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Transforming Growth Factor (TGF)-β1 Internalization

MODULATION BY LIGAND INTERACTION WITH TGF-β RECEPTORS TYPES I AND II AND A MECHANISM THAT IS DISTINCT FROM CLATHRIN-MEDIATED ENDOCYTOSIS*

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Transforming growth factor-β (TGF-β) internalization was studied by monitoring the uptake of ^{125}I-TGF-β1 in Mv1Lu cells, which endogenously express TGF-β receptors types I (RI), II (RII), and III (RIII), and 293 cells transfected with RI and RII. At 37 °C internalization occurred rapidly, within 10 min of ligand addition. Internalization was optimal in 293 cells expressing both RI and RII. Internalization was prevented by phenylarsine oxide, a nonspecific inhibitor of receptor internalization, but was not affected by reagents that interfere with clathrin-mediated endocytosis such as monodansylcadaverine, K44A dynamin, and inhibitors of endosomal acidification. Electron microscopic examination of Mv1Lu cells treated with ^{125}I- TGF-β1 at 37 °C indicated that internalization occurred via a noncoated vesicular mechanism. Internalization was prevented by prebinding cells with TGF-β1 at 4 °C for 2 h prior to switching the cells to 37 °C. This was attributed to a loss of receptor binding, as indicated by a rapid decrease in the amount of TGF-β1 bound to the cell surface at 37 °C and by a reduction in the labeling intensities of RI and RII in ^{125}I-TGF-β1-cross-linking experiments. Mv1Lu or 293 (RI+RII) cells, prebound with TGF-β1 at 4 °C and subsequently stripped of ligand by an acid wash, nevertheless initiated a signaling response upon transfer to 37 °C, suggesting that prebinding promotes formation of stable RI·RII complexes that can signal independently of ligand.

Receptor-mediated endocytosis is triggered by ligand binding to its receptor at the cell surface and results in internalization of both ligand and receptor. Depending on the ligand-receptor system, internalization can serve to sequester the receptors rapidly, promote access of activated receptor to intracellular substrates, or in some cases, target the ligand and/or receptor to specific organelles such as the nucleus (1–5). Internalized receptors can be recycled back to the cell surface, or in certain cases, internalized receptor and/or ligand can be targeted for lysosomal degradation. The latter process results in a net reduction in the number of surface receptors, a process termed down-regulation.

The endocytic mechanisms underlying receptor-mediated endocytosis can be divided into two main types: endocytosis via clathrin-coated pits and non-clathrin-mediated internalization such as caveolae-mediated or "noncoated" vesicle-mediated endocytosis (6). Clathrin-mediated endocytosis is by far the best characterized mechanism and is utilized by many receptors including G protein-coupled, seven-transmembrane receptors (*e.g.* β2-adrenergic and neurokinin 1), tyrosine-kinase receptors such as those for epidermal growth factor (EGF),[1] platelet-derived growth factor, and insulin, as well as other non-kinase, single transmembrane receptors (*e.g.* transferrin) (7–11). In contrast, very little is known about the mechanism utilized for internalization or down-regulation of the more recently discovered family of serine/threonine kinase receptors for transforming growth factor-β (TGF-β).

TGF-β-signaling requires the interaction between two functionally distinct Ser/Thr kinase receptors, TGF-β receptor types I and II (RI and RII, respectively). TGF-β binds directly to RII which induces the formation of an activated TGF-β·RII·RI complex. RII transphorylates RI which in turn phosphorylates intracellular substrates such as Smad2/3 (12, 13). An additional TGF-β receptor, type III (RIII), can form a complex with RI and RII. RIII has no kinase function but is thought to facilitate ligand binding to RII (14, 15). Studies utilizing chimeric receptors, consisting of the extracellular domain of granulocyte/macrophage colony-stimulating factor (GM-CSF) α or β receptor fused to the transmembrane and cytoplasmic domains of RI or RII, have shown that both homomeric and heteromeric combinations of RI or RII are capable of internalizing GM-CSF (16). This indicates that both receptors possess internalization motifs. Nevertheless, transphorylation of RI by RII was required for optimal internalization, and down-regulation was observed only for the heteromeric complex (17). These results suggest that functional interactions between RII and RI set up distinct endocytic responses. Our own studies, using cells expressing full-length TGF-β receptors, confirmed the dual requirement of RI and RII for receptor down-regulation and also showed that RIII can enhance this process (18). In addition, affinity labeling and microscopic analysis of cells expressing RII and RI tagged with green fluorescent protein (GFP) indicated that receptor internalization may be preceded by ligand-induced receptor aggregation/modulation at the cell surface (18). Taken together these findings illustrate the po-

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[1] The abbreviations used are: EGF, epidermal growth factor; TGF-β, transforming growth factor-β; RI, RII, RIII, TGF-β receptors type I, II, and III, respectively; GM-CSF, granulocyte/macrophage colony-stimulating factor; GFP, green fluorescent protein; MDC, monodansylcadaverine; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; BSA, bovine serum albumin; D-PBS, Dulbecco's phosphate-buffered saline; PAO, phenylarsine oxide; LM, light microscopy; EM, electron microscopy.

tential contribution of both the cytoplasmic and extracellular domains of the TGF-β receptors in endocytosis.

Internalization of the chimeric GM-CSF/TGF-β receptors in GM-CSF-treated cells was inhibited by potassium depletion or cytosolic acidification of cells using NH_4Cl and amiloride (16, 17). These treatments are known to interfere with clathrin-mediated internalization. However, as noted above, TGF-β binding promotes receptor modulation events that may be unique and are not necessarily mimicked by a foreign ligand and chimeric receptors. In addition, it has been shown for other receptor systems that endocytic routing can be altered depending on ligand-receptor affinities. For example, EGF and transforming growth factor-α (TGF-α), which both bind to the EGF receptor with similar affinities at neutral pH, show different endosomal sorting patterns, and this was demonstrated to be a function of the different dissociation constants of these ligands in the endosome where the pH is more acidic (19). We have therefore elected to preserve all possible aspects of TGF-β/receptor interactions and their effects on endocytosis by examining the internalization of native TGF-β in cells expressing full-length TGF-β receptors.

In this study we have monitored internalization of ^{125}I-TGF-β1 in Mv1Lu cells, which endogenously express RI, RII, and RIII. Internalization was also studied in 293 cells transfected with RII and RI. Our results indicate that TGF-β1 is internalized rapidly at 37 °C and confirm that RI and RII are both required for optimal internalization. Our results demonstrate that known inhibitors of clathrin-mediated endocytosis, such as monodansylcadaverine (MDC) or K44A dynamin, did not interfere with internalization and therefore suggest that TGF-β internalization occurs through a distinct mechanism. In support of this, electron microscopic examination of ^{125}I-TGF-β1-treated Mv1Lu cells indicated that internalization is mediated via noncoated vesicles. Intriguingly, prebinding of Mv1Lu cells or 293 (RI+RII) cells with ^{125}I-TGF-β1 at 4 °C prevented subsequent internalization of ligand at 37 °C. However, cells that were prebound with TGF-β1 at 4 °C and then stripped of ligand were able to signal upon transfer to 37 °C. This indicates that ligand binding at 4 °C induces formation of stable RI·RII complexes that are capable of signaling independently of ligand.

EXPERIMENTAL PROCEDURES

Cell Culture

Mink lung epithelial cells (Mv1Lu CCL-64) and 293 cells (CRL-1573) were obtained from American Type Culture Collection (Rockville, MD). DR-26 cells were a gift from J. Massagué (Sloan-Kettering Cancer Center, New York). MLEC-32 cells were a gift from D. B. Rifkin (Kaplan Cancer Center, New York). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS).

