Specific Activation of Mitogen-activated Protein Kinase by Transforming Growth Factor- β Receptors in Lipid Rafts Is Required for Epithelial Cell Plasticity

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Transforming growth factor (TGF)- β regulates a spectrum of cellular events, including cell proliferation, differentiation, and migration. In addition to the canonical Smad pathway, TGF- β can also activate mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/Akt, and small GTPases in a cell-specific manner. Here, we report that cholesterol depletion interfered with TGF- β -induced epithelial-mesenchymal transition (EMT) and cell migration. This interference is due to impaired activation of MAPK mediated by cholesterol-rich lipid rafts. Cholesterol-depleting agents specifically inhibited TGF- β -induced activation of extracellular signal-regulated kinase (ERK) and p38, but not Smad2/3 or Akt. Activation of ERK or p38 is required for both TGF- β -induced EMT and cell migration, whereas PI3K/Akt is necessary only for TGF- β -promoted cell migration but not for EMT. Although receptor heterocomplexes could be formed in both lipid raft and nonraft membrane compartments in response to TGF- β , receptor localization in lipid rafts, but not in clathrin-coated pits, is important for TGF- β -induced MAPK activation. Requirement of lipid rafts for MAPK activation was further confirmed by specific targeting of the intracellular domain of TGF- β type I receptor to different membrane locations. Together, our findings establish a novel link between cholesterol and EMT and cell migration, that is, cholesterol-rich lipid rafts are required for TGF- β -mediated MAPK activation, an event necessary for TGF- β -directed epithelial plasticity.

INTRODUCTION

Transforming growth factor (TGF)- β is a polypeptide that regulates a variety of cell events, including cell growth, death, differentiation, and migration. Two transmembrane serine/threonine kinase receptors, known as type I (T β RI) and type II receptors (T β RII) are required for TGF- β signal transduction. Ligand binding promotes the formation of receptor complex where T β RII phosphorylates T β RI. The activated T β RI in turn activates R-Smads, Smad2 and Smad3, via phosphorylation at their C-terminal serine residues. As a result, activated R-Smads form a heterocomplex with Smad4 and are accumulated in the nucleus to regulate gene expression (Massague and Chen, 2000; Feng and Derynck, 2005). In addition to this canonical Smad2/3 pathway, TGF- β has been reported to activate other signaling molecules, such as mitogen-activated protein kinases (extracellular signal-regulated kinase [ERK]), p38 and c-Jun N-terminal kinases [JNKs]), and phosphatidylinositol 3-kinase (PI3K)/Akt and p21-activated kinase at a cell-specific manner (Derynck and Zhang, 2003; Moustakas and Heldin,

2005). Despite activated at a relatively low level, these non-Smad pathways could make great contribution to total TGF- β signal output (Moustakas and Heldin, 2005). Unlike the Smad pathway, TGF- β activates non-Smad pathways in a cell-type- and context-dependent manner.

TGF- β signaling is regulated at multiple layers including TGF- β receptor trafficking. On the plasma membrane, TGF- β receptors bind to clathrin-associated adaptor complex AP2 and are constitutively internalized via clathrin-coated pits (Lu *et al.*, 2002; Yao *et al.*, 2002). When reached to early endosomes, TGF- β receptors can interact with scaffold proteins SARA (Tsukazaki *et al.*, 1998) and endofin (Chen *et al.*, 2007b), which in turn promote the interaction between TGF- β receptors and Smad2 or Smad4, respectively. Although it is still in debate whether clathrin-mediated endocytosis of TGF- β receptors is required for TGF- β signaling (Hayes *et al.*, 2002; Lu *et al.*, 2002; Penheiter *et al.*, 2002; Runyan *et al.*, 2005), it is clear that this process can enhance Smad-mediated TGF- β signaling (Lu *et al.*, 2002; Panopoulos *et al.*, 2002; Di Guglielmo *et al.*, 2003).

TGF- β receptors can also be found in lipid rafts, specified membrane microdomains in the plasma membrane that are enriched in cholesterol and sphingolipids (Simons and Toomre, 2000; Anderson and Jacobson, 2002). These microdomains usually function to bring different signaling receptors into proximity with its downstream substrates, resulting in signal specificity of signaling cascades with influence on cellular physiological responses (Munro, 2003). For example, promoting the lipid raft localization of insulin receptors could potentiate the insulin-induced activation of MAPK, but not Akt, and elicit cellular responses in cytoskeletal structure and ruffle formation (He *et al.*, 2003). Lipid

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Abbreviations used: EMT, epithelial-mesenchymal transition; F-actin, filamentous actin; ICD, intracellular domain; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; T β R, TGF- β receptor; TGF, transforming growth factor.

rafts have been shown to play an essential role in MAPK activation by nerve growth factor (Peiro *et al.*, 2000) and by platelet-derived growth factor (Stehr *et al.*, 2003). TGF- β receptors are also reported to localize in lipid rafts where they may be in proximity to the Smad7–Smurf2 complex and facilitated for degradation (Di Guglielmo *et al.*, 2003). Using single molecule imaging, we have recently shown that lipid rafts can facilitate the heterocomplex formation of T β RI and T β RII (Ma *et al.*, 2007b). Interestingly, although both type I and type II receptors of bone morphogenetic protein (BMP) undergo clathrin-mediated endocytosis, only the type II receptor can be internalized via caveola microdomains (Hartung *et al.*, 2006). Furthermore, BMP-mediated Smad1/5 phosphorylation occurs in nonraft regions, whereas BMP-induced alkaline phosphatase expression requires receptor location in lipid rafts, implying that BMP signaling might be specified by receptor localization at the plasma membrane.

