To examine signaling pathways underlying transforming growth factor-β (TGF-β)-mediated changes in cell morphology, we used a microarray system to identify downstream target genes that may play a role in this process. Through this approach, we found that the NET1 gene was induced upon TGF-β treatment in several cell types. NET1 is a guanine nucleotide exchange factor for RhoA whose activity has been implicated in stress fiber formation. In the Swiss 3T3 cell line, TGF-β induces NET1 expression, and this correlated with an increase in stress fiber formation. Overexpression of the wild type NET1 gene increases stress fiber formation, and overexpression of a dominant negative NET1 mutant (L392E) prevented TGF-β-dependent increase in stress fiber formation. Furthermore, treatment of the cells with a RhoA kinase inhibitor Y-27632 blocks TGF-β-induced stress fiber formation. By using a stable cell line expressing dominant negative Smad3, we found that the Smad signaling pathway is essential for the induction of NET1, which in turn leads to the increase of Rho activity. Taken together, those data suggest that induction of NET1 is important for the increase of Rho activity upon TGF-β treatment, which may represent the critical trigger for a variety of downstream events in different cells. Our results support the presence of a novel signaling pathway by which TGF-β may regulate the formation of stress fibers and reorganization of cytoskeletal structures.

TGF-β1 is a growth factor with a diverse range of biological activities such as growth inhibition, cellular migration, wound healing, immune regulation, and bone remodeling (1–4). One important but relatively unexplored process is the ability of TGF-β to modulate cell morphology and motility. Cell morphologic change and elevated migration in response to TGF-β have been observed in a number of cell types (5–9), but the mechanism underlying this TGF-β induced process remains largely unknown.

Cell morphologic change and migration are processes that involve dynamic cytoskeleton reorganization. In addition to providing a structural framework around which cell shape and polarity are defined, actin cytoskeleton provides a driving force for the cells to move and to divide (10). Recent works demonstrate that the Rho family of small GTPases are key regulatory molecules linking surface receptors to the organization of actin cytoskeleton (10–12). The Rho family consists of Rho, Rac, and Cdc42 subfamilies. Among them, Rho is the molecular switch for stress fiber formation, and Rac and Cdc42 regulate lamellipodia and filopodia formation, respectively (10, 13–16).

Rho GTPase can bind and hydrolyze guanosine nucleotides. The exchange of GDP for GTP results in a conformational change that unmasks structural domains involved in the binding of Rho to its downstream target proteins. This mechanism allows Rho GTPase to exchange between active GTP-bound and inactive GDP-bound forms, a cycling process that is regulated by three main classes of proteins as follows: 1) guanine nucleotide exchange factors (GEFs), which stimulate the exchange of bound GDP for GTP, leading to activation of GTPase; 2) GTPase-activating proteins (GAPs), which promote the inactivation of GTPase by increasing the rate of GTP hydrolysis; and 3) GDP dissociation inhibitors (GDIs), which can inhibit the dissociation of GDP from the GTPase, thus keeping GTPase in an inactive state (10, 11, 17). Although TGF-β can induce cell morphologic change and stress fiber formation, it is still unclear how the TGF-β signal regulates these pathways.

To understand better the mechanism by which TGF-β modulates the change in cell morphology, in this study we used a microarray system to search for candidate genes associated with this TGF-β-dependent activity. We found one gene in particular, NET1, whose mRNA and protein levels increase upon treatment with TGF-β. NET1 is a specific guanine nucleotide exchange factor for RhoA (18). By increasing RNA and protein levels of NET1, more RhoA may be in its GTP-bound active state to induce stress fiber formation. Furthermore, the expression of NET1 appears to be controlled by Smads, as the presence of a dominant negative form of Smad3 blocks the TGF-β-mediated induction of NET1. Our results suggest that NET1 may be a critical link between the activation of RhoA and the TGF-β and Smad-dependent induction of stress fiber formation.