Plasmids

The RI and RII plasmids contained the coding regions for rat RI and human RII, respectively. Each gene, minus the 5′- and 3′-untranslated regions, was amplified and inserted separately into pcDNA-3. K44A dynamin (in pcDNA-3) was donated by M. Bouvier (University of Montreal) and initially provided by A. M. van der Bliek (San Diego). pcDNA3/erbB1 (the EGF receptor plasmid) was a kind gift from Y. Yarden (Rehovot, Israel). The Smad2 plasmid was a gift from L. Attisano (Univ. of Toronto, Ontario)

Internalization Assays

Internalization of TGF-β1 in Mv1Lu Cells at 37 °C—Mv1Lu cells (or DR-26 cells) were seeded in DMEM (plus 10% FBS) onto 12-well plates (2.2×10^5 cells/well) for 18–20 h. The cells were washed twice with TGF-β-binding medium (200 mM HEPES-buffered DMEM, pH 7.4, 0.2% bovine serum albumin (BSA)) and then incubated in binding medium for 30 min at 4 °C. The cells were then treated with 100 pM ^{125}I-TGF-β1 (NEN Life Sciences Products) in cold binding medium plus or minus 10 nM unlabeled TGF-β1 and immediately transferred to a 37 °C water bath for the indicated time periods. After each time period the cells were transferred on ice and washed twice with Dulbecco's phosphate-buffered saline containing 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$ (D-PBS++), and 0.1% BSA. Surface ligand was removed from the cells by washing once with 150 mM NaCl, 0.1% acetic acid, 2 M urea at 4 °C for 3 min. Internalized ligand was then extracted by treating the cells with Triton X-100 solubilization buffer (1.0% Triton X-100, 10% glycerol, 1 mM EDTA, 20 mM Tris-HCl, pH 7.5) for 30 min at 4 °C. The amounts of surface and internalized ligand were quantitated in a gamma counter. Specific counts (specific cpm) were determined by subtracting competed samples (plus unlabeled TGF-β1) from noncompeted samples.

Internalization of TGF-β1 in 293 Cells at 37 °C—293 cells were seeded in DMEM (plus 10% FBS) onto 12-well plates (1×10^5 cells/well) for 18–20 h. The cells were transfected with the indicated plasmids (*e.g.* RII, RI+RII, or pcDNA-3) using Superfect (Qiagen) according to the manufacturer's specifications. After 20 h the cells were tested for internalization of ^{125}I-TGF-β1 as indicated above for Mv1Lu cells except after incubation at 37 °C and subsequent washing with D-PBS++, surface ligand was removed from the 293 cells by washing twice with 150 mM NaCl, 0.1% acetic acid (minus urea) for 2 min at 4 °C. Urea was not included in the acid wash in this case because 293 cells were less adherent to the plates in the presence of urea.[2]

Effect of Inhibitors of Endocytosis on TGF-β1 or EGF Internalization at 37 °C—Mv1Lu (or 293 (RI+RII)) cells were washed once with TGF-β-binding medium at 4 °C. The cells were then treated with 50 μM phenylarsine oxide (PAO) (Sigma) for 10 min, 100 μM MDC (Sigma) for 30 min, or 500 μM chloroquine (Sigma) for 30 min at 37 °C. The cells were then transferred on ice and incubated with fresh binding medium at 4 °C for 15 min. This was replaced with binding medium containing 100 pM ^{125}I-TGF-β1 (plus or minus unlabeled TGF-β1), and the cells were then transferred to 37 °C for 60 min (for Mv1Lu cells) or 90 min (for 293 RI+RII) cells. In the case of K44A dynamin, 293 cells were transfected with either RI +RII plasmids or pcDNA3/erbB1 plasmid along with equal molar amounts of K44A dynamin plasmid or empty vector (pcDNA-3). After transfection the cells were washed and then treated with either 100 pM ^{125}I-TGF-β (plus or minus 10 nM unlabeled TGF-β1) for 90 min or 150 pM ^{125}I-EGF (NEN Life Sciences Products) (plus or minus 75 nM unlabeled EGF) for 60 min at 37 °C.

Internalization after Prebinding Cells with TGF-β1 at 4 °C—Mv1Lu or 293 (RI+RII) cells were seeded and washed as indicated above. The cells were then incubated with 100 pM ^{125}I-TGF-β1 in binding media (plus or minus 10 nM unlabeled TGF-β1) for 2 h at 4 °C. The cells were then transferred to a 37 °C bath and incubated for the indicated time periods. Surface and internalized TGF-β was determined as described above.

Cross-linking of ^{125}I-TGF-β1 to Receptors after Prebinding at 4 °C

293 (RI+RII) cells were washed twice on ice with D-PBS++, 0.1% BSA. The cells were prebound with 100 pM ^{125}I-TGF-β1 at 4 °C for 2.5 h. The cells were then either kept at 4 °C or transferred to 37 °C for 0, 30, 60, or 120 min. At the end of each time point the cells were washed once with D-PBS++ at 4 °C and then treated with 1 mM bis(sulfosuccinimidyl) suberate (Pierce) for 5 min at 4 °C to cross-link bound ligand to the surface receptors. The reaction was quenched by the addition of glycine (final concentration 100 mM) for 5 min, and then the cells were washed twice with D-PBS++ and solubilized with Triton X-100 buffer for 30 min at 4 °C. The samples were electrophoresed in a 3–11% polyacrylamide gradient gel under denaturing conditions and the labeled receptors were detected using a PhosphorImager.

TGF-β-Luciferase Reporter Assay after Prebinding Ligand at 4 °C

MLEC-32 (mink lung epithelial) cells stably express a luciferase reporter gene under the control of the TGF-β-responsive plasminogen activator inhibitor promoter (20). MLEC-32 cells were seeded in DMEM (plus 5% FBS) onto 24-well plates (8×10^4 cells/well) for 18–20 h. The cells were washed twice with D-PBS++, 0.1% BSA at 4 °C and then incubated with 0 or 10 pM TGF-β1 at 4 °C for 2 h. The cells were then washed three times with D-PBS++, 0.1% BSA, twice with 150 mM NaCl, 0.1% acetic acid (for 3 min), and again two times with D-PBS++, 0.1% BSA at 4 °C. The cell were then treated with or without a second dose of TGF-β1 (10 pM) in DMEM (minus FBS) at 37 °C for 4 h. The cells were then washed once with D-PBS++, lysed, and analyzed for luciferase activity using cell lysis buffer and luciferase substrate (Promega kit, Madison, WI) in a Lumat LB9501 luminometer (Berthhold).

[2] J. C. Zwaagstra, M. El-Alfy, and M. D. O'Connor-McCourt, unpublished observations.

Western Blot Detection of Smad2 Phosphorylation

Transfected 293 cells (1×10^5 cells/well in a 12-well plate), expressing RI, RII, and Smad2, were prebound with 10 pM TGF-β1 at 4 °C for 3 h. The cells were then acid stripped (as indicated above for MLEC-32 cells) and subsequently incubated in the absence or presence of 10 pM TGF-β1 at 37 °C for 4 h. Cell lysates were prepared, electrophoresed in a 8% acrylamide gel, and then transferred onto nitrocellulose. Phosphorylated Smad2 was then detected with an antibody specific for phospho-Smad2 (Geneka Biotechnology Inc., Montreal, Quebec) followed by electrochemical luminescence (Renaissance Kit, NEN Life Science Products).

