Involvement of Cell-Cell Interactions in the Rapid Stimulation of Cas Tyrosine Phosphorylation and Src Kinase Activity by Transforming Growth Factor-β1*

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Transforming growth factor-β (TGF-β) regulates a wide range of physiological and pathological cellular processes, including cell migration, mesenchymal transition, extracellular matrix synthesis, and cell death. Cas (Crk-associated substrate, 130 kDa), an adaptor protein localized at focal adhesions and stress fibers, is also known to have important functions in cell migration and the induction of immediate-early gene expression. Here, we report that a rapid and transient tyrosine phosphorylation of Cas is induced by TGF-β1 and that E-cadherin-mediated cell-cell interaction and the Src kinase pathway are involved in this early TGF-β1 signaling. The addition of TGF-β1 to epithelial cells rapidly induced tyrosine phosphorylation of Cas and promoted the formation of complexes between focal adhesion molecules. Cas phosphorylation required the integrity of the actin cytoskeleton but was not dependent on cell adhesion, implying that Cas-dependent signaling may be distinct from integrin signaling. TGF-β1 also stimulated Src kinase activity, and specific inhibitors of Src completely blocked the induction of Cas phosphorylation by TGF-β1. The Cas phosphorylation and Src kinase activation seen in our results were induced in an epithelial phenotype-specific manner. Stable transfection of E-cadherin to L929 cells and L cells as well as E-cadherin blocking assay revealed that E-cadherin-mediated cell-cell interactions were essential for both Cas phosphorylation and Src kinase activation. Taken together, our data suggest that rapid Cas phosphorylation and Src kinase activation may play a novel role in TGF-β1 signal transduction.

Transforming growth factor-β (TGF-β)1 regulates a wide range of physiological and pathological cellular processes, including differentiation, immune response, inflammation, extracellular matrix synthesis, angiogenesis, and wound healing in humans (1–3). In epithelial and endothelial cells, TGF-β strongly inhibits cell growth (4, 5) and, in fibroblasts, acts in both growth stimulatory and growth inhibitory manners depending on the stage of differentiation and culture conditions (6, 7). TGF-β exhibits a tumor suppressor activity, and components of its signaling pathway are frequently mutated or silenced in colon and pancreatic cancers (8). However, accumulating data indicate that TGF-β can positively affect tumorigenesis and contribute to the progression and invasiveness of tumors (9–11). This tumor-activating activity of TGF-β is associated with its ability to induce an epithelial to mesenchymal transition and stimulate cell migration (12). The epithelial to mesenchymal transition induced by TGF-β results in the disruption of the polarized morphology of epithelial cells, the formation of stress fibers, and an enhancement of cell migration (11, 13–15).

The cell migration and reorganization of the actin cytoskeleton induced by many extracellular factors are accompanied by dramatic changes in the tyrosine phosphorylation of several signaling proteins localized at the focal adhesion plaques (16, 17). The rapid increase in the tyrosine phosphorylation of the nonreceptor tyrosine kinase focal adhesion kinase (FAK) and the adaptor proteins Cas (Crk-associated substrate) and paxillin has been identified as a prominent early event in cells stimulated by several signaling molecules that regulate cell growth, differentiation, migration, and apoptosis (18, 19).

Cas initially identified as a highly phosphorylated protein of 130 kDa in v-Src and v-Crk transformed cells (20, 21). Cas contains an N-terminal SH3 domain followed by a stretch of proline-rich sequences, a central substrate domain composed of a cluster of potential SH2-binding sites, and a C-terminal domain, which contains consensus binding sites for the SH3 and SH2 domains of c-Src (22, 23). The N-terminal SH3 domain mediates the interaction of Cas with several proteins, including FAK, related adhesion focal tyrosine kinase/PYK2, and FAK-related nonkinases (24, 25), two protein tyrosine phosphatases, PTP1B and PTP-PEST (26, 27), and the guanine nucleotide exchange factor C3G (28). Following tyrosine phosphorylation, the central substrate domain of Cas interacts with a number of SH2-containing signaling molecules, such as the adapter proteins Crk and Nck, possibly recruiting these molecules to focal adhesions (29, 30). All of these structural features indicate that Cas is a docking molecule that can assemble and transmit cellular signals through SH2 and SH3 containing intracellular proteins. Recently, Cas has been shown to be essential for cell migration (31, 32) and actin reorganization (33, 34) and for the mediation of the transcriptional activation of the serum response element by Src tyrosine kinase (35). In addition, Src and Cas mediate c-Jun N-terminal kinase (JNK) activation (36).
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which is regulated through Cas-Crk complex (37, 38).