EXPERIMENTAL PROCEDURES

Reagents—Human TGF-β1 was a generous gift from R & D Systems. ROCK inhibitor Y-27632 was kindly provided by Yoshitomi Pharmaceutical Ind., Ltd. Rhodamine-labeled phalloidin was purchased from Molecular Probes (Eugene, OR). Ribonuclease Protection Assay kit RPAII and Ribo-probe DNA for mouse cyclophilin, JunB, were from Ambion Inc. (Austin, TX). Human cDNA Expression Array I was from CLONTECH Laboratories Inc. (Palo Alto, CA). Anti-NET1 antibody (N-17) and anti-Smad2/3 antibody (H-2) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Smad2 antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY).

Cell Culture—Human HaCaT cells were a generous gift from Drs. P.
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FIG. 1. Protein synthesis is required for TGF-β-mediated changes in cell morphology. HaCaT cells were either untreated or TGF-β-treated for 24 h, with or without the protein synthesis inhibitor, cycloheximide. The cells were subsequently fixed in 4% paraformaldehyde and stained using a Dif-Quik staining kit (DADE, Behring). Pictures were taken using an Olympus microscope. A, no treatment. B, TGF-β-treated for 24 h. C, TGF-β plus 10 mg/ml cycloheximide for 24 h.

FIG. 2. TGF-β induces NET1 expression in HaCaT cells. A, HaCaT cells grown under normal serum conditions and treated with TGF-β from 30 min to 24 h. After the treatment, total RNAs were extracted from these cells for the RPA. Radiolabeled NET1, p15 (positive control), and GAPDH (internal loading control) riboprobes were included in the assay. Protected RNAs were analyzed by 6% denatured gel followed by fluorography. B, identical experiments were performed as in A except that the cells were grown in serum-free medium. Three riboprobes, NET1, p15, and GAPDH, were used for RPAs. C, HaCaT cells were grown under normal serum conditions and treated with TGF-β for different time points. Cell lysates were made, and NET1 protein level was analyzed by Western blot using a specific antibody (Santa Cruz Biotechnology). Arrow indicates the approximate position of NET1 in 8% SDS-polyacrylamide gel.

Baukamp and N. Fusenig (Institute of Biochemistry and Molecular Biology, Heidelberg, Germany) and were maintained in minimum Eagle's medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine (Life Technologies, Inc.). The HaCaT stable cell line overexpressing a Smad3 mutant (D407E), a generous gift from Dr. M. Kato (Japanese Foundation for Cancer Research, Tokyo, Japan) (19), was cultured in MCDB153 medium (Sigma) supplemented with 0.1 mM CaCl₂, 5% dialyzed fetal bovine serum, 5 ng of epidermal growth factor, and 300 μg/ml G418. Swiss 3T3, A-549, and PANC-1 cells were from American Type Culture Collection (Manassas, VA) and were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. BJ human diploid fibroblast was a gift from Dr. C. Counter (Duke University, Durham, NC).

Plasmids—Human NET1 cDNA construct was a generous gift from Dr. T. Miki at the NCI, National Institutes of Health. NET1 cDNA was cloned into the Bluescript vector to generate pNet1-1. pNet1-1 was linearized with StuI and used to make riboprobe with T7 RNA polymerase. FLAG-tagged NET1 constructs were generated by polymerase chain reaction and cloned into the pFLAG-CMV2 vector from Eastman Kodak Co. The NET1 mutation (L392E) was created by a standard polymerase chain reaction-mediated mutagenesis method.

Ribonuclease Protection Assays (RPAs)—RNAs from TGF-β-treated or untreated cells were extracted with the RNeasy mini kit from Qiagen Inc. (Valencia, CA). Riboprobe were labeled with [γ-32P]UTP using T7 RNA polymerase and eluted at 37 °C overnight. 8 μg of total RNA was used for one reaction, and a standard RPA protocol from the manufacturer was followed. Briefly, total RNA was co-precipitated with radiolabeled probes and hybridized overnight at 56 °C. The next day, reactions were treated with RNase A/T1 at room temperature for 1 h to digest unhybridized RNA. Final products were separated on a 6% polyacrylamide gel containing 7% urea, and the gel was exposed to film at −80 °C.

