with TGF-β1 (200 pM, 37 °C) for the indicated times, fixed/permeabilized, and labeled for Smad2/3 as described under “Experimental Procedures.” A, representative micrographs. Bar, 15 μm. B, quantification of Smad2/3 nuclear translocation. The percentage of cells with predominantly nuclear Smad2/3 was derived by scoring 300 cells in each sample. The S.E. (n = 3) is indicated. C and D, surface biotinylation assay of TβRI down-regulation. Mv1Lu cells in 10-cm plates were transfected with myc-TβRI or pcDNA3 (control) and subjected to surface biotinylation followed by incubation at 37 °C for the indicated times as detailed under “Experimental Procedures.” Immunoprecipitates of myc-TβRI and cell lysates (6% v/v of the lysate) were resolved by SDS-PAGE and Western blotting using the indicated probes. C, Western blots of a representative experiment (one out of four). D, quantification (means ± S.E., n = 4) of the intensities of the biotinylated myc-TβRI immunoprecipitated at the different time points, after normalization to the myc-TβRI levels in the lysates. The normalized level in myc-TβRI transfected cells at time 0 (2nd lane) in each experiment was taken as 100%. Identical results were obtained in the presence or absence of ligand; thus, only the latter are shown.

emerged as the major TβRI internalization pathway, as evidenced by the blockade of TβRI endocytosis in cells subjected to several CME inhibitory treatments and by the insignificant effect of nystatin, a caveolar endocytosis inhibitor (Figs. 2 and 3; supplemental Fig. S1). The identification of CME as the prominent TβRI internalization pathway is in line with the large majority of earlier reports on the endocytosis of TGF-β receptors or their chimeric constructs (24–27, 34, 35, 40), and it is further supported by the strong inhibitory effect of RNAi-mediated clathrin knockdown on TβRI internalization (27). It differs from a previous publication that suggested that TGF-β receptors also undergo caveolar endocytosis (26); however, the latter report did not measure directly TβRI endocytosis and employed a higher nystatin concentration (50 μg/ml), which may also inhibit CME (27).

Following the identification of CME as the main route for TβRI internalization, we conducted a mutational analysis of myc-TβRI in search of its CME-targeting signal(s). To this end, we first screened a series of cytoplasmic truncation mutants and then focused on alanine substitution mutations in regions whose omission inhibited TβRI endocytosis (for schematics of the mutants, see Fig. 1). The results (Figs. 3 and 5) implicate two signals in TβRI endocytosis as follows: (i) Leu180-Ile181, a di-leucine family signal from the same family as the Ile218-Ile219-Leu220 sequence identified earlier as the sole CME targeting signal in TβRII (34); (ii) a three-residue Glu161-Glu162-Asp163 negatively charged cluster in the membrane-proximal region preceding the GS domain, reminiscent of acidic cluster CME-targeting motifs (65–67). Interestingly, a combination of a di-leucine sequence and acidic cluster was shown to be required for the dynamic retention of insulin-regulated aminopeptidase in endosomes (67). Notably, the contribution of the two signals to TβRI endocytosis differs between the COS7 and Mv1Lu-derived cells, with an equal weight for each signal in COS7 cells (Fig. 3), but a near-complete dependence on the Leu180-Ile181 signal in R1B-L17 cells (Fig. 5). The impact of the two signals is in accord with the reported cell type differences in the internalization of chimeric TGF-β receptors (32, 33), and it may reflect distinct repertoires of clathrin adaptor proteins in different cell lines.

The identification of the CME-targeting signals on TβRI and the preparation of endocytosis-defective TβRI mutants lacking these signals enabled direct investigation of the roles of TβRI endocytosis in signaling, without resorting to treatments that induce a general block of endocytic pathways, thus avoiding potential side effects due to the altered cellular distribution of multiple signaling proteins. To this end, we expressed endocytosis-defective (TβRI-2A, TβRI-2A3A), endocytosis-enhanced (TβRI-YRIL, TβRI-2A-YRIL), or TβRI(WT) in R1B-L17 cells (devoid of TβRI), and we measured their effects on Smad-dependent transcriptional activation of TGF-β luciferase reporter constructs. The results (Fig. 6) lead to two important conclusions. First, TβRI endocytosis is not required for signaling; in fact, the activation of the endocytosis-defective mutants is higher than that of TβRI(WT), although the endocytosis-enhanced TβRI-YRIL induces a lower level of transcriptional activation. This suggests that TβRI internalization via coated pits plays a role in signal termination. Second, the endocytosis-defective mutants display partial constitutive activity; their expression in R1B-L17 cells results in high activation levels in the absence of ligand, whereas TGF-β can still induce further stimulation, unlike the constitutively active TβRI-T204D mutant, whose activity is ligand-independent (Fig. 6). The constitutively active character of the endocytosis-defective
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