TGF- β induces epithelial-mesenchymal transition (EMT) in normal epithelial cells and tumor cells, which is characterized as loss of cell–cell contacts and acquisition of fibroblastic phenotype as well as increased mobility and may account for tumor invasion and tissue fibrosis (Zavadil and Bottinger, 2005; Derynck and Akhurst, 2007). It is well established that TGF- β acts in a context-dependent manner and diverse cellular responses induced by TGF- β may result from coordination of different downstream pathways (Siegel and Massague, 2003). Alteration of the balance between distinct TGF- β signaling pathways may account for the difference in TGF- β responsiveness (Wakefield and Roberts, 2002).

Although both Smad- and non-Smad-mediated signaling pathways are important for TGF- β -provoked cellular responses, how these pathways are specified is unclear. Whether cholesterol has any effect on TGF- β -induced EMT and cell migration also awaits investigation. In this article, we report that depletion of cholesterol disrupts TGF- β -induced EMT and cell migration. This effect is due to interference of lipid raft localization of TGF- β receptors and to impaired TGF- β -mediated activation of MAPK. However, cholesterol depletion has no profound effect on TGF- β -induced Smad phosphorylation or Akt activation. Those results are confirmed by targeting active intracellular domain of T β RI to specific location. These results for the first time suggest that the localization at different plasma membrane regions of TGF- β receptors specifies the downstream signaling of TGF- β and thus influences the signaling output consequence on cellular responses. Our findings also reveal a link between cholesterol and EMT/cell migration through modulation of TGF- β signaling.

MATERIALS AND METHODS

Cell Culture, Reagents, and Antibodies

The human keratinocyte HaCaT cells were kindly provided by Dr. Xin-Hua Feng (Baylor College of Medicine); mouse mammary epithelial NMuMG cells were a gift from Dr. Ying E. Zhang (National Cancer Institute), and mouse embryonic cells were prepared from C57/BL6 mice according to standard protocol. All cells were grown in DMEM supplemented with 10% fetal bovine serum. Transfection was performed with calcium phosphate for human embryonic kidney (HEK) 293 cells, Vigorous (Vigorous Biotechnology, Beijing, China) for NMuMG cells, and Lipofectamine (Invitrogen, Carlsbad, CA) for HaCaT cells. Nystatin, chlorpromazine hydrochloride, filipin, lovastatin, cholesterol, and LY294002 were from Sigma-Aldrich (St. Louis, MO); PD98059 and SB203580 were from Calbiochem (San Diego, CA). Rhodamine-phalloidin was from Invitrogen. Anti-phospho-Smad3 antibody was a generous gift from Dr. Edward B. Leof (Mayo Clinic College of Medicine, Rochester, MN). Anti-early endosomal antigen-1 and anti-E-cadherin were purchased from BD Biosciences Transduction Laboratories (Lexington, KY). Anti-phospho-Smad2 was from Millipore (Billerica, CA). Anti- α -adaptin was from Affinity BioReagents (Golden, CO). Anti-p38 was from Beyotime Institute of Biotechnology (Beijing, China). Anti-phospho-Akt (Thr308), anti-phospho-p38, and anti-Akt were from Cell Signaling Technology (Beverly,

CA). Anti-Smad2/Smad3 was made in house. Other primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

DNA Constructs

Eps15 Δ III-green fluorescent protein (GFP) plasmid was a gift from Dr. Alexandre Benmerah (Université Paris Descartes). The constitutively active T β RI (caT β RI) harbors three point mutations (L193G, P194G, and T204D). Plasmids expressing the intracellular domain (ICD) harboring the three mutations were generated from caT β RI, and raft-ICD was made by fusing the N terminus of ICD to the N-terminal 16 amino acids of Fyn (Chen and Resh, 2001); nonraft-ICD was made by fusing of ICD to the N-terminal 10 amino acids of Lck, which contains one mutation (cysteine to alanine) and an additional five lysine residues (Lillemeier *et al.*, 2006); and endo-ICD was made by fusing of ICD to the FYVE domain of human SARA (2076–2282) (Itoh *et al.*, 2002). The validity of all constructs was confirmed by DNA sequencing. All the constructs were C-terminally tagged with hemagglutinin (HA) epitopes.

Cell Migration Assay

The migration assay were carried out as reported previously (Ma *et al.*, 2007a). Image were captured at room temperature using a camera (Roper Scientific, Trenton, NJ) on an inverted microscope (TE2000-U; Nikon, Tokyo, Japan), with a Nikon Plan Fluor 4 $\times$ 0.13 objective and Image-ProPlus 5.1 software (Media Cybernetics, Bethesda, MD).