Protein-tyrosine kinases of the Src family play pivotal roles in various signal transduction processes that contribute to the regulation of cell growth, differentiation, and cell migration (39). Several lines of evidence indicate that intrinsic Src kinase activity is required for the mitogenic and migratory effects of various growth factors (39, 40). Previously, TGF-β has been reported to regulate Src kinases in cell growth inhibition. TGF-β1 induces degradation of activated Src tyrosine kinase in v-Src transformed rat fibroblasts (41) and decreases Src kinase activity in HepG2 carcinoma cells, whereas it increases its activity and protein levels in Malignant epithelial cell lines (42). In human prostatic carcinoma cell line PC3, TGF-β1 was reported to down-regulate Src tyrosine kinases (43). However, a role for TGF-β in the regulation of Src kinase activity is not currently well defined.

Although Smad proteins are considered important mediators in the regulation of target gene expression induced by TGF-β, Smad-independent signaling involving extracellular signal-regulated kinase (ERK), p38, and JNK pathways in epithelial cells has been demonstrated. Because there is a similarity among TGF-β, Src, and Cas signaling that mediate the reorganization of actin cytoskeleton, stimulation of cell migration, and JNK activation, we hypothesized that TGF-β would stimulate the tyrosine phosphorylation of Cas and Src kinase activity. In recent reports, treatment with TGF-β for 48 h was shown to stimulate the tyrosine phosphorylation of focal adhesion molecules in normal murine mammary epithelial cells (48) and rabbit corneal keratinocyte (49). However, the mechanism responsible for Cas phosphorylation in TGF-β signaling remains unclear. In this report, we demonstrate that TGF-β induces a rapid and transient increase in tyrosine phosphorylation of Cas in an epithelial cell-specific manner and promotes rapid formation of complexes among focal adhesion molecules. TGF-β also activates Src kinase activity, which is essential for the tyrosine phosphorylation of Cas. E-cadherin-mediated cell-cell interactions and the intact actin cytoskeleton in epithelial cells are required for this event.

EXPERIMENTAL PROCEDURES

Materials—TGF-β1 was obtained from PeproTech. Fluorescein isothiocyanate (FITC)-conjugated and rhodamin-conjugated anti-mouse IgG, horseradish peroxidase-conjugated anti-mouse, and anti-rabbit IgG, rhodamin-conjugated, insulin-like growth factor-1 (IGF-1), and epidermal growth factor (EGF) were from Sigma. C2 and cytochalasin D were from Calbiochem-Novabiochem Ltd. Cas (C-20), c-Src (SRC2), and FAK (C-20) polyclonal antibodies (pAbs), protein G-agarose, and ECL reagent were from Santa Cruz Biotechnologies. Monoclonal anti-human E-cadherin IgG (HECD-1) was obtained from Zymed Laboratories, Inc., monoclonal anti-FAK 2A7 and anti-Src (GD11) antibodies and the Src kinase assay kit were from Upstate Biotechnologies, Inc. The dual luciferase reporter assay system was from Promega. All of the culture reagents were purchased from Invitrogen. The other reagents used were of the purest grade available.

Cell Lines—Human embryonic kidney epithelial cells (HEK293), human keratinocytes (HaCaT), and mouse fibroblasts (L929 cells, NIH3T3, and Swiss3T3) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO2. The original L cell strain and EL1 cells overexpressing mouse E-cadherin (50) were obtained from Dr. M. Takeichi (Kyoto University). Madin-Darby canine kidney (MDCK) epithelial cells, L cells, and EL1 cells were grown in Eagle’s minimal essential medium with Eagle’s salts containing 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 10% FBS.

Establishment of Stable E-cadherin-expressed L929 Cells—Mouse E-cadherin inserted into the plasmid pBl176 was kindly provided by Dr. M. Takeichi. The E-cadherin fragment was reisolated by double dige-
were performed as described above. Aggregation assays were performed at 37 °C at 100 rpm for the indicated times in triplicate wells, in 24-well ultra low attachment plates (Corning Costar Cooperation, Cambridge, MA). The assays were stopped after 0 and 60 min by fixing the cells in 1% glutaraldehyde. The extent of cell-cell binding was monitored by measuring the disappearance of single cells using a Coulter counter. The standard deviations of the mean values are included.

Src Kinase Assay—Tyrosine kinase activity of Src was assayed by immunoprecipitation using GD11 as described above. The immunoprecipitates from ~500 µg of total protein were washed three times with Triton X-100 lysis buffer, and the reactions were carried out using components of a commercially available Src kinase assay kit (Update Biotechnology Inc.). The assay is based on Src-dependent phosphorylation of a substrate peptide (KVEKIGETYGVRKYK) derived from p34^cdc2. In some experiments to measure Src kinase activity using acridine deanol as a substrate, immunoprecipitates were washed twice with HNTG buffer (50 mM Tris-HCl, pH 7.2, 10 mM MnCl₂, 10 mM MgCl₂, 150 mM NaCl, 0.2 mM sodium orthovanadate, 1 mM dithiothreitol) and incubated with HNTG buffer plus 5 µCi of [γ-32P]ATP and 4 µg/reaction enolase for 10 min at 30 °C. The reaction was stopped by adding 4× sample buffer and boiling and was analyzed by SDS-polyacrylamide gel electrophoresis. The presence of equivalent amounts of enolase was verified by Coomassie staining of the gel and equivalent amounts of Src by immunoblotting. The labeled enolase was visualized by autoradiography.