Light Microscope (LM) and Electron Microscope (EM) Autoradiography

Mv1Lu cells were seeded onto 10-cm plates for 18 h. The cells were incubated with 100 pM ^{125}I-TGF-β1 (plus or minus 10 nM unlabeled TGF-β1) in D-PBS++, 0.1% BSA at 37 °C for 10 or 60 min. The cells were then washed four times with cold PBS++, fixed in 2.5% gluteraldehyde, 0.1 M cacodylate at 4 °C, and then scraped from the plate and pelleted by centrifugation. The pellet was embedded in Epon-812, and semithin (0.5-μm thick) sections were prepared and placed on slides for the LM study. The slides were then coated with Kodak NTP2 emulsion for autoradiography and exposed at 4 °C for 3 or 12 days. Straw-colored thin sections (~100 nm) of the same Epon blocks were collected for the EM study. These were coated with Ilford L4 emulsion, exposed for 2 or 3 months, and developed for filamentous silver grains (21).

RESULTS

Rapid Internalization of TGF-β1 in Mv1Lu Cells at 37 °C—The time course for TGF-β1 internalization was compared in Mv1Lu cells (which express RI, RII, and RIII) and DR-26 cells (a mutant Mv1Lu cell derivative that lacks RII). DR-26 cells bind ligand to RIII, but because they lack RII, they are incapable of binding ligand to RI and signaling (22, 23). They serve here as a negative control for RII-dependent TGF-β internalization. The cells were treated with 100 pM ^{125}I-TGF-β1 for increasing time periods at 37 °C (in the absence or presence of 100 fold excess unlabeled ligand to determine nonspecific binding) and then washed twice with PBS to remove unbound ligand. Ligand bound to surface receptors was removed using an acid wash and counted (surface TGF-β1) (see "Experimental Procedures"). This was followed by extraction of internalized ligand by solubilizing the cells with buffer containing Triton X-100 (internalized TGF-β1) (Fig. 1, *A* and *B*). Internalization occurred rapidly, within the first 10 min after the addition of ^{125}I-TGF-β1 to Mv1Lu cells (Fig. 1*A*). The amount of internalized TGF-β1 increased rapidly and reached a maximum level by 40 min. Similar kinetics were observed in at least four repeat experiments with maximum values ranging between 3,000 and 4,400 specific cpm internalized (data not shown). A lower amount of internalized TGF-β1 was also detected in DR-26 cells. The levels in these cells, however, never exceeded 30% of that seen for Mv1Lu cells and may be the result of partial internalization mediated by RIII. These results therefore indicate that RI and RII are the primary mediators of internalization. The surface levels of TGF-β1 on Mv1Lu cells remained relatively constant between 10 and 40 min (~2,000 specific cpm) and then declined (Fig. 1*B*). In contrast, surface TGF-β1 for DR-26 cells remained at basal levels for the first 60 min (ranging between 0 and 500 specific cpm) and then increased slowly. The constant level of surface TGF-β1 seen for Mv1Lu cells during the first 40 min presumably reflects an equilibrium state between internalized and recycled receptors at physiological temperature. The subsequent decline in both surface and internalized TGF-β1 after 40 min may be the result of ligand depletion from the binding buffer and intracellular degradation of ligand in these cells. Degradation of internalized TGF-β1 has been noted previously in another cell line (24).

Interactions between RI and RII and TGF-β1 Are Required for Optimal Internalization—Recent studies using chimeric GM-CSF/TGF-β receptors indicated that GM-CSF-induced homodimers of RI or RII could result in internalization of GM-CSF. However, in the case of full-length TGF-β receptors, RI requires RII for binding to TGF-β. Therefore, in cells expressing both of these receptors, it is probable that heteromeric RI·RII complexes and potentially homodimeric RII complexes mediate internalization of TGF-β. To estimate the relative contributions of these receptor combinations we measured TGF-β1 internalization in 293 cells transfected with either RII alone or RII plus RI.

Fig. 2*A* shows that, as expected from our above results for Mv1Lu cells, 293 cells expressing RI and RII readily internalized TGF-β1. The level of TGF-β1 internalized in RI+RII cells increased steadily between 0 and 60 min and then started to level off. Internalization was specifically the result of the transfected TGF-β receptors because no internalized TGF-β1 could be detected in control cells transfected with empty vector (control). In cells expressing RII alone, the amount of internalization was reduced compared with RI+RII cells. In several repeat experiments, the maximum levels of TGF-β1 internalized by RII cells were somewhat variable but consistently lower than for RI+RII cells, ranging between 50 and 70% at the 90 min time point (data not shown). This indicates that RII can mediate internalization but does so less efficiently than when this

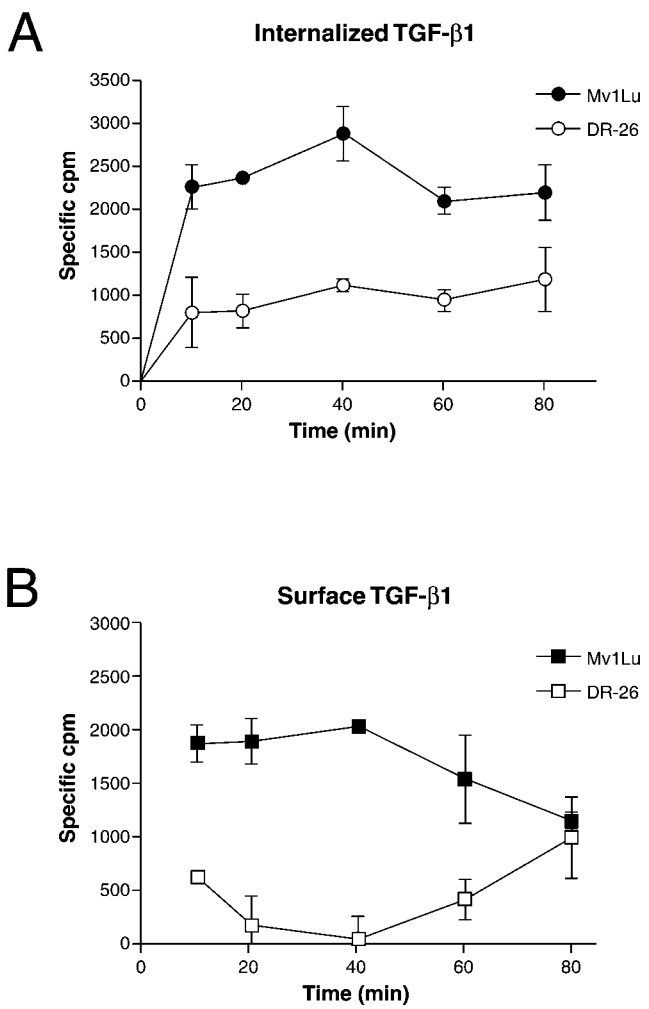

FIG. 1. **Internalization of TGF-β1 at 37 °C.** Mv1Lu or DR-26 cells were treated with 100 pM ^{125}I-TGF-β1 for the indicated time periods at 37 °C. The graphs show the amounts of internalized TGF-β1 (*panel A*) and surface TGF-β1 (*panel B*) expressed as specific cpm (total cpm minus the cpm of competed samples) for triplicate samples. These results are representative of four separate experiments.