Immunostaining and Western Blot Analysis—Swiss 3T3 cells were plated on cover slides and grown to sub-confluency. The cells were washed and grown in serum-free medium for 48 h before treatment with TGF-β for 4 h. Then the cells were fixed in 4% paraformaldehyde for 10 min at room temperature and incubated with 1:100 fluorescein-labeled phalloidin diluted in 2% bovine serum albumin in phosphate-buffered saline solution in the dark for 30 min. Cells were washed 4 times with phosphate-buffered saline solution before mounting on glass slides. Photographs were taken with a Zeiss confocal microscope. The immunostaining on Smad2/Smad3 was performed as described previously.

The transfection of COS cells was performed by using a standard DEAE-dextran protocol, and routine Western blots were performed as described before. Swiss 3T3 cells were transfected with LipofectAMINE PLUS reagent by Life Technologies Inc.

RESULTS

TGF-β-mediated Cell Morphologic Change Requires Protein Synthesis in HaCaT Cells—TGF-β modulates cell morphologic changes in many cell types (5–9). In the spontaneously immortalized human keratinocyte cell line, HaCaT, TGF-β treatment leads to a dramatic morphologic change after 24 h (Fig. 1, A and B). Contrary to the normal compact appearance for keratinocytes, TGF-β treatment leads to a bigger and less compact cell shape in HaCaT cells. In addition, TGF-β-treated cells have significantly more membrane ruffling compared with un-

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A

Cell lines: HaCaT A549 BJ PANC-1
TGF-β: + + + + +

B

TGF-β: +

Fig. 3. *NET1* mRNA level is increased in additional cell types. A, using HaCaT cells as a positive control, several TGF-β-responsive human cell lines, A549, BJ, and PANC-1, were examined for *NET1* induction after TGF-β treatment. RNA samples from the indicated cell lines, either untreated or TGF-β-treated for 2 h, were prepared and analyzed by RPA. B, RNA from Swiss 3T3, a mouse fibroblast cell line, was extracted after 2 h TGF-β treatment or no treatment and analyzed by RPA. Three riboprobes, *NET1*, *JunB* (positive control), and cyclophilin (internal loading control) were included in the reaction.

Fig. 4. TGF-β-mediated stress fiber formation in Swiss 3T3 cells is RhoA-dependent. A, Swiss 3T3 cells were grown in normal serum and untreated or treated with TGF-β for 30 min. Cells were then stained with α-Smad2/3 (Santa Cruz Biotechnology) and fluorescence-labeled secondary antibody to determine Smad2/3 localization. B, Swiss 3T3 cells, after growing in serum-free medium for 48 h, were treated with TGF-β for 4 h or left untreated. Stress fibers were visualized using a fluorescence-labeled phalloidin, and pictures were taken under a Zeiss confocal microscope.

treated cells (data not shown). When cells were treated with cycloheximide, a protein synthesis inhibitor, the dramatic morphologic change of HaCaT cells induced by TGF-β was no longer observed (Fig. 1C). Since cycloheximide blocks protein synthesis, the result suggests that TGF-β may cause morphologic change of these cells through inducing the expression of certain TGF-β downstream target genes.

**TGF-β Induces NET1 Expression**—To better understand how TGF-β modulates cell morphology and to identify downstream genes critical for this process, we performed experiments using a microarray system containing about 600 genes. mRNA extracted from treated and untreated HaCaT cells were reverse-transcribed and hybridized with the microarray system. In the samples treated with TGF-β for 1 h, several genes were found to be significantly up-regulated (data not shown). Among these genes, several of them (e.g. *c-jun* and *junB*) have been previously reported (20, 21). One novel gene that fit our search criteria was *NET1*, a guanine nucleotide exchange factor (GEF) that was previously shown to increase specifically the activity of the small GTPase RhoA (18). In other studies, *NET1* was isolated in a yeast two-hybrid screen using RhoA as bait, and overexpressed *NET1* was shown to transform NIH 3T3 cells, cause stress fiber formation, and lead to JNK activation (22).