Immunoprecipitation—HaCaT, E-cadherin transfected L929 cells (L9E12), and control vector transfected cells (L9M3) were grown on Lab-Tek chambered glass slides (Nunc). After 24 h, the cells grown in DMEM containing 10% FBS were serum-starved for 18 h in the absence or presence of 25 µg/ml HECD-1 or control mouse IgG. The cells were then fixed in freshly made 4% paraformaldehyde/PBS at room temperature for 15 min, incubated with 100 mM glycine in PBS for 20 min to block aldehydes, rinsed three times for 10 min in 0.5 mM glycine/PBS, permeabilized in 0.5% Triton X-100, 0.5 mM glycine/PBS for 10 min, rinsed three times for 10 min in 0.5 mM glycine/PBS, and blocked in PBS containing 2% bovine serum albumin for 1 h. For E-cadherin staining, the cells were incubated with anti-mouse E-cadherin (C20920) in blocking buffer for overnight at 4 °C. As secondary antibodies, FITC-conjugated or rhodamin-conjugated anti-mouse antibodies were used. Finally, the cells were washed, mounted in anti-fade medium (Molecular Probes, Eugene, OR), and examined by a conventional inverted fluorescence microscopy (model S 100; Carl Zeiss, Inc., Oberkochen, Germany), and the captured images were processed by Adobe Photoshop.

RESULTS

Rapid and Transient Stimulation of Tyrosine Phosphorylation of Cas, FAK, and Paxillin by TGF-β1—To investigate whether TGF-β1 induces tyrosine phosphorylation of Cas in HEK293 cells, serum-starved cells were stimulated with various concentrations of TGF-β1 for 10 min and lysed, and the extracts were immunoprecipitated with anti-Cas antibody. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with an anti-phosphotyrosine mAb (PY20). As shown in Fig. 1A, TGF-β1 induced a marked increase in the tyrosine phosphorylation of Cas following a bell-shaped dose-response relationship with maximum effect at 1–5 ng/ml. At a higher concentration (5 ng/ml) of TGF-β1, tyrosine phosphorylation of Cas was nearly at the base-line level.

The kinetics of tyrosine phosphorylation of Cas stimulated by TGF-β1 in HEK293 is shown in Fig. 1B. An increase in tyrosine phosphorylation of Cas could be detected as early as 2 min after treatment with 2 ng/ml TGF-β1, reaching a maximum after 5–10 min. Subsequently, tyrosine phosphorylation of Cas declined gradually to almost base-line levels. TGF-β1 also induced a rapid and increased tyrosine phosphorylation of FAK and the adapter protein paxillin. The increase in the tyrosine phosphorylation of FAK was dose-dependent (Fig. 1C) and could be detected as early as 2 min after addition of 2 ng/ml TGF-β1, peaking at 5 min (Fig. 1D). In the event of paxillin, the maximal increase was reached at 10 min (Fig. 1E).

Cas contains a tyrosine kinase substrate domain consisting of 15 potential SH2-binding motifs, nine of which conform to the SH2-binding motif for Crk (22), and cell adhesion to extracellular matrix (ECM) proteins promotes FAK and e-Src kinase activity leading to tyrosine phosphorylation of Cas and its association with Crk or Nck (29, 30). The C-terminal 150 amino acids (focal adhesion targeting sequence) of FAK contain binding sites for paxillin, and the major site of FAK autophosphorylation, tyrosine 397, is potentially a high affinity binding site for the SH2 domain of Src (18). Consequently, we examined whether TGF-β1-induced tyrosine phosphorylation of these focal adhesion molecules could lead to the formation of complexes among the focal adhesion molecules Cas, Crk, FAK, and paxillin in HEK293 cells. The immunoprecipitates of Crk and FAK were analyzed by immunoblotting probed with anti-Cas and anti-paxillin antibodies, respectively. As shown in Fig. 2, TGF-β1 induced the rapid and transient formation of Crk/Cas (Fig. 2A) and FAK/paxillin (Fig. 2B) complexes that were time-dependent and parallel to the TGF-β1-induced tyrosine phosphorylation of these molecules. The formation of these complexes reached a maximum after 5–10 min of TGF-β1 stimulation and then declined. Taken together, TGF-β1 induces a rapid and parallel increase in the tyrosine phosphorylation of focal adhesion molecules and concomitantly promotes the complex formations among these molecules in HEK293 cells.