Immunoblotting, Coimmunoprecipitation, Immunofluorescence, and Reporter Assay

For immunoblotting, the cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 1 mM NaVO₄, 10 mM NaF, and protease inhibitors), and the protein concentration in the lysates was determined by a spectrophotometer. Equal amount of the lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and the immunoblotting was performed with primary antibodies as indicated in the figures and secondary anti-mouse antibodies conjugated to horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Proteins were visualized by chemiluminescence. The signal density of phosphorylated proteins was normalized to the corresponding unphosphorylated proteins. For coimmunoprecipitation, cells were lysed in 500 mM sodium carbonate followed by homogenization with 10 strokes of loose Dounce homogenator and sonication. After 12,000 $\times$ g centrifugation for 10 min, the lysates were immunoprecipitated with specific antibody and protein A-Sepharose (Zymed Laboratories, South San Francisco, CA). The precipitates were analyzed by immunoblotting. Immunofluorescence and reporter assay were performed as described previously (Zhang *et al.*, 2007). Immunofluorescent images were viewed at room temperature by fluorescence microscopy with a confocal microscope (FV500; Olympus, Tokyo, Japan) by using a 60 $\times$ numerical aperture 1.4 oil objective, and images were analyzed using FluoView (Olympus) and Photoshop software (Adobe Systems, Mountain View, CA).

Lipid Raft Fractionation

Cells were grown to near confluence in four 100-mm dishes. After two washes with ice-cold phosphate-buffered saline (PBS), cells were scraped with 0.75 ml of 500 mM sodium carbonate, pH 11.0, and placed on ice for 10 min. Homogenization was carried out with 10 strokes of loose Dounce homogenator followed by three 20-s burst of sonication on ice. The homogenates were adjusted to 42.5% sucrose by addition equal volume of 85% sucrose in 2 $\times$ HBS (25 mM HEPES, pH 6.5, and 150 mM NaCl). A discontinuous sucrose gradient was generated by overlaying with 3.5 ml of 35% sucrose in 1 $\times$ HBS and 250 mM sodium carbonate and the 3.5 ml of 5% sucrose in 1 $\times$ HBS and 250 mM sodium carbonate. The solution was then centrifuged at 260,000 $\times$ g for 16 h at 4°C. Twelve 1-ml fractions were collected from the top of the tube, and a portion of each fraction was analyzed by immunoblotting.

RESULTS

Cholesterol Depletion Inhibits TGF- β -induced Epithelial–Mesenchymal Transition and Cell Migration

When stimulated with TGF- β , several cell lines, including HaCaT cells, undergo EMT, a process characterized by loss of E-cadherin from the plasma membrane, replacement of cortical actin filaments by actin stress fibers, and acquirement of spindle-like cell morphology (Zavadil and Bottinger, 2005; Thiery and Sleeman, 2006). In the absence of TGF- β , HaCaT cells occurred as typical epithelial cells with the epithelial marker E-cadherin and actin cytoskeleton arranged in a cortical pattern at cell–cell junctions; after 36 h stimulation with TGF- β , cells acquired spindle-shaped fibro-

Figure 1. TGF- β -induced EMT is sensitive to cholesterol depletion. (A and B) HaCaT cells were cultured in 0.5% serum and incubated with 25 μ g/ml nystatin in the absence or presence of 5 ng/ml TGF- β 1 for 36 h, followed by phase-contrast microscopy imaging or immunofluorescence with anti-E-cadherin antibody (green) and rhodamine-phalloidin to stain filamentous actin (F-actin) (red). Bar, 20 μ m. (C) HaCaT cells were cultured in 0.5% serum and incubated with 25 μ g/ml nystatin (Nys) together with or without 50 μ g/ml cholesterol (Chole) in the presence of 5 ng/ml TGF- β 1 for 36 h, followed by immunofluorescence with anti-E-cadherin antibody (green) and rhodamine-phalloidin to stain F-actin (red). Bar, 20 μ m. (D) HaCaT cells were cultured in 0.5% serum and incubated with 25 μ g/ml nystatin in the presence or absence of 5 ng/ml TGF- β 1 for 36 h, followed by anti-E-cadherin immunoblotting. Dishevelled-3 served as loading controls. The numbers underneath each gel indicate the relative band densities after normalized to controls.

blast-like morphology with down-regulation/delocalization of E-cadherin as well as formation of actin stress fibers, as reported previously (Figure 1, A and B). Furthermore, when HaCaT cells were treated with nystatin, a cholesterol sequestering agent (Simons and Toomre, 2000), the TGF- β -induced EMT was blocked. Similar effect was observed when HaCaT cells were treated with filipin, another cholesterol sequestering agent (data not shown). To extend our study to other cell types, we assessed the effect of nystatin on TGF- β -induced EMT in murine mammary gland epithelial NMuMG cells and obtained similar results (Supplemental Figure S1). To test whether the inhibitory effect of nystatin was due to its cholesterol-sequestering activity, we supplemented HaCaT cells with cholesterol and found cholesterol addition could restore TGF- β -induced EMT (Figure 1C). We also examined E-cadherin protein levels in the EMT process, and in agreement with the above-mentioned immunofluorescence data, nystatin inhibited the TGF- β -induced down-regulation of E-cadherin (Figure 1D).