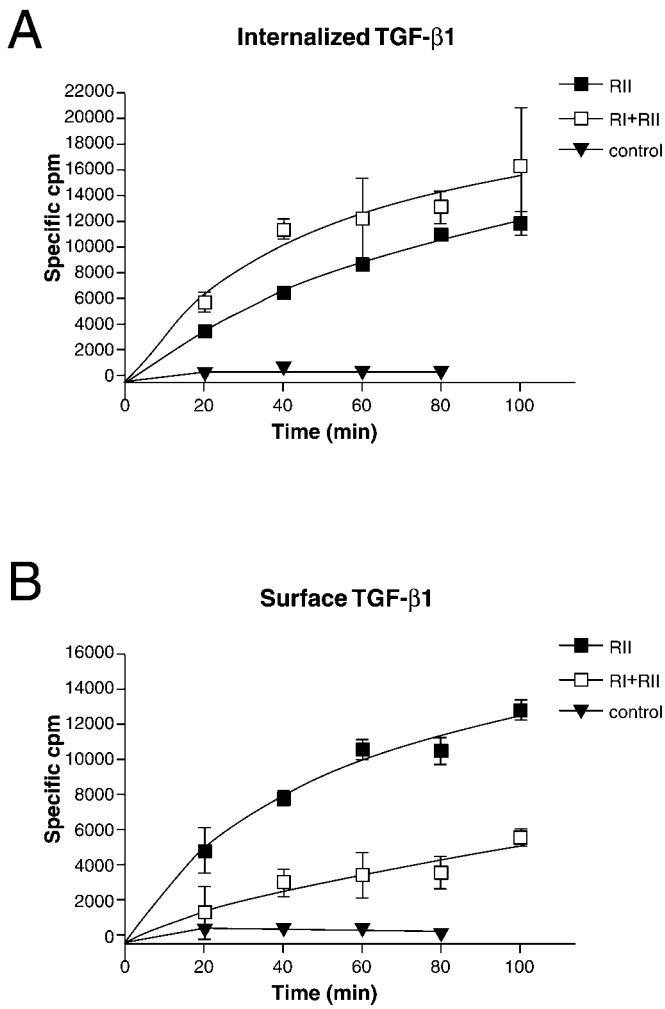

FIG. 2. **Internalization of TGF-β1 at 37 °C in 293 cells expressing RI and/or RII.** 293 cells transfected with RII, RI+RII, or pcDNA-3 (control) were treated with 100 pM ^{125}I-TGF-β1 for the indicated time periods at 37 °C. The graphs show the amounts of internalized TGF-β1 (*panel A*) and surface TGF-β1 (*panel B*) expressed as specific cpm (total cpm minus the cpm of competed samples) for triplicate samples. The curves were generated by nonlinear regression analysis. These results are representative of four separate experiments.

receptor is combined with RI. Together these results imply that interactions between RI and RII and TGF-β1 are required for optimal internalization. It should be noted that the amount of TGF-β internalized by RII alone might be overestimated in Fig. 2*A* because 293 cells express a low level of endogenous RI (as detected by cross-linking of radiolabeled-TGF-β1).[2] Hence a percentage of the TGF-β1 internalized by RII-transfected cells is likely also mediated by heteromeric RI·RII complexes.

The surface TGF-β1 levels were also monitored for the above cells (Fig. 2*B*). In general it can be seen that compared with cells expressing RII alone, RI+RII cells had the least amount of surface TGF-β1 at any given time point, indicating a lower number of receptors remaining at the cell surface. This is consistent with the data in Fig. 2*A* and indicates that cells with both receptors internalize TGF-β ligand and receptors more rapidly.

Prebinding of TGF-β1 Modulates Receptor Binding and Internalization—In the procedure used for the TGF-β internalization assays shown in Figs. 1 and 2, internalization is initiated by the addition of TGF-β1-containing medium to the cells and immediate incubation at 37 °C. An alternative method is to prebind ligand to surface receptors at 4 °C for an extended period of time and then switch the cells to 37 °C to initiate internalization. This second method has been used successfully for other ligand-receptor systems to assess whether increased receptor occupancy influences internalization (25).

Fig. 3*A* shows an example of a 37 °C time course for both surface and internalized ligand after prebinding of Mv1Lu cells with 100 pM ^{125}I-TGF-β1 for 2 h at 4 °C. Control experiments done at 4 °C indicated that binding of TGF-β1 to the surface of these cells reached a maximum by about 1.5 h (between ~3,000 and 4,000 specific cpm), whereas internalized ligand did not exceed basal levels (~1,000 specific cpm) during the prebinding period (data not shown). After transfer to 37 °C, surface TGF-β fell quickly from an initial level of ~3,000 specific cpm (at time 0) to a lower level of ~1,000 specific cpm by 20 min and remained low for 60 min thereafter (Fig. 3*A*). This reduction could not be attributed to internalization because no corresponding net increase in internalized TGF-β1 was observed and must therefore reflect a loss of ligand from the surface receptors. In contrast, in control experiments in which cells were maintained constantly at 4 °C, surface ligand did not fall below the level established after 2 h of binding (data not shown and Fig. 3*C*). Together these results indicate that prebinding at 4 °C modulates the receptors such that their ability to bind ligand decreases when the cells are transferred to 37 °C (see "Discussion").

Loss of surface TGF-β1 accompanied by no internalization of ligand was also observed for 293 (RI+RII) cells after prebinding with 100 pM ^{125}I-TGF-β1 at 4 °C (Fig. 3*B*). To visualize directly the effect of prebinding ligand on surface receptors, we used a cross-linking agent (bis(sulfosuccinimidyl) suberate) to label the surface receptors of 293 (RI+RII) cells radioactively with ^{125}I-TGF-β1 after prebinding for 2.5 h (time 0) and at successive time points (30, 60, and 120 min) after switching the cells to 37 °C (Fig. 3*C*). This was compared with cells that were maintained at 4 °C after prebinding. On the cells maintained at 4 °C, no reduction was observed in the labeling intensities for RI and RII between 0 and 120 min, confirming that these receptors remain on the cell surface and continue to bind ligand at this temperature (Fig. 3*C*, compare *second* and *fourth lanes* with *first lane*). In the cells that were transferred to 37 °C, however, there was a progressive reduction in labeling intensities for both receptors (Fig. 3*C*, compare *fifth* and *sixth lanes* with *first lane*). Similar results were obtained for RI and RII on prebound Mv1Lu cells (data not shown), confirming that these prebound receptors lose their ability to bind ligand at 37 °C.

Prebound TGF-β Receptors Signal Independently of Ligand—Our above results indicate that receptors that are prebound at 4 °C readily release TGF-β1 at 37 °C. A possible consequence of ligand release would be disruption of preformed RI·RII complexes and loss of receptor signaling activity. The signaling ability of prebound receptors was therefore assessed, as shown in Fig. 4*A*. Mv1Lu cells stably expressing a TGF-β-responsive luciferase reporter gene (MLEC-32 cells) were prebound with 0 or 10 pM TGF-β1 at 4 °C for 2 h. The cells were then acid washed to remove bound ligand, followed by a second incubation with (+TGF-β1, *black bars*) or without ligand (−TGF-β1, *clear bars*) at 37 °C for 4 h. The cells were then lysed and assayed for luciferase activity. We reasoned that any decrease in receptor activity resulting from disruption of receptor complexes would be reflected in a reduced luciferase response at 37 °C in a short term assay, in this case 4 h. The +TGF-β1 response for cells pretreated at 4 °C without ligand (0 pM) was only slightly reduced compared with control cells that were kept at 37 °C (*37 °C control*), indicating that preincubation at 4 °C itself did not alter TGF-β signaling capacity significantly. Surprisingly, the cells prebound with 10 pM TGF-β1 but not treated subsequently with ligand at 37 °C