The Integrity of the Actin Cytoskeleton Is Essential for the Tyrosine Phosphorylation of Cas Induced by TGF-β1, but Cell Adhesion Is Not—Accumulating data indicate that the integrity of the actin cytoskeleton is required for stimulation of Cas tyrosine phosphorylation by cell adhesion (51), growth factors (52, 53), or G protein-coupled receptor agonists (54, 55). To examine whether a disruption of the actin cytoskeleton could affect the TGF-β1-induced phosphorylation of Cas, serum-starved HEK293 cells were exposed for 2 h to increasing concentrations of cytochalasin D and subsequently stimulated with 0.5 or 2 ng/ml TGF-β1 for another 10 min. Consistent with previous reports, treatment with cytochalasin D blocked Cas tyrosine phosphorylation induced by TGF-β1 in a concentration-dependent manner, blocking completely at 2 µM (Fig. 3A). We also found that the amount of Cas that could be immunoprecipitated increased after treatment with increasing concentrations of cytochalasin D (Fig. 3A, lower panel).

To determine whether the TGF-β1-induced tyrosine phosphorylation of Cas is dependent on cell adhesion, HEK293 cells were detached and suspended for 60 min at room temperature and then stimulated with 2 ng/ml TGF-β1 for the times indicated in Fig. 3B. An increase in tyrosine phosphorylation of Cas could be detected as early as 2 min after treatment of 2 ng/ml TGF-β1, reaching a maximum after 5–10 min. Thereafter, tyrosine phosphorylation of Cas declined gradually to almost base-line levels after 60 min of incubation. These findings indicate that the tyrosine phosphorylation of Cas induced by TGF-β1 requires the integrity of the actin cytoskeleton but may not be dependent on integrin-mediated cell adhesion.

An Epithelial Phenotype Is Important in Tyrosine Phosphorylation of Cas—Accumulating data demonstrate that TGF-β has contradictory effects in the proliferation and transformation of epithelial and fibroblast cells with different origins (4–7, 56). These findings allow us to examine the ability of TGF-β1 to induce Cas phosphorylation in cells with different phenotypes. We also compared Cas phosphorylation induced by TGF-β1 and by other growth factors. As shown in Fig. 4A, Cas phosphorylation increased in HEK293 epithelial cells following treatment with TGF-β1 and EGF for 10 min. In HaCaT cells, Cas phosphorylation was induced by TGF-β1 to a greater level than by
IGF-1 or EGF. On the other hand, Cas phosphorylation in mouse fibroblast cells (L929) was induced by IGF-1 and EGF but not TGF-β1. Similarly, Cas phosphorylation was not stimulated by TGF-β1 in other fibroblast cell lines, including NIH3T3 and Swiss3T3 (Fig. 4B). In addition, Cas phosphorylation in these fibroblast cells was not observed in response to TGF-β1 until 60 min (data not shown). Because no observation of Cas phosphorylation in these fibroblast cells could be due to the nonresponsiveness of these cells to TGF-β, we assessed the reporter gene activity, using 3TP-Lux reporter construct, which is utilized widely to determine the responsiveness of mammalian cells to TGF-β. As shown Fig. 4C, three fibroblast cell lines used in these experiments were found to be TGF-β-responsive. Taken together, the ability of Cas to be phosphorylated in response to treatment with TGF-β1 may be a characteristic of epithelial cells.

To further examine the requirement of an epithelial phenotype in tyrosine phosphorylation of Cas, we utilized a mesen-
chymal epithelial transition model, as mentioned in previous reports (50, 57). L cells are frequently used for studies of cadherin adhesion because they do not express any endogenous cadherins. In this study, L929 cells, derivatives of L cells, were stably transfected with pcDNA3 encoding mouse E-cadherin or a control plasmid. The transfected cells were isolated after G418 selection and were then analyzed for expression of E-cadherin by immunofluorescence staining (Fig. 5A) using anti-mouse E-cadherin antibody. E-cadherin transfected L929 cells (L9E12) were highly expressing exogenous E-cadherin, well located at the boundary of cells, whereas E-cadherin was not

FIG. 2. Time course of TGF-β1-induced associations of Cas with c-Crk and paxillin with FAK. Serum-starved HEK293 cells were treated at 37 °C with 2 ng/ml TGF-β for various times as indicated and subsequently lysed. Associations of Cas with c-Crk (A) and paxillin with FAK (B) were analyzed by immunoprecipitation (IP) using anti-CrkII (A) and anti-FAK (B) antibodies followed by Western blotting (WB) with the corresponding antibodies. The results shown are representative of two independent experiments.