EMT has been associated with enhanced cell migration (Thiery and Sleeman, 2006), and TGF- β promotes cell migration in both epithelial cells and fibroblast cells. To test whether cholesterol is essential for TGF- β -induced cell migration, we measured the mobility of HaCaT cells by scratch wound assay. The TGF- β -promoted cell migration could be observed at 24–32 h after TGF- β treatment. Nystatin suppressed TGF- β -induced cell migration (Figure 2A). Furthermore, lovastatin, a potent 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor that is clinically used as a cholesterol-lowering agent (Tobert, 2003), had the similar effect as nystatin (Figure 2B). These data together strongly suggest that cholesterol is required for both TGF- β -induced EMT and cell migration.

Cholesterol Is Required for TGF- β -induced MAPK Activation

It has been shown that in HaCaT cells, TGF- β treatment leads to rapid activation of various cytoplasmic molecules, including Smad2, Smad3, PI3K/Akt, and MAPK (ERK and

p38) (Zavadil *et al.*, 2001; Wilkes *et al.*, 2005; Lin *et al.*, 2006). To test whether cholesterol is required for TGF- β signaling, HaCaT cells were pretreated with nystatin before TGF- β stimulation. The activation of various signaling molecules was measured by detection of their phosphorylation at endogenous levels by immunoblotting with respective anti-phospho-antibodies. TGF- β induced phosphorylation of Smad2, Smad3, p38, ERK, and Akt in HaCaT cells (Figure 3A) but not JNK (data not shown). Interestingly, Smad2 phosphorylation induced by TGF- β was slightly enhanced by nystatin treatment, whereas Smad3 and Akt activation were unaffected. However, despite slightly increasing basal

Figure 2. Cholesterol is required for TGF- β -induced cell migration. Subconfluent HaCaT cells were wounded by a 20- μ l size pipette tip and then treated with 25 μ g/ml nystatin (A) or 15 μ M lovastatin (B) in the absence or presence of 10 ng/ml TGF- β 1 for indicated time. Experiments were repeated four times with similar results and a representative micrograph for each condition is shown. Initial boundaries of wound were marked with black lines. Bar, 100 μ m.

Figure 3. Cholesterol is essential for TGF- β -induced activation of ERK and p38. (A–C) HaCaT cells were pretreated with 50 μ g/ml nystatin (A), 4 μ g/ml filipin for 1 h (B), or 25 μ M lovastatin for 24 h (C), and then treated with 10 ng/ml TGF- β 1 for indicated times (minutes). The cells were harvested for immunoblotting with various antibodies as indicated. (D and E) NMuMG (D) and NIH3T3 (E) were treated similarly as described in A before harvested for immunoblotting analysis. The numbers underneath each gel indicate the relative band densities. (F) HaCaT cells transfected with *Renilla* and together with ARE-luciferase, CAGA-luciferase, or with pFA-Elk and pFR-luciferase were incubated with 50 μ g/ml nystatin together with (closed bars) or without (open bars) 5 ng/ml TGF- β 1 for 16 h. Each experiment was performed in triplicate, and the data represent the mean $\pm$ SEM after normalized to *Renilla* activity. Asterisk (*) indicates a significant difference with or without TGF- β 1 treatment ($p < 0.05$).

phosphorylation of p38 and ERK, nystatin abolished TGF- β -induced MAPK phosphorylation. Moreover, nystatin also abolished TGF- β -induced JNK phosphorylation in Mv1Lu cells (data not shown).

To confirm the specific cholesterol-depletion effect of nystatin, another lipid rafts disrupter, filipin, which can also

sequester cholesterol (Simons and Toomre, 2000), was tested in our experiment, and we found that filipin treatment significantly inhibited TGF- β -mediated ERK activation but had minimal effect on Smad2 phosphorylation in HaCaT cells (Figure 3B). Furthermore, lovastatin had similar effect on TGF- β -mediated activation of ERK and Smad2

(Figure 3C). These data further support that cholesterol is required for TGF- β -induced MAPK activation.

Because TGF- β elicits distinct responses and activates various signaling pathways in a cell-specific manner (Derynck and Zhang, 2003; Rahimi and Leof, 2007), we wanted to evaluate our results in other cell types. To this end, several TGF- β -responsive cell lines such as NMuMG cells and NIH3T3 fibroblasts were considered, and they have been widely used to study both Smad and non-Smad signaling pathways (Moustakas and Heldin, 2005). NMuMG was responsive to TGF- β -induced EMT but not migration, whereas NIH3T3 was responsive to TGF- β -induced cell migration but not EMT (data not shown). In both cells, nystatin abolished TGF- β -induced MAPK activation but had no effect on Smad2 activation (Figure 3, D and E), suggesting a common mechanism of TGF- β -mediated MAPK activation. Together, these data suggest that the requirement of cholesterol in TGF- β -induced MAPK activation is preserved in various cell types.