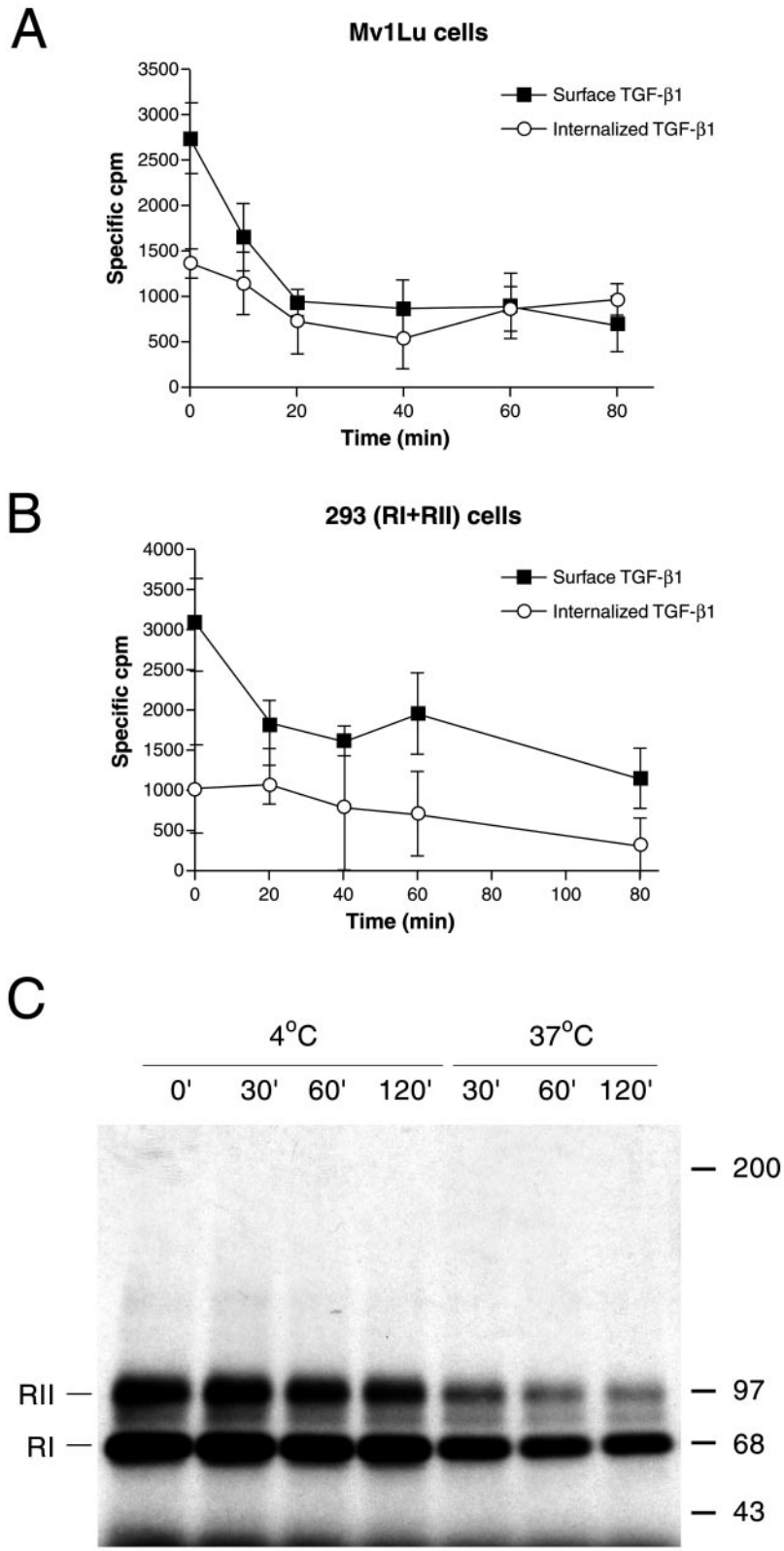

FIG. 3. **Loss of surface TGF-β1 and absence of internalized TGF-β1 after pretreatment of cells with ligand at 4 °C.** Mv1Lu cells (*panel A*) or 293 (RI+RII) cells (*panel B*) were preincubated at 4 °C with 100 pM ^{125}I-TGF-β1 for 2 h. The cells were then incubated at 37 °C for the indicated time periods. The amounts of surface and internalized TGF-β1 were determined for each time point. *Panel C*, the ^{125}I-TGF-β1 labeling intensities of RI and RII become reduced at 37 °C after pretreatment of 293 (RI+RII) cells with ligand at 4 °C. 293 (RI +RII) cells were pretreated at 4 °C with 100 pM ^{125}I-TGF-β1 for 2.5 h. The cells were then either left at 4 °C or incubated at 37 °C for 0, 30, 60, or 120 min. Bound radioactive ligand was chemically cross-linked to surface RI and RII, followed by solubilization of the receptors and gel electrophoresis. The labeled receptors were detected using a PhosphorImager. The positions of RI and RII are indicated on the *left*. The positions of the molecular size standards (in kDa) are indicated on the *right*.

(−TGF-β1, *clear bar*) still showed a luciferase response. The luciferase activity level for these cells was in fact similar to the +TGF-β1 response of the cells that were not pretreated with ligand (37 °C control and 0 pM at 4 °C, *black bar*). This response was not the result of residual ligand from the prebinding step because we have determined by cross-linking residual prebound ^{125}I-TGF-β1 to surface receptors or counting surface radioactivity that our acid wash procedure removed 70–80% of prebound ligand (data not shown). Control experiments also indicated that the luciferase response of these cells to lower amounts of ligand (*i.e.* 2–3 pM, the 20–30% of the TGF-β1 that would be left as residual) was close to basal levels and could be distinguished easily from 10 pM (data not shown). These results therefore indicate that prebound receptors remain active even after TGF-β1 is removed and can signal independently of ligand. This was confirmed using 293 (RI+RII) cells expressing