FIG. 3. TGF-β-induced Cas tyrosine phosphorylation requires the integrity of the actin cytoskeleton and is independent on cell adhesion. A, serum-starved HEK293 cells were pretreated for 2 h in the absence (−) or in the presence of increasing concentration of cytochalasin D (CytoD), as indicated and then stimulated without (−) or with indicating concentration of TGF-β for a further 10 min. The treated cells were lysed and analyzed by immunoprecipitation with anti-Cas antibody followed by Western blotting with anti-PY20 phosphotyrosine antibody. B, confluent HEK293 cultured in 10% FBS containing DMEM were detached with calcium free Hanks’ balanced salt solution containing 5 mM EDTA and 5 mM EGTA, washed twice with serum-free media, and suspended in DMEM containing 0.5% bovine serum albumin and 100 μg/ml DNase at room temperature with continuous rotating for 20 rpm for 60 min. Cells in suspension were then treated with 2 ng/ml TGF-β at 37 °C for various times, dipped into ice for 5 min, rinsed with stop buffer at 4 °C, and then lysed. The cellular lysates were processed for immunoprecipitation (IP) and Western blotting (WB), as above. The data shown are representative of three independent experiments. DMSO, dimethyl sulfoxide.

FIG. 4. TGF-β-induced Cas tyrosine phosphorylation in epithelial and fibroblast cell lines. A, Cas tyrosine phosphorylation induced by various growth factors in epithelial (HEK293 and HaCaT) and fibroblast cell lines (L929). Serum-starved cells were treated in the absence (C) or in the presence of 2 ng/ml TGF-β (T), 10 nM IGF-1 (I), and 100 nM EGF (E) at 37 °C for 10 min. The cells were then lysed, and the lysates were analyzed by immunoprecipitation with anti-Cas antibody followed by Western blotting (WB) with anti-PY20 phosphotyrosine antibody. B, Cas tyrosine phosphorylation induced by TGF-β1 in fibroblast cell lines. Serum-starved fibroblast cell lines (NIH 3T3 and Swiss 3T3) were treated in the absence (−) or in the presence (+) of 2 ng/ml TGF-β1 at 37 °C for 10 min. Equal amounts of cellular lysates were used in analysis of Cas tyrosine phosphorylation. C, L929, Swiss3T3, and NIH3T3 fibroblast cells were transiently co-transfected with 1 μg of 3TP-Lux, and 0.1 μg of SV40-RL. After 18 h, the cells were incubated in the absence (closed bars) or presence of TGF-β1 (2 ng/ml) (open bars) for an additional 24 h. The cells were lysed, and the luciferase activity was determined using the Promega dual luciferase reporter assay according to the manufacturer’s instructions. The luciferase activity is expressed as the ratio of specific luciferase activity divided by the luciferase activity of the internal standard. Shown are the means ± S.D. of triplicates from a representative experiment.
detected in L929 (L9M3) transfected with a control plasmid. To analyze the effect of E-cadherin expression on the tyrosine phosphorylation of Cas induced by TGF-β1, we examined the time dependence of Cas phosphorylation status following TGF-β1 treatment in serum-starved L9E12 and L9M3 cells. In L9E12 cells, TGF-β1 induced a transient increase in tyrosine phosphorylation of Cas, which could be detected as early as 2 min after the addition of 2 ng/ml, reaching a maximum at 5 min (Fig. 5, B, upper panels, and C). In contrast to the L9E12 cells, L9M3 cells did not display Cas phosphorylation in response to TGF-β1 throughout the time course examined (Fig. 5B, lower panels). To confirm the effect of E-cadherin expression in this event, we utilized E-cadherin-deficient L cells and ELβ1 cells, which highly express mouse E-cadherin (50). Similar to the results in L9M3 and L9E12 cells, Cas phosphorylation induced by TGF-β1 was displayed in ELβ1 but not in L cells (Fig. 5D). In addition, the basal levels of Cas phosphorylation in L9E12 and ELβ1 cells were higher than those in L9M3 and L cells, respectively (Fig. 5, C and D). All of these transfected cells were also confirmed to be responsive to TGF-β1 (Fig. 5E).

**Mechanism of Cas Tyrosine Phosphorylation by TGF-β**

The preceding data show that E-cadherin expression is essential to Cas phosphorylation induced by TGF-β1. E-cadherin is a calcium-dependent cell-cell interaction molecule that mediates homophilic interactions between many types of epithelial cells and is essential for tissue morphogenesis. Cell-cell interaction via E-cadherin involves the coordination of extracellular binding and intracellular anchorage to the actin-based cytoskeleton (58, 59). Because the integrity of the actin cytoskeleton and E-cadherin expression are essential to the tyrosine phosphorylation of Cas, we further investigated the effect of E-cadherin-mediated cell-cell interaction on Cas phosphorylation. As illustrated in Fig. 6A, to block E-cadherin-mediated cell-cell interaction, HaCaT cells were pretreated for 18 h with HEC-1, an antibody against an epitope in the extracellular domain of E-cadherin. The distribution of E-cadherin was then analyzed with immunofluorescence staining using monoclonal antibody against an epitope in the cytoplasmic domain of E-cadherin (Fig. 6A, upper panels, C20820). In addition, the distribution of HEC-1 bound to the cells was also analyzed using...
FITC-labeled anti-mouse IgG without treatment of a primary antibody (Fig. 6A, lower panels, α-Ms). In HaCaT cells pretreated with HECD-1, E-cadherin appeared to be diffusely distributed in the cytoplasm as well as at sites of cell-cell interaction (Fig. 6A, right panels). In contrast, control cells pretreated with normal mouse IgG showed a typical pattern of staining for E-cadherin only at the cell-cell contacts (Fig. 6A, left and lower panel). In this case, a remarkable disappearance of E-cadherin at cell-cell contacts was most likely due to their redistribution throughout the cell surface. These results demonstrate that HECD-1 is effective in interfering E-cadherin-mediated cell-cell interaction as asserted in previous reports (60, 61). Under these conditions, we determined that Cas was not phosphorylated on tyrosine by TGF-β1 (Fig. 6B).