We next performed RPA to confirm the up-regulation of *NET1* messenger RNA levels by TGF-β. HaCaT cells, grown in both serum-starved and normal serum conditions, were treated with TGF-β for different times, and total RNA samples were extracted. As shown in Fig. 2, A and B, TGF-β induced the expression of *NET1* mRNA in both serum-free and normal serum conditions. The induction started at 30 min following TGF-β treatment and peaked at 2 h. As a positive control, the cyclin-dependent kinase inhibitor (CDKI) p15 was also induced by TGF-β treatment (23) but with slower kinetics. Consistent with an increase in mRNA level, *NET1* protein levels were also increased in TGF-β-treated cells (Fig. 2C).

To test if *NET1* expression is subject to TGF-β-mediated regulation in a broader range of cell types, we examined several other TGF-β-responsive cell lines for an increase in *NET1* mRNA expression. As shown in Fig. 3A, human lung carcinoma A549 and human diploid fibroblast BJ cell lines were found to increase *NET1* expression after 2 h of TGF-β treatment. A more moderate level of induction of *NET1* expression was also observed in a mouse fibroblast cell line, Swiss 3T3, after 2 h of TGF-β treatment (Fig. 3B). The human pancreatic carcinoma cell line, PANC-1, was found to express *NET1* at a very low basal level, and it is not further induced by TGF-β (Fig. 3A). Taken together, we conclude that the up-regulation of *NET1* expression by TGF-β is not restricted to the human keratinocyte HaCaT cells, but instead is a general response shared by other TGF-β-responsive cell lines.

**TGF-β Induces Stress Fiber Formation in Swiss 3T3 Cells**—As a guanine exchange factor, *NET1* is known for its specific activity toward RhoA (18). Since RhoA activity is critical in modulating the process of stress fiber formation and up-regulation of *NET1* expression is likely to increase RhoA activity, we decided to investigate the role of *NET1* in TGF-β-mediated stress fiber formation in a suitable system. We examined the cell lines in which *NET1* is induced by TGF-β to see if TGF-β could also enhance stress fiber formation in these cells. In HaCaT, A549, and PANC-1 cells, stress fibers were difficult to visualize both before and after TGF-β treatment. A significant amount of stress fiber was seen in BJ fibroblast cells, but the basal level of stress fiber was too high to allow an assessment of the TGF-β effect on the process in these fibroblasts even with 2 days of growing the cells under serum-starved conditions. Finally, we found the Swiss 3T3 cell line to be an ideal model system for this study. As shown in Fig. 4A, upon TGF-β treatment for 30 min, a significant nuclear accu-
mulation of two effector proteins of the TGF-β signaling pathway, Smad2 and Smad3, was observed, suggesting that those cells are responsive to TGF-β treatment and the TGF-β-Smad signaling pathway is intact. In normal serum-containing media, the presence of high levels of stress fibers in Swiss 3T3 cells was probably due to the activation of RhoA pathway by various growth factors in the serum. In contrast, a much lower level of stress fibers was seen when the cells were cultured in medium without serum for 48 h and stained with rhodamine-labeled phalloidin (Fig. 4B). After TGF-β was added into the medium and incubated for 4 h prior to staining, a dramatic increase in stress fiber formation was observed with phalloidin staining (Fig. 4B). The formation of stress fibers could be observed as early as 2 h post-TGF-β treatment.