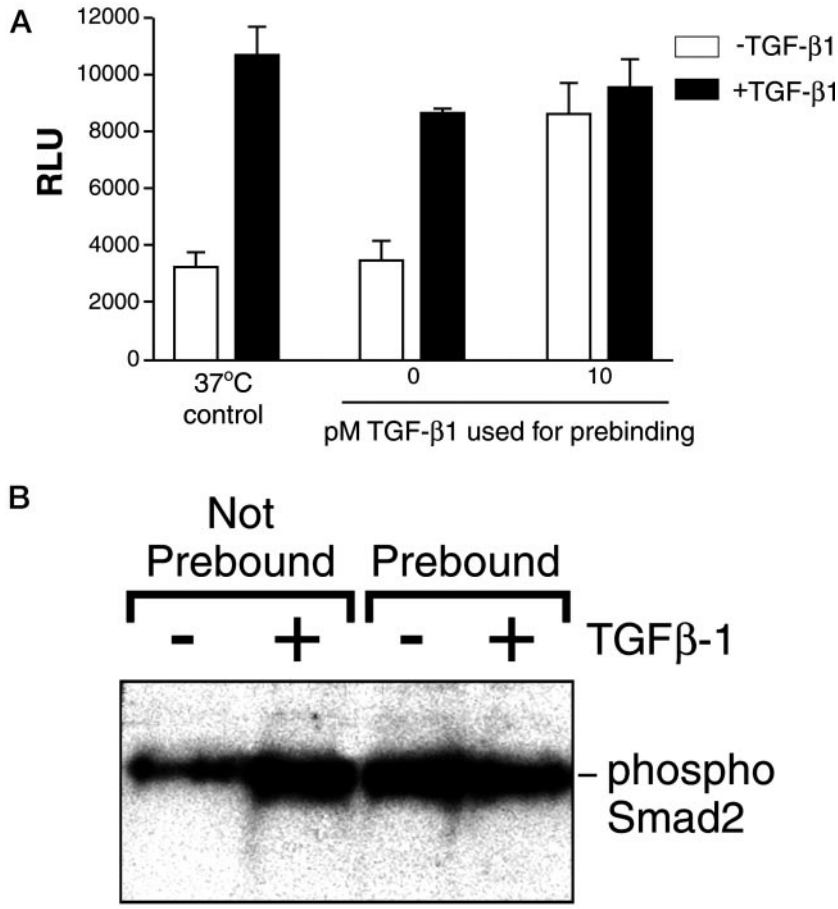

FIG. 4. *Panel A*, Mv1Lu cells show a ligand-independent signaling response after prebinding with TGF-β1 at 4 °C. MLEC-32 cells (Mv1Lu cells stably expressing a TGF-β-responsive luciferase reporter gene) were pretreated with 0 or 10 pM TGF-β1 at 4 °C for 2 h. The cells were then washed and acid stripped at 4 °C to remove bound ligand and then incubated with (+*TGF-β1*) or without (−*TGF-β1*) a second dose of TGF-β1 at 37 °C for 4 h. The cells were then lysed and analyzed for luciferase activity. The graph shows the signaling response, in relative luciferase units (*RLU*), for triplicate samples. The response for control cells that were not preincubated at 4 °C is shown on the *left*. These results are representative of four separate experiments. *Panel B*, Smad2 is phosphorylated in the absence of ligand after prebinding 293 (RI+RII) cells with TGF-β1 at 4 °C. 293 cells expressing RI, RII, and Smad2 were either prebound with 10 pM TGF-β1 or incubated in the absence of ligand (not prebound) at 4 °C for 3 h. The cells were then acid treated to remove prebound ligand and subsequently treated with (+) or without (−) TGF-β1 at 37 °C for 4 h. Cell lysates were prepared, and samples were Western blotted and probed with phospho-Smad2 antibody. Equal amounts of protein were loaded in each lane. These results are representative of duplicate samples for two separate experiments.

Smad2, the primary substrate phosphorylated by RI after its activation. These cells were either left unbound or prebound with 10 pM TGF-β1 at 4 °C for 3 h followed by acid removal of ligand and then assessed for their ability to phosphorylate Smad2 at 37 °C using an antibody specific for phospho-Smad2 (Fig. 4*B*). A low level of phospho-Smad2 was detected in control cells, not prebound with ligand, perhaps because of partial receptor interaction and activation resulting from overexpression (*first lane*). However, the level of phospho-Smad2 was augmented by subsequent treatment of these cells with TGF-β1 (compare *second lane* with *first lane*). In contrast, in cells prebound with TGF-β1, elevated phospho-Smad2 was observed even for cells that were not treated subsequently with ligand (*third lane*). These results therefore strongly suggest that prebinding facilitates the formation of stable RI·RII complexes that no longer require TGF-β1 to signal (see "Discussion").

Effect of Inhibitors of Endocytosis on TGF-β Internalization—To examine the underlying mechanism of TGF-β receptor-mediated internalization of ligand we tested the effects of known inhibitors of receptor endocytosis on TGF-β1 internalization in Mv1Lu cells or 293 cells expressing RI and RII. Internalization assays were in this case performed only at 37 °C, without a prebinding step (Fig. 5, *A* and *B*). PAO reacts with vicinal sulfhydral groups, forming stable ring structures, and has been used widely as a general inhibitor of receptor-mediated endocytosis (26–29). MDC, a potent inhibitor of transglutaminases, prevents internalization of many receptors including those for transferrin, hepatocyte growth factor, and interleukin-8 and is thought to block clathrin-mediated endocytosis at the receptor invagination step (30–33). K44A dynamin is a dominant-negative mutant form of dynamin which interferes with internalization of several receptors, including the EGF receptor. K44A dynamin blocks clathrin-coated pit constriction and coated-vesicle budding (34–37).

Treatment of Mv1Lu cells with 50 μM PAO virtually abolished TGF-β1 internalization (Fig. 5*A*). Quantification of surface TGF-β1 on these cells showed that PAO treatment resulted in at least a 2-fold increase in the levels of surface ligand compared with untreated cells after 60 min at 37 °C (data not shown). This verifies that PAO did not interfere with ligand binding and indicates that the TGF-β receptors were not internalized in these cells. However, treatment of Mv1Lu cells with 100 μM MDC had no effect on TGF-β1 internalization (Fig. 5*A*). A similar treatment of A431 cells (which endogenously express the EGF receptor) with 100 μM MDC caused a 70% reduction in EGF internalization (data not shown). Similarly, K44A dynamin had no effect on TGF-β1 internalization in 293 cells expressing RI and RII, whereas EGF internalization in 293 cells expressing ErbB1 (the EGF receptor) was reduced by at least 50% (Fig. 5*B*). We were unable to assess the effect of K44A dynamin in Mv1Lu cells because of the poor transfectability of these cells. Cytosolic acidification using NH_4Cl followed by amiloride treatment, another method commonly used to prevent constriction and endocytosis of clathrin-coated vesicles (38), also proved ineffective in preventing TGF-β1 internalization in both Mv1Lu cells and 293 (RI+RII) cells (data not shown).

Chloroquine is a mild basic compound commonly used to interfere with receptor down-regulation. Its activity results from its ability to neutralize the acidic endosome and lysosome and thus inhibit degradation of receptors and/or ligands by lysosomal proteases (39–41). We have observed that at doses higher than 100 μM, chloroquine can interfere with EGF internalization in 293 and A431 cells, presumably because of interference with endosomal acidification, a process essential to

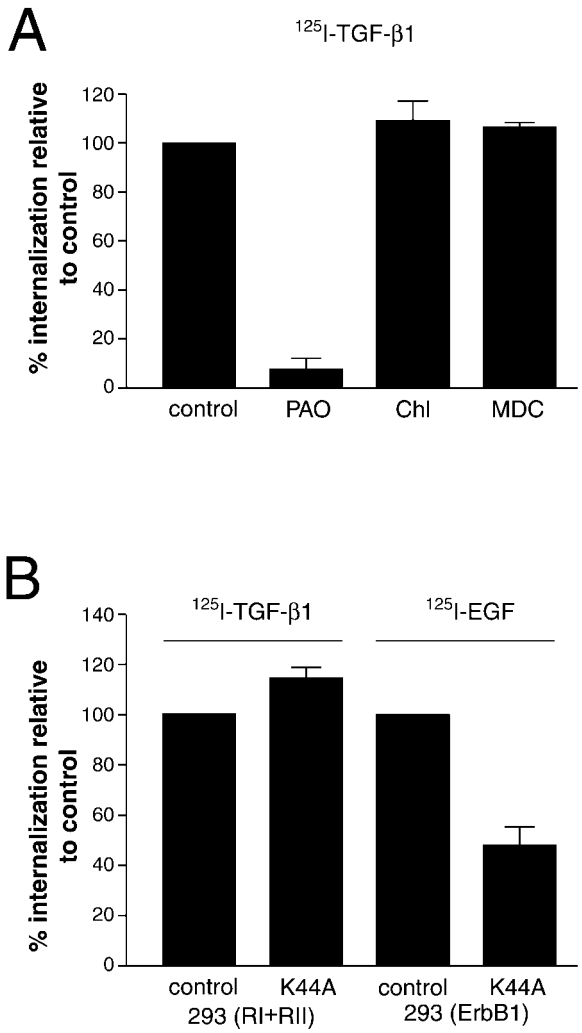

FIG. 5. **Effect of various inhibitors of endocytosis on TGF-β1 internalization.** *Panel A*, effect of PAO, chloroquine (*Chl*), or MDC treatment on TGF-β1 internalization in Mv1Lu cells. The amounts of TGF-β1 internalized (after 60 min at 37 °C) are shown on the graph as percentages relative to untreated controls. *Panel B*, effect of K44A dynamin on TGF-β1 internalization in 293 cells expressing RI+RII or on EGF internalization in 293 cells expressing ErbB1 (the EGF receptor). The amounts of TGF-β1 internalized (after 90 min at 37 °C) or EGF internalized (after 60 min at 37 °C) are shown as percentages relative to the controls.

clathrin-mediated receptor endocytosis (6, 42).[2] Treatment of Mv1Lu or 293 cells with 500 μM chloroquine, a concentration that was sufficient to reduce EGF internalization by 60%, had no effect on TGF-β1 internalization (Fig. 5*A* and data not shown).