To validate the importance of cholesterol in TGF- β -mediated MAPK activation, we then investigated whether cholesterol is essential for TGF- β -induced reporter gene transcription. Both ARE-luciferase and CAGA-luciferase are TGF- β -responsive transcriptional reporters and are activated by the Smad pathway. As shown in Figure 3F, TGF- β induced their expression in HaCaT cells, and nystatin had no apparent effect on TGF- β responsiveness. pFR-luciferase is responsive to ERK activation in the presence of Elk (Path-Detect *trans*-Reporting System; Stratagene, La Jolla, CA). Nystatin significantly inhibited TGF- β -induced expression of this reporter (Figure 3F). These data indicated that cholesterol is required for MAPK-mediated gene transcription in response to TGF- β .

MAPK Activation Is Essential for TGF- β -induced EMT and Cell Migration in HaCaT Cells

Because cholesterol is essential for TGF- β -induced EMT and cell migration and MAPK activation, we further investigated whether MAPK activation is responsible for TGF- β -induced EMT and migration. Here, we found in HaCaT cells, both the p38 inhibitor SB203580 and the mitogen-activated protein kinase (MEK)/ERK inhibitor PD98059 abolished TGF- β -induced EMT. However, the PI3K/Akt inhibitor LY294002 had no effect, indicating that two MAPKs but not PI3K/Akt activation are necessary for TGF- β -induced EMT (Figure 4A). In TGF- β -induced migration, inhibition of p38 or MEK/ERK completely blocked TGF- β -enhanced cell migration. Noticeably, inhibition of PI3K/Akt also suppressed cell migration, indicating that two MAPKs and Akt are necessary for TGF- β -promoted cell migration (Figure 4B). These results indicated MAPK activation contribute to TGF- β -induced EMT and migration of HaCaT cells.

Cholesterol-dependent Localization of TGF- β Receptors in Lipid Raft Microdomains

Cholesterol is an essential structural component of cell membrane. In agreement with the previous reports that both T β RII and T β RI have been reported to be dynamically localized in cholesterol-rich membrane microdomains (also called lipid rafts) of epithelial cells (Di Guglielmo *et al.*, 2003; Zhang *et al.*, 2005; Ma *et al.*, 2007b), TGF- β -induced receptor complexes could be found in both lipid rafts and nonraft membrane with more in lipid raft fractions (Figure 5A). We further examined the membrane localization of endogenous receptors in several cell lines. Lipid rafts from nonraft membrane were separated using sucrose density gradient centrifugation, and receptor protein levels in lipid raft (fractions

Figure 4. Inhibition of MAPK activation blocks TGF- β -induced EMT and cell migration. (A) HaCaT cells were cultured in 0.5% serum and incubated with 10 mM p38 kinase inhibitor SB203580, 20 mM MEK/ERK inhibitor PD98059, or 40 mM PI3K/Akt inhibitor LY294002 in the absence or presence of 5 ng/ml TGF- β for 36 h, followed by immunofluorescence with anti-E-cadherin antibody (green) and rhodamine-phalloidin to stain F-actin (red). (B) Subconfluent HaCaT cells were wounded by a 20- μ l size pipette tip and then treated with 25 μ g/ml nystatin, 10 mM SB203580, 20 mM PD98059, or 40 mM LY294002 in the absence or presence of 10 ng/ml TGF- β for 32 h. Wound width at 32 h postwounding was normalized to width at the start time and plotted as the mean $\pm$ SEM of triplicates. Asterisk (*) indicates a significant difference with or without TGF- β treatment ($p < 0.05$).

4~6) and nonlipid raft (fractions 9~12) were analyzed. Caveolin-1 and α -adaptin were used as lipid raft and nonlipid raft markers, respectively. Our results showed that in HaCaT (Figure 5B), NMuMG (Figure 5C), and HEK293 (Supplemental Figure S2A) cells, ~80% of T β RI were localized in lipid rafts (fractions 4~6), and a small amount of T β RII could also be detected in those fractions. TGF- β treatment caused notable receptor redistribution by shifting T β RI to nonraft regions and T β RII to lipid rafts in HaCaT and NMuMG cells. Importantly, cholesterol depletion by nysta-

Figure 5. TGF- β receptors are localized in both lipid rafts and nonlipid raft regions. (A) HEK293 cells were transfected with T β RII-GFP together with T β RII-HA or empty vector and treated with 10 ng/ml TGF- β 1 for 1 h as indicated. The cells were harvested for anti-HA immunoprecipitation and then anti-GFP immunoblotting (left, top). The receptor expression was confirmed by immunoblotting of the total cell lysates. To examine receptor complexes in different membrane fractions, the transfected cells were treated with 10 ng/ml TGF- β 1 for 1 h and subjected to sucrose density gradient centrifugation. Fractions 3–6 and 9–12 were pooled, and receptor complexes were analyzed by anti-HA immunoprecipitation and then anti-GFP immunoblotting (right, top). Caveolin-1 and α -adaptin were detected as lipid raft and nonraft markers, respectively. (B) HaCaT cells were pretreated with 50 μ g/ml nystatin for 1 h and incubated with or without 10 ng/ml TGF- β 1 for another hour as indicated. The cell lysates were subjected to sucrose density gradient centrifugation, and endogenous proteins from each sucrose fraction were analyzed by immunoblotting. Arrowhead indicates T β RI. (C and D) NMuMG (C) and NIH3T3 (D) cells were incubated with or without 5 ng/ml TGF- β 1 for 1 h. The cell lysates were subjected to sucrose density gradient centrifugation, and endogenous proteins were analyzed by immunoblotting. Arrowhead indicates T β RI.