**NET1 Is Involved in TGF-β-mediated Stress Fiber Formation**—Since overexpression of NET1 was previously shown to be sufficient to cause stress fiber formation, it is possible that NET1 serves as a link between the activation of TGF-β signaling pathway and the activation of RhoA and consequently stress fiber formation. To test this, we first determined if overproduction of NET1 could also lead to stress fiber formation in Swiss 3T3 cells. We constructed expression plasmids containing either a FLAG-tagged wild type NET1 or a mutant NET1 with a point mutation on the conserved Dbl domain (L392E) which abolishes its guanine exchange activity toward RhoA. The two constructs express equal amounts of NET1 protein in transfected COS cells, as shown by Western blot analysis on Fig. 5A. We then introduced the NET1 constructs by transient transfection into Swiss 3T3 cells and subsequently stained cells with rhodamine-labeled phalloidin. As shown in Fig. 5B, the NET1 wild type construct-transfected cells, indicated by the co-transfected GFP marker, have significantly more stress fibers when compared with the surrounding non-transfected cells or GFP alone-transfected cells, suggesting that increased expression of NET1 is sufficient to cause enhancement of stress fiber formation in Swiss 3T3 cells. TGF-β treatment did not lead to further increase in stress fiber formation (data not shown). Most important, transfection of the cells with the mutant NET1 construct led to significantly less stress fibers upon TGF-β treatment (Fig. 5C), suggesting that the function of NET1 is critical in the TGF-β-mediated stress fiber formation.

To confirm the involvement of RhoA in TGF-β-mediated...
stress fiber formation, we used a specific inhibitor Y27632 for Rho kinase (ROCK), a Rho downstream kinase that is required for RhoA-mediated stress fiber formation. We found that Y-27632 potently inhibited TGF-β-dependent stress fiber formation (Fig. 5D). Taken together, our results suggest that in Swiss 3T3 cells NET1 induction is both necessary and sufficient for stress fiber formation to occur, probably through increasing RhoA activity.

Smad Signaling Pathway Is Essential for NET1 Induction and Rho Activation—As transcription factors, Smad proteins have been shown to mediate the transcriptional activation of a number of TGF-β target genes. To determine if Smads are also involved in the induction of NET1 by TGF-β, we took advantage of a HaCaT stable cell line that overexpresses a Smad3 dominant negative mutant (D407E). Earlier studies indicated that the phosphorylation of endogenous Smad2 and Smad3, as well as the inhibition of cell proliferation, in response to TGF-β treatment was significantly blocked in this cell line (19). To test if TGF-β is capable of inducing the expression of NET1 in this cell line, we performed RPA analysis. As shown in Fig. 6A, the induction of NET1 in the Smad3D407E cell line was significantly lower than that of the control HaCaT cell line after TGF-β treatment for 2 h. As a demonstration for the inhibition of Smad activity, the phosphorylation of endogenous Smad2 in response to TGF-β was shown to be blocked significantly in Smad3D407E by using a phospho-specific antibody against Smad2 (Fig. 6B).

To test if the inhibition of Smad signaling and induction of NET1 by TGF-β lead to changes in downstream signaling, we next studied whether the activation of Rho is altered in Smad3D407E stable line. To do this, we used a serum-responsive element luciferase reporter (SRE-luc) that has been used previously for assaying Rho activity. As shown in Fig. 6C, TGF-β treatment for 24 h leads to a 10-fold induction of the SRE reporter gene in the control HaCaT cell line, indicating that a significant increase in Rho activity was induced by TGF-β. Similar activation of Rho also occurred in control stable line HaCaT-pCDNA3. However, in the Smad3D407E cell line, it was unchanged upon TGF-β treatment, suggesting that Rho activity may not be affected by TGF-β treatment in the absence of functional Smads and the induction of NET1.