To confirm the involvement of cell-cell interaction in Cas phosphorylation, we used a suspended culture system as shown in Fig. 3B. HaCaT cells were detached and incubated at room temperature for 60 min (referred to as 0 min) with continuous rotation at 20 rpm, followed by the pretreatment with 50 μg/ml of normal mouse IgG or HECD-1 for another 60 min. As shown in Fig. 6C, the aggregation in HaCaT cells pretreated with control IgG for 60 min increased by 50%, compared with that seen at 0 min. Aggregation decreased below baseline in cells treated with HECD-1. Under these conditions, we examined Cas phosphorylation induced by TGF-β1. When HaCaT cells in suspension at 0 min or after 60 min in control IgG were treated with TGF-β1 for another 10 min, Cas phosphorylation was induced (Fig. 6D, 0 min and IgG). However, prevention of aggregation by treatment with HECD-1 blocked the effect of TGF-β1 on Cas phosphorylation (Fig. 6D, HECD-1). These results show that E-cadherin-mediated cell-cell interaction is required for TGF-β1 to stimulate Cas phosphorylation.

The Activity of Src Kinase Is Involved in Cas Phosphorylation Induced by TGF-β—Several lines of evidence show that Src family kinases are involved in the tyrosine phosphorylation of Cas: 1) Cas is a major tyrosine-phosphorylated protein in cells transformed by v-Src (22); 2) Cas is tyrosine-phosphorylated upon cell adhesion in a Src-dependent manner (30); and 3) tyrosine phosphorylation of Cas is decreased in Src−/− mouse fibroblasts and increased in Csk (C-terminal Src kinase)-deficient cells (Csk−/− cells) in parallel with Src activity (29). Therefore, we examined the effect of Src family kinases on the
tyrosine phosphorylation of Cas induced by TGF-β1, using the specific inhibitors PP2 and herbimycin A (62, 63). Both inhibitors significantly reduced TGF-β1-induced Cas phosphorylation (Fig. 7A), and PP2 was confirmed to inhibit it in a dose-dependent manner with an IC_{50} value of ~2 μM (Fig. 7B).

However, TGF-β has been reported to regulate the activity of Src kinases in HepG2 and PC3 cells in a negative manner (42, 43). Therefore, we examined Src kinase activity in HaCaT cells treated with TGF-β1. Src kinase activities were measured by immunocomplex kinase assays using two specific substrates, a substrate peptide (KVEKIGEGTYGVVYK) derived from pS4^{calc} (Fig. 8, A and C) and acid-denatured enolase (Fig. 8, B and D). As shown in Fig. 8 (A and B), TGF-β1 significantly induced the activity of c-Src in a dose-dependent manner, parallel to the tyrosine phosphorylation of Cas (Fig. 8B, lower panels). The kinetics of c-Src activity stimulated by TGF-β1 in serum-starved HaCaT is shown in Fig. 8 (C and D). An increase in c-Src activation could be detected as early as 2 min after treatment with 3 ng/ml TGF-β1, reaching a maximum after ~5–7 min. In parallel experiments, Cas phosphorylation was induced in a delayed manner, peaking at 10 min, similar to HEK293 cells (Fig. 1B). Thereafter, c-Src activity was nearly at base-line levels after 15 min of incubation. To further verify the activation of c-Src by TGF-β, we additionally examined whether TGF-β1 induced the activity of c-Src in another epithelial cell line, MDCK. An increase in c-Src activation and Cas phosphorylation in MDCK was also found after treatment with 3 ng/ml TGF-β1 in a similar manner in HaCaT (Fig. 8E).