tin shifted T β RI from lipid rafts to nonlipid raft fractions, as observed previously (Yamamoto *et al.*, 2006). A different distribution pattern of endogenous receptors was observed in NIH3T3 fibroblasts (Figure 5D) as well as in mouse embryonic fibroblasts (Supplemental Figure S2B), in which most of T β RI and T β RII were located in nonraft fractions. Interestingly, in the HaCaT cells that had been treated with TGF- β for 36 h and had undergone EMT, we observed more T β RI in nonraft fractions (Supplemental Figure S2C), resembling the distribution pattern of T β RI in fibroblasts. The dynamic and cell type-specific distribution of TGF- β receptors in lipid rafts suggest that cholesterol-rich microdomains may play an important role in TGF- β signaling.

Specific Targeting of the Intracellular Domain of T β RI to Lipid Rafts Activates MAPK and Induces EMT

The above-mentioned studies using pharmacological drugs indicate that cholesterol-rich lipid rafts are required for TGF- β -induced MAPK activation and EMT. To directly investigate whether the localization of T β RI at lipid rafts is important to activate MAPK and to induce EMT, we sought to manipulate the membrane localization of active TGF- β receptors. We generated three chimeric TGF- β receptor constructs with distinct membrane targeting sequences fused to

the N terminus of the ICD harboring three point mutations (L193G, L194G, and T204D) that lose the FKBP12-binding activity and thus render T β RI superactive (Charnig *et al.*, 1996; Chen *et al.*, 1997). To target ICD to the lipid rafts or nonraft regions of the plasma membrane, the N terminus of Fyn tyrosine kinase (Chen and Resh, 2001) or a mutated N terminus of Lck tyrosine kinase (Lillemeier *et al.*, 2006) was fused to the ICD to generate raft-ICD and nonraft-ICD, respectively (Figure 6A). Because TGF- β receptors are also localized in intracellular vesicles, we constructed another early endosome targeting chimera by fusing the FYVE domain of SARA (Itoh *et al.*, 2002) onto ICD to generate endo-ICD. Immunofluorescence and sucrose gradient centrifugation analysis confirmed the correct localization of these constructs (Supplemental Figure S3), whereas the ICD alone was diffusely distributed in the cytoplasm.

Next, we assessed whether the chimeric constructs still possess T β RI activity by measuring CAGA-luciferase activation in HEK293 cells. As shown in Figure 6B, the reporter expression was significantly increased by the membrane-anchored constructs, whereas the cytosolic ICD slightly activated the reporter. The activation by raft-ICD and nonraft-ICD was comparable with that by the full-length caT β RI. These data support our observation that TGF- β receptors

Figure 6. Lipid raft-targeted TβRI(ICD) specifically activates ERK and induces EMT. (A) Schematic illustration of the ICD of caTβRI tagged with a HA epitope at C terminus and different membrane targeting motifs at N terminus: fyn, N terminus of Fyn tyrosine kinase containing a myristoylation (myr) and a palmitoylation (pal) sites; lck, mutated N terminus of Lck tyrosine kinase containing a myristoylation site; FYVE, FYVE domain of human SARA. caTβRI, full-length constitutively active TβRI. (B) Activation of CAGA-luciferase reporter by the membrane-anchored ICD constructs in HEK293 cells. Each experiment was performed in triplicate, and the data represent the mean $\pm$ SEM after normalized to *Renilla* activity. The expression of HA-tagged constructs were shown by anti-HA immunoblotting. Arrow indicates a nonspecific band (ns band). (C) NMuMG cells were transfected with various constructs as indicated. Phosphorylation of Smad2 and ERK and protein expression were examined by immunoblotting. Arrow indicates a nonspecific band (ns band). The numbers underneath each gel indicate the relative band densities. (D) HaCaT cells were transfected with various constructs as indicated. At 24 h after transfection, the cells were cultured in 0.5% serum for additional 36 h followed by immunofluorescence for HA epitope (green) and F-actin (red). Bar, 20 μ m.

can activate Smad-dependent transcription at lipid rafts or nonraft regions of the cell membrane.

We further examined the ability of these constructs to phosphorylate Smad2 and ERK (Figure 6C). Consistent with the reporter assay, the expression of the membrane-anchored constructs apparently increased Smad2 phosphorylation. However, the ERK activation pattern was distinct from that of Smad2: only raft-ICD enhanced ERK phosphorylation, whereas nonraft-ICD or endo-ICD had little effect (Figure 6C).