Thus, although internalization of TGF-β can be blocked by PAO modification of surface proteins, reagents that interfere with the initial stages of clathrin-mediated endocytosis (MDC and K44A dynamin) or subsequent stages of the endosomal pathway (chloroquine) cannot prevent internalization of TGF-β1.

Microscopic Evidence for Endocytosis of TGF-β via a Noncoated Vesicular Pathway in Mv1Lu Cells—Our above results indicate that TGF-β is not internalized by clathrin-coated vesicles. To visualize endocytosis of TGF-β1 directly we performed both LM and EM autoradiography of Mv1Lu cells at different times after treatment with 100 pM ^{125}I-TGF-β1 at 37 °C (Fig. 6, *A* and *B*). *Panels A* and *B* (Fig. 6*A*) are representative LM autoradiographs at 10 and 60 min, respectively, showing radiolabel (silver grains, seen as *tiny dots*) over the cells. The specificity of the radiolabel is shown by comparison with *panels C* and *D* (Fig. 6*A*), which show control samples treated with ^{125}I-TGF-β in the presence of 100-fold excess unlabeled TGF-β1 for 10 and 60 min, respectively. Fig. 6*B* shows representative EM autoradiographs detecting progressive stages of entry of radiolabeled TGF-β1 at 10 min (*panels A* and *B*) and 60 min (*panels C–E*). At 10 min, silver grains (seen as *filamentous coils*) can be seen both at the cell surface (*panel A*) and within noncoated vesicles inside the cell (*panel B*). At 60 min a higher percentage of silver grains (relative to 10 min) was detected within intracellular vesicles located either close to the cell surface (*panels C* and *D*) or deeper within the cytoplasm (*panel E*). Silver grains are formed within 50 nm of their radioactive source (43); therefore, it is likely that TGF-β1 is inside these vesicles. In these and all other autoradiographs we have examined, there was no evidence for ^{125}I-TGF-β1 in clathrin-coated pits or clathrin vesicles (*panels A–E* and data not shown). Instead, our results indicate that TGF-β1 is internalized via a noncoated vesicular mechanism.

DISCUSSION

Our results show that TGF-β internalization is optimal in cells expressing both RI and RII, indicating cooperativity between these receptors (Fig. 2*A*). It is likely that both the endo and ectodomains contribute to this cooperativity. In recent experiments, using 293 cells expressing mutant receptors, we have determined that certain single amino acid substitutions in the ectodomain of RI which partially disrupt its interaction with RII (but do not interfere with TGF-β binding) cause a reduction in internalization by as much as 50%.[3] This further implies that a productive interaction between the extracellular domains of RI and RII is required for functional alignment of their intracellular domains and optimal internalization.

TGF-β1 was readily internalized in Mv1Lu or 293 (RI+RII) cells maintained at 37 °C (Figs. 1*A* and 2*A*). However, internalization was impaired by prebinding TGF-β1 to these cells at 4 °C for 2 h (Fig. 3, *A* and *B*). In contrast, we and others have observed that prebinding of other ligands such as EGF to cells expressing the EGF receptor (293 and A431 cells, for example) did not interfere with internalization (data not shown and Ref. 24). Similarly, internalization was not impaired after prebinding of GM-CSF to cells expressing chimeric receptors comprised of the cytoplasmic domains of RI or RII fused to the extracellular domains of the GM-CSF α and β receptors (16, 17). Our results therefore indicate that TGF-β1 uniquely modulates its receptors at the cell surface.

Modulation at the cell surface by TGF-β may reflect a preliminary stage in sequestration/internalization which occurs when TGF-β is bound to the heteromeric RI·RII complex. Our experimental procedure of prebinding receptors with ligand at 4 °C likely captures this stage by trapping receptors at the surface membrane. This stage likely involves a ligand-induced reconfiguration of the receptors at the cell surface. Evidence for this was seen in our previous study using 293 cells expressing RII plus RI fused to a GFP tag (RI-GFP) (18). Pretreatment of these cells with TGF-β1 at 4 °C resulted in the formation of receptor aggregates or patches at the cell surface, presumably because of the association of multiple RI-GFP·RII complexes.

Our results indicate that release of TGF-β1 by the surface receptors after prebinding is promoted at 37 °C. On prebound 293 (RI+RII) or Mv1Lu cells maintained at 4 °C the surface receptors continued to bind ligand (Fig. 3*C*, *first four lanes* and data not shown). However, when prebound cells were transferred to 37 °C, the level of surface TGF-β1 fell immediately

[3] A. Guimond, T. Sulea, J. C. Zwaagstra, I. Ekiel, and M. D. O'Connor-McCourt, submitted for publication.