**The Activation of c-Src Is Also Inhibited by Blocking E-cadherin-mediated Cell-Cell Interaction**—The preceding data showed that Cas phosphorylation induced by TGF-β1 requires E-cadherin-mediated cell-cell interaction and c-Src kinase activity. Therefore, we examined whether E-cadherin-mediated cell-cell interaction was required for the activation of c-Src kinase by TGF-β1 under conditions illustrated in Figs. 5 and 6. Unlike L9M3 and L cells, E-cadherin-expressed L9E12 and ELβ1 cells displayed a remarkable increase in the activity of c-Src kinase after treatment with 2 ng/ml TGF-β1 for 5 min (Fig. 9, A and B). To confirm the effect of E-cadherin-mediated cell-cell interaction on the activation of c-Src kinase by TGF-β1, we utilized E-cadherin blocking assays as mentioned in Fig. 6B. As shown in Fig. 9C, the c-Src kinase activity induced by TGF-β1 was completely blocked by the disruption of cell-cell interactions. These data suggest that E-cadherin-mediated cell-cell interaction has a role as an upstream mediator in the activation of c-Src kinase and Cas phosphorylation by TGF-β1.

**DISCUSSION**

The results presented here demonstrate that TGF-β1 in epithelial cells stimulates the rapid and transient tyrosine phosphorylation of Cas via a pathway that is dependent on c-Src kinase. Recently, the treatment of TGF-β for 2 or 3 days was shown to induce epithelial to mesenchymal transition, accompanied by the stimulation of the tyrosine phosphorylation of focal adhesion molecules in normal mammary epithelial cells (48) and corneal keratinocytes (49). However, the mechanism for Cas phosphorylation in TGF-β1 signaling remains unclear. In our experiments, the TGF-β1 stimulation of epithelial cells results in the rapid tyrosine phosphorylation of Cas as early as 2 min and in a bell-shaped dose response, similar to Cas phosphorylation induced by EGF (53) and platelet-derived growth factor (54). The changes in tyrosine phosphorylation of Cas paralleled the TGF-β1-induced changes in tyrosine phosphorylation of other focal adhesion molecules (FAK and paxillin) and the associations of focal adhesion molecules. These data suggest a novel function for focal adhesion molecules as a signaling component in TGF-β1-mediated early signal transduction.

Epithelia are characterized by a high degree of cellular asymmetry and have been used extensively to study how cell polarity is developed (64). In epithelial cells, the full establishment of this polarity requires both cell-cell and cell-ECM interactions, which are mediated by adhesion mechanisms involving different types of cell surface receptors. Among them, cadherins and integrins play a major role, because they are able to recognize and interact with other cell adhesion receptors on neighboring cells or with proteins of the ECM, respectively. The integrity of the actin cytoskeleton in epithelial cells is also essential to the maintenance of this cell polarity, connecting with cytoplasmic domains of cell adhesion receptors. We found here that the induction of Cas phosphorylation by TGF-β requires the presence of an intact actin cytoskeleton because the tyrosine phosphorylation can be prevented by cytochalasin D treatment. A similar situation has been observed when Cas phosphorylation was induced by EGF, IGF-1, platelet-derived growth factor, bombesin, or integrin-mediated cell adhesion (51–54).

The structure of the actin cytoskeleton in nonpolarized fibroblast cells is distinct from that seen in epithelial cells. These facts, together with the contradictory effects of TGF-β on the proliferation of both cells, prompted us to examine whether Cas phosphorylation would be induced by TGF-β1 in a variety of fibroblast cell types. In contrast to other growth factors (IGF-1 and EGF), the present study shows that TGF-β seems to have an ability to specifically phosphorylate the tyrosine residues of Cas in epithelial cells, although not in fibroblast cell lines. This result is supported by our results demonstrating that the induction of Cas phosphorylation by TGF-β1 occurs in exogenously E-cadherin-expressing cells (L9E12 and ELβ1) but not in E-cadherin deficient cells (L9M3 and L). In addition, the basal levels of Cas phosphorylation as well as Src kinase activity in
L9E12 and ELβ1 cells were found to be higher than those in L9M3 and L cells. This implies that the persistent occupancy of E-cadherins by their recruitment to areas of cell-cell contact may contribute to the activity of these signal molecules, likely a result of mitogen-activated protein kinase and cdc42 activations induced by cell-cell clustering (65, 66).

Additionally, the present study provides evidence that TGF-β in HaCaT and MDCK cells induces a rapid and transient increase in Src kinase activity. TGF-β-induced Src kinase was also found to mediate the regulation of Cas phosphorylation as well as to be regulated by E-cadherin-mediated cell-cell interaction, parallel to the induction of Cas phosphorylation. On the other hand, TGF-β has been previously reported to negatively regulate Src kinases in HepG2 and PC3 carcinoma cells (42, 43). The reason for the differing results remains unknown. Because E-cadherins do not exhibit any enzymatic activity in their short cytoplasmic tail, it is conceivable that their ability to function as signal transducing receptors or scaffold proteins may depend on their physical interaction with other signal transduction molecules, such as catenins. This assumption may explain the contradictory regulation of Src kinase by TGF-β. Indeed, although HepG2 and PC3 cells have polarized epithelial phenotypes and also express E-cadherin, the catenins associated with E-cadherin in both cells differ from those of normal epithelial cells. HepG2 cells have a mutation of the β-catenin gene, leading to its accumulation in the nucleus (67), and PC3 cells do not express the α-catenin gene (68). Therefore, these facts allow us to suggest that other signal molecules in addition to E-cadherin may be involved in the regulation of Src kinase activity by TGF-β and may display cell type-specific differences in TGF-β signaling.