We then studied the ability of these chimeric constructs to induce EMT in HaCaT cells. As shown in Figure 6D, stress fibers were observed only in caTβRI- or raft-ICD-expressing

cells, but not in the nontransfected cells or the cells expressing other constructs, indicating that both caTβRI and raft-ICD can induce EMT. Together, these results indicate that membrane-anchorage is critical to preserve receptor activity and that the lipid raft distribution of the receptor is crucial for ERK activation and EMT induction.

Membrane Clathrin-coated Pits Are Dispensable for TGF- β -induced MAPK Activation and EMT

Because TGF- β receptors are dynamically distributed in lipid rafts and nonraft compartments (Di Guglielmo *et al.*, 2003; Zhang *et al.*, 2005), we then examined the role of clathrin-coated pits in TGF- β -induced MAPK activation and

Figure 7. Disruption of clathrin-coated pits has minimal effect on TGF- β -stimulated phosphorylation of Smad2 and -3, whereas it enhances phosphorylation of p38 and ERK. HaCaT cells were pretreated with 50 μ M chlorpromazine (CPZ) for 1 h and then treated with 10 ng/ml TGF- β 1 for indicated times (minutes). The cells were harvested for immunoblotting with various antibodies as indicated. The numbers underneath each gel indicate the relative band densities.

EMT. Chlorpromazine is an agent that disassembles clathrin-coated pits on plasma membrane and inhibits clathrin-mediated endocytosis of TGF- β receptors (Yao *et al.*, 2002). In HaCaT cells, TGF- β -induced phosphorylation of Smad2, Smad3, and Akt were barely impaired by chlorpromazine treatment, whereas p38 and ERK activation were enhanced (Figure 7). Besides, chlorpromazine treatment had no influence on TGF- β -induced EMT or cell migration (data not shown). Moreover, expression of an Eps15 mutant, which disassembles clathrin-coated pits and inhibits transferrin internalization (Benmerah *et al.*, 2000), did not interfere with TGF- β -induced EMT either (Supplemental Figure S4). These data suggest that membrane lipid rafts, but not clathrin-coated pits, are required for TGF- β -induced MAPK activation and EMT.

DISCUSSION

It has been documented that in addition to the canonical Smad pathway, TGF- β can also signal via other pathways by activating MAPKs (ERK, p38, and JNK), PI3K/Akt, PAK2, protein kinase A, and small GTPases (Ras, Rho, Rac, and Cdc42) in a cell-specific way (Derynck and Zhang, 2003; Moustakas and Heldin, 2005). To date, how these signal pathways are specifically activated by TGF- β is poorly understood. In the current study, we provided evidence that cholesterol-depleting drugs block TGF- β -induced MAPK activation. We found that both T β RI and T β RII can be detected and form a complex in lipid rafts and nonlipid raft regions. We further showed that although TGF- β -induced

cell migration needs cooperation of multiple signal pathways, activation of MAPK in lipid rafts is specially required for TGF- β -induced EMT, which is confirmed by the expression of the constructs that specifically targeted the active T β RI ICD to different membrane regions.

Cholesterol and Integration of Multiple Signal Pathways Are Required for TGF- β -induced EMT and Cell Migration

EMT plays an essential role in embryogenesis and tumorigenic- and fibrotic-related pathogenesis (Thiery and Sleeman, 2006; Derynck and Akhurst, 2007). In this study, we demonstrated that cholesterol is essential for TGF- β -induced EMT and cell migration because both of them are inhibited by the cholesterol-depleting agent nystatin. Furthermore, nystatin-impaired EMT could be rescued by cholesterol addition. These results were further supported by targeting the T β RI ICD to specific membrane regions—only raft-ICD, which is localized in cholesterol-rich lipid rafts, could induce EMT. The pivotal role of cholesterol in TGF- β -induced EMT suggests a possibility of cholesterol lowering as a potential therapeutic strategy against pathological EMT during tumor progression and tissue fibrosis (Demierre *et al.*, 2005; Santana *et al.*, 2008).

TGF- β has been shown to cooperate with other signaling pathways such as Ras/Raf/MAPK, Notch, PI3K/Akt, and Wnt/ β -catenin to control the activity or expression of the factors related to EMT (reviewed by Zavadil *et al.*, 2005 and Moustakas and Heldin, 2007). It is well accepted that in addition to the canonical Smad pathway, TGF- β also activate MAPK, PI3K/Akt, and small GTPases in a context-dependent manner (Derynck and Zhang, 2003; Moustakas and Heldin, 2005). We found that inhibition of ERK or p38, but not PI3K/Akt, is required for TGF- β -induced EMT in HaCaT cells, which is in accordance with the previous reports that ERK and p38 functions are necessary for TGF- β -induced EMT (Zavadil *et al.*, 2001; Bakin *et al.*, 2002). In contrast, we found that TGF- β -promoted migration of HaCaT cells need coordination among ERK, p38 and PI3K/Akt as inhibition of any of their activities impaired cell migration. In either TGF- β -induced EMT or cell migration, Smad activation should be essential (Roberts *et al.*, 2006). These data together suggest that differential integration of various pathways account for different TGF- β -evoked cellular responses. Detailed dissection of the TGF- β -initiated signaling network is crucial for our understanding of the physiopathological function of TGF- β as a multifunctional factor.