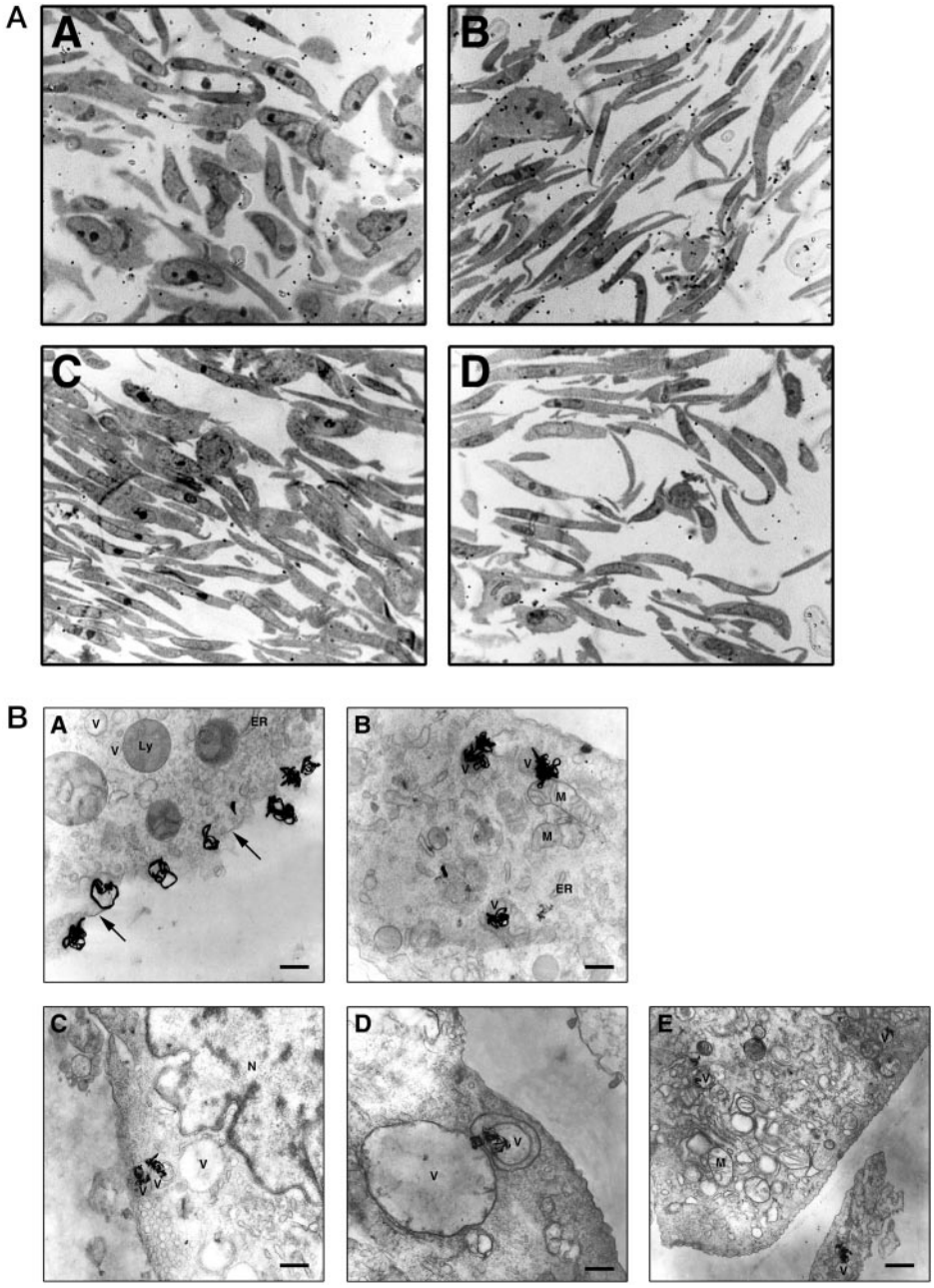

FIG. 6. *Part A*, internalized TGF-β1 is detected by LM autoradiography. Mv1Lu cells were treated with 100 pM ^{125}I-TGF-β1 at 37 °C for 10 min (*panel A*) or 60 min (*panel B*). Surface-bound and internalized radiolabeled ligand is detected as silver grains (seen as *tiny dots*) over the cells. The specificity of the radiolabel is demonstrated by comparison with samples treated with radiolabeled ligand in the presence of 100-fold excess unlabeled ligand for 10 or 60 min (*panels C* and *D*, respectively). *Part B*, internalized TGF-β1 is detected in noncoated vesicles by EM autoradiography. *Panels A–E* show electron autoradiographs of Mv1Lu cells after treatment with 100 pM ^{125}I-TGF-β1 at 37 °C for 10 min (*panels A* and *B*) or 60 min (*panels C–E*). *Panel A*, in this cell, radiolabeled ligand (seen as *filamentous silver grains*) is predominantly along the cell membrane (*arrows*). Magnification, × 33,000. *Bar*, 240 nm. *Panel B*, silver grains are seen in the cytoplasm overlying or near noncoated vesicles. Magnification, × 25,000. *Bar*, 320 nm. *Panels C–E*, at 60 min, silver grains are detected within noncoated vesicles near the cell membrane (*panels C* and *D*) or deep in the cytoplasm (*panel E*). Magnification in *panel C*, × 25,000; *bar*, 320 nm. *Panel D*, × 33,000; *bar*, 240 nm. *Panel E*, × 15,200; *bar*, 525 nm. *N*, nucleus; *Ly*, lysosome; *M*, mitochondria; *ER*, endoplasmic reticulum; *V*, small or large noncoated vesicle.

(Fig. 3, *A* and *B*, and data not shown). This occurred even though, in these experiments, an excess amount of ligand was present in the medium. Together, these results suggest that the loss in the ability of the receptor to bind ligand is not solely caused by a change in the conformation of the receptors resulting from their aggregation at 4 °C but is facilitated by subsequent events that occur at 37 °C. This could potentially involve phosphorylation of these receptors since phosphorylation at specific residues on the intracellular domains of other receptors (*e.g.* the EGF, insulin, and lipotoxin A_4 receptors) has been shown to down-modulate their binding affinity (44–46).

Despite their loss of ligand, prebound receptors retain their capacity to signal. This is indicated by our results showing that 293 (RI+RII) cells phosphorylate Smad2 and that Mv1Lu cells initiated a signaling response at 37 °C even after prebound TGF-β1 was removed from these cells by acid (Fig. 4, *A* and *B*). This suggests that preformed complexes of RI and RII remain intact and are signaling-competent even after ligand release. It also raises the possibility that signaling occurs, at least initially, at the cell surface because our results indicate that internalization of ligand is impaired after prebinding TGF-β1 to Mv1Lu cells at 4 °C. However, our present approach, using radioactive TGF-β1 to monitor internalization, does not rule out the alternative possibility that, after transfer to 37 °C, prebound receptors internalize without ligand. Because these receptors appear to be signaling-competent, they may also be internalization-competent. Further experiments, using cell fractionation procedures and TGF-β receptor-specific antibodies, will be required to trace directly the routing of receptors after prebinding to ligand.

Our experiments using various inhibitors of endocytosis indicate that TGF-β internalization in Mv1Lu or 293 cells does not occur via a clathrin-mediated pathway. This conclusion agrees with our EM results that localized TGF-β1 within noncoated vesicles and to regions on the surface membrane which are devoid of clathrin-coated pits. This is in contrast to previous reports indicating that chimeric GM-CSF/TGF-β receptors utilize a clathrin-mediated pathway (16, 17). This could be attributed to a difference in the cell types utilized in our study, *i.e.* Mv1Lu and 293 cells (epithelial) instead of AKR-2B cells (mes-

enchymal), which were used to express these chimeric receptors. Alternatively, as noted above, this difference may reflect unique ligand-promoted associations among TGF-β receptors, which include their native extracellular domains, and membrane and/or signaling molecules. For example, the initial steps in TGF-β signaling and/or internalization may involve ligand-induced translocation and segregation of RI and RII into discrete membrane domains. A similar mechanism has been hypothesized for certain G protein-coupled receptors, such as the B2 bradykinin receptor and the M2 muscarinic acetylcholine receptor, which become localized within caveolin/cholesterol-rich membrane domains upon treatment with their corresponding agonists (47, 48). These "microdomains" participate in endocytosis and are thought to facilitate recruitment and organization of downstream effector molecules into specialized signaling complexes (49, 50). At present, only preliminary information is available as to how and where in the cell TGF-β signaling complexes, including SARA (*S*mad *A*nchor for *R*eceptor *A*ctivation) and Smads, are assembled (51). Further biochemical analysis will be required to determine whether aggregated TGF-β receptors associate with caveolin or other distinct membrane components.

It is conceivable that TGF-β receptors are internalized by more than one mechanism. For example, the M2 muscarinic acetylcholine receptor can be internalized by either a dynamin-dependent or dynamin-independent mechanism, depending on the cell type in which it is expressed (5, 52). Presumably this is because of the presence of more than one type of internalization motif on this receptor which corresponds to separate endocytic mechanisms. Recent studies on the entry of certain bacterial toxins into mammalian cells have provided evidence for alternate endocytic pathways (53, 54). Ricin and Shiga toxins, for example, after binding to their receptors on the cell surface, are transported in a retrograde fashion to the Golgi and the endoplasmic reticulum. This transport is not dependent on low endosomal pH and, in the case of ricin, is clathrin/dynamin-independent. The molecular mechanisms behind clathrin-independent endocytosis have not yet been clarified, largely because of the lack of known inhibitors of this process. Our EM data indicate that for TGF-β1, internalization occurs via a noncoated vesicular pathway. Some of the TGF-β1-containing vesicles appear distinct from typical endosomes in that they harbor one or more internal vesicles, suggesting some stage involving endosomal invagination (Fig. 6*B*, see *panels C* and *D*). Further investigation will be required to delineate the evolution of these structures.

In summary, we have demonstrated first that TGF-β is internalized rapidly at 37 °C in an RI/RII-dependent manner. Second, this internalization does not have the characteristics of classical dynamin-dependent, clathrin-mediated endocytosis. Finally, prebinding of ligand at 4 °C captures receptors at a novel stage of complex formation such that they cannot subsequently internalize ligand yet are able to signal.

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Transforming Growth Factor (TGF)-β1 Internalization: MODULATION BY LIGAND INTERACTION WITH TGF- β RECEPTORS TYPES I AND II AND A MECHANISM THAT IS DISTINCT FROM CLATHRIN-MEDIATED ENDOCYTOSIS

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