Although our biochemical approaches showed obvious evidence concerning the tyrosine phosphorylation of focal adhesion molecules and their associations, the formation of focal adhesion or stress fiber induced by TGF-β for 10–30 min were not observed in HaCaT cells using immunofluorescence staining for focal adhesion molecules and F-actin (data not shown). Focal adhesions are known to be regions of a cell in direct contact with the extracellular matrix, providing anchorage sites for actin stress fibers and forming a link between the extracellular matrix and the actin cytoskeleton via the integrin family of cell surface receptors (69). There may be a reason for the poor observation of focal adhesion and stress fibers; the events induced by TGF-β may be independent of integrin activation. The finding that supports this assumption is that Cas phosphorylation induced by TGF-β also occurs in suspended cells (Fig. 3B), implying that this TGF-β signaling is not dependent on cell adhesion. Furthermore, the present study shows that E-cadherin-mediated cell-cell interactions play a major role in Cas phosphorylation induced by TGF-β. Overall, it can be concluded that the induction of Cas phosphorylation by TGF-β is dependent on E-cadherin-mediated cell-cell interaction and independent of the cell-ECM adhesion. However, considering that a variety of the stimuli leading to the stimulation of tyrosine phosphorylation of Cas have been shown to induce a rapid increase in stress fibers and in focal adhesions (19, 51, 70) and that we cannot rule out the possibility that the
HaCaT cells in our study may not be suitable in staining for focal plaques and F-actin, further study is required to elucidate the exact mechanism of integrin-independent Cas phosphorylation in the early event of TGF-β signaling.

Recent studies have shown other functions of Cas in cellular signaling processes that are distinct from integrin signaling. Src/Cas signaling mediates the transcriptional activation of the serum response element through Ras/mitogen-activated protein kinase/extracellular signal-regulated kinase/extracellular signal-regulated kinase pathways (35). Considering that TGF-β-mediated induction of c-Fos for 1 h proceeds through extracellular signal-regulated kinase-dependent signal transduction in HaCaT cells (45), the rapid Src/Cas activation by TGF-β may mediate the expression of immediate-early genes such as c-Fos (71). Further, Cas has been reported to activate the JNK pathway through coupling with Src and Crk (36–38, 72). Engel et al. (47) demonstrated that the JNK activation stimulated by TGF-β is bimodal in mink lung epithelial cells. The rapid JNK activation by TGF-β for 5–30 min is Smad-independent, followed by a sustained, Smad-dependent JNK activation. These findings, together with our observations, indicate that TGF-β-induced Src/Cas signaling may be involved in this rapid JNK activation in epithelial cells. Interestingly, recent microarray studies revealed that the pattern of gene expression by TGF-β in MCF-10A normal epithelial cells is different from that in E-cadherin-deficient MDA-MB-231 cells (73). Therefore, the present study provides a possibility that E-cadherin-dependent Src/Cas signaling may regulate the expressions of early TGF-β-responsive genes in epithelial cells, through mitogen-activated protein kinase pathways.

In addition, there is an interesting report showing that HEF1, one of the Cas family proteins, is quickly degraded by Smad3 and then restored rapidly in epithelial cell lines A549, HepG2, and HaCaT (74). This study suggests that Smad3 can regulate proteasomal degradation of HEF1 and that these degradation events are followed by negative feedback of HEF1 to shut off the nuclear signaling activities of Smad3 efficiently. However, in this study, the endogenous Cas degradation by TGF-β was not observed up to 24 h of TGF-β treatment in HaCaT cells (data not shown). It thus appears that Cas, unlike HEF1, is not affected by the mechanism of Smad3-regulated proteasomal degradation.

In conclusion, TGF-β induces a rapid and transient increase in the tyrosine phosphorylation of Cas in epithelial cells, which is regulated by TGF-β-induced Src kinase activity. Furthermore, the induction of Cas phosphorylation and Src kinase activity requires E-cadherin-mediated cell-cell interaction but is not dependent on cell-ECM adhesion. These findings provide a novel signal transduction of TGF-β linked to Src/Cas cellular signaling in its related multi-cellular processes of epithelial cells. Further studies will be necessary to elucidate fully how engagement of E-cadherins results in the regulation of Src kinase and what downstream signaling pathways of Src/Cas regulated by TGF-β participate in the regulations of immediate-early genes, ECM production, cell death, and mesenchymal transition of normal epithelial cells.

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