Specific Activation of MAPK by TGF- β in Cholesterol-rich Lipid Rafts

Lipid rafts/caveolae have been regarded as a signaling platform on cell membrane where multiple signal pathways are initiated (Simons and Toomre, 2000; Patra, 2008). For example, receptor tyrosine kinases, including insulin, nerve growth factor, and platelet-derived growth factor receptors are constitutively localized in lipid rafts or translocated into lipid rafts upon ligand binding, and their lipid raft localization is essential for MAPK activation (Liu *et al.*, 1997; Peiro *et al.*, 2000; He *et al.*, 2003; Limpert *et al.*, 2007). We found that TGF- β -mediated activation of MAPK requires intact cholesterol-rich lipid rafts as this activation was impaired by cholesterol-depleting drugs nystatin and filipin or by the cholesterol biosynthesis inhibitor lovastatin, whereas TGF- β -mediated activation of Smad2, Smad3, and Akt can take place in both lipid rafts and nonlipid raft regions. The importance of lipid rafts in TGF- β -induced MAPK activation was also confirmed by raft-ICD that contains the T β RI ICD specifically targeted to lipid rafts. Of note, nystatin

treatment resulted in a higher basal activation of ERK and p38. This could be because MAPK can be activated by many other signals in lipid rafts and depletion of cholesterol may have a profound effect on all these signals. Nonetheless, TGF- β could not enhance MAPK activation in the presence of nystatin.

At this moment, it is unclear how MAPK is activated by TGF- β receptors in lipid rafts. The cholesterol-rich microdomains may provide an environment or retain some factors that prefer MAPK activation. This may be similarly applied to receptor tyrosine kinases that require their residency in lipid rafts to activate MAPK. Interestingly, T β RI was shown to have intrinsic tyrosine kinase activity that contributes to ERK activation via direct phosphorylation of ShcA (Lee *et al.*, 2007). Src, which is enriched in lipid rafts (Liang *et al.*, 2001), was recently suggested to mediate integrin-induced Tyr284 phosphorylation of T β RII, and this phosphorylation may provide a docking site for adapter proteins Grb2 and Shc that in turn lead to p38 activation (Gallagher and Schiemann, 2007). It was recently reported that the membrane-associated protein TRAF6 interacts with TGF- β receptors and mediates the TGF- β -induced activation of p38 and JNK MAPKs (Sorrentino *et al.*, 2008; Yamashita *et al.*, 2008). It remains to be determined whether the recruitment of these adapters to TGF- β receptors occurs in lipid rafts. Although internalization of TGF- β receptors to early endosomes has been shown to promote Smad activation, our study with endo-ICD indicated that TGF- β -induced MAPK activation may not occur in these compartments.

Cell Type-specific and Dynamic Distribution of TGF- β Receptors on the Cell Membrane

Our sucrose gradient centrifugation analysis revealed that the distribution of TGF- β receptors on the cell membrane varies in different cell types, although both T β RI and T β RII can be found in lipid rafts and nonraft fractions. In epithelial cells such as HaCaT, NMuMG, and HEK293 cells, most T β RI was detected in caveolin-containing lipid raft fractions and most T β RII in α -adaptin-containing nonraft fractions. In contrast, in fibroblast cells such as NIH3T3 and primary mouse embryonic fibroblasts, and in HaCaT cells that have undergone full EMT, both receptors were mainly found in nonraft fractions. It remains to be studied whether the different receptor distribution patterns are due to the loss of cell polarity in mesenchymal/fibroblast cells. Nonetheless, it is apparent that cholesterol is important for TGF- β -induced activation of MAPKs because nystatin disrupts this activation in both epithelial (HaCaT and NMuMG) and fibroblast (NIH3T3) cells.

TGF- β treatment caused notable, albeit not dramatic, receptor redistribution by shifting T β RI to nonraft regions and T β RII to lipid rafts in HaCaT and NMuMG cells. Consistently, TGF- β -induced receptor complex formation could be detected at both lipid raft and nonraft fractions. Manipulation of the cholesterol levels can alter the distribution of TGF- β receptors in the membrane microdomains (Chen *et al.*, 2007a). Furthermore, several lines of evidence indicate that the distribution of TGF- β receptors on the cell membrane is dynamically regulated. For example, the cell surface proteoglycan heparin sulfate and the extracellular polysaccharide hyaluronan were reported to promote TGF- β receptors trafficking into lipid rafts that lead to fast receptor turnover (Ito *et al.*, 2004; Chen *et al.*, 2006), whereas interleukin-6 can partition TGF- β receptors from lipid rafts to non-lipid raft regions (Zhang *et al.*, 2005). In accordance with previous reports, we found that the localization in lipid rafts can facilitate rapid turnover of T β RI, but it only occurs in the

presence of caveolin-1 (data not shown). Together, our findings indicate that the distribution of TGF- β receptors on the plasma membrane is dynamically regulated.

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