DISCUSSION

As a multifunctional growth factor, TGF-β regulates a wide range of biological processes, including cell morphologic change and migration, likely through the modulation of expression of downstream target genes. To explore the signaling pathway that may mediate the specific effect of TGF-β on changes in stress fiber formation, we have identified the NET1 gene as a candidate target gene whose function may be important for this TGF-β-induced process. We have demonstrated that NET1, a specific GEF for RhoA, is rapidly induced by TGF-β in several TGF-β-responsive cell lines. In Swiss 3T3 cells, TGF-β induces the formation of stress fibers in a RhoA-dependent manner, a process correlated with the induction of NET1 expression. To test if this increased expression of NET1 is functionally important for TGF-β-regulated stress fiber formation, we overexpressed a dominant negative NET1 mutant in Swiss 3T3 cells and found that it could block stress fiber formation induced by TGF-β. These results, together with previous findings that implicate NET1 as an activator of RhoA, support a model that TGF-β-dependent NET1 induction could lead to the activation
of RhoA, which in turn causes increased stress fiber formation.

Although NET1 is the first gene so far identified in TGF-β signaling pathway whose function may be directly associated with the process of cytoskeletal reorganization, we suspect that there may be other related genes that can mediate the TGF-β effect involved in the regulation of cell motility and morphologic changes. For example, we have found that NET1 expression is not induced in primary mouse fibroblasts (data not shown), even though TGF-β has an effect on the migration of those cells, a process that involves dynamic cytoskeletal change. This observation suggests that TGF-β may use alternative pathways to activate RhoA, as small GTP-binding proteins could be regulated by GEFs, GAPs, and GDIs. It is likely that in different cell types, different GEFs, or GAPs and GDIs serve as targets that can be regulated by the TGF-β signaling pathway.

On the other hand, activation of stress fiber formation may not be the only function of NET1 induction. Consistent with this notion, we failed to observe significant increases in stress fiber formation in certain types of cells, such as HaCaT cells that showed a dramatic NET1 induction in response to TGF-β treatment. One possible consequence of NET1 induction is the activation of the JNK pathway, as a previous report demonstrated that overexpression of NET1 could lead to JNK activation (18). JNK activation by TGF-β has been reported to occur in various cell types (24, 25). In one recent study, TGF-β was found to induce JNK activation in a two-wave kinetic manner as JNK kinase activity peaked at an early time point (10 min) and a late time point (12 h) following TGF-β treatment (24). Although the pathway by which JNK is activated by TGF-β remains unknown, it was speculated that the immediate-early phase of JNK activation is probably a process independent of Smad proteins, known effectors of the TGF-β signaling pathway involved in transcription regulation of target genes, whereas the later phase of induction is Smad-dependent. Thus, the ability of NET1 to activate JNK pathway may provide a possible link between TGF-β and the later phase of JNK activation.

From the rapid time course of NET1 mRNA accumulation (Fig. 2A), we noticed a close correlation between the induction of NET1 expression and the known kinetics of phosphorylation...
and nuclear accumulation of Smad2 and Smad3 (26, 27). To examine if NET1 serves as one of the direct target genes for these two Smads, we used recombinant adenoviruses to determine if Smad3 and Smad4 overexpression would be able to stimulate the expression of the endogenous NET1 gene. The result was negative (data not shown), suggesting that Smad overexpression may not be sufficient to activate the expression of NET1, and other signaling events may have to be initiated by the TGF-β receptor complex simultaneously. Alternatively, the overexpression of Smad proteins clearly may not accurately reflect the signaling process of Smad activation and action. To explore this possible link further, we have used a HaCaT stable line overexpressing Smad3D407E, a mutant that can block the phosphorylation of endogenous Smad2 and Smad3 upon TGF-β treatment. The induction of NET1 in Smad3D407E cells was dramatically decreased in comparison to that of normal HaCaT cells, suggesting that Smads are essential for NET1 induction. The detailed mechanism for the Smad-mediated transcriptional regulation of NET1 expression will have to be revealed with the cloning and characterization of the NET1 promoter in the future.

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The Activity of Guanine Exchange Factor NET1 Is Essential for Transforming Growth Factor-β-mediated Stress Fiber Formation
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