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Integrin Signaling and Cell Spreading Mediated by Phorbol 12-Myristate 13-Acetate Treatment

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Abstract Spreading of SNU16mAd gastric carcinoma cells was previously shown to be regulated via a signaling network from transforming growth factor β1 (TGFβ1) to integrins signaling, through a mediation of protein kinase C δ (PKCδ). However, in the previous study, the roles of PKCδ appeared complicated. In this study to clarify the roles of PKCδ in the spreading of the gastric carcinoma cells, we questioned if PKC activation via phorbol 12-myristate 13-acetate (PMA) treatment could mimic the TGFβ1 effects. An acute PMA treatment increased phosphorylations of focal adhesion (FA) kinase, paxillin, c-Src, and cofilin, just as TGFβ1 did. Furthermore, cell spreading mediated by TGFβ1- or acute PMA treatment correlated with activation of RhoA, which regulates actin reorganization and FA formation. However, stress fiber formation was prominent in TGFβ1-treated cells, compared to cortical actin organization in PMA-treated cells. Altogether, these observations indicate that acute PMA treatment could mimic the TGFβ1 mechanisms for cell spreading through subtly different effects on actin reorganization. J. Cell. Biochem. 99: 88–95, 2006.

Key words: cell spreading; integrin; RhoA; PMA; TGFβ1

Cell spreading is a prerequisite of efficient response of normal epithelial cells to extracellular stimuli for proliferation, migration, invasion, and gene transcription. For this to happen, integrins consisting of an α and a β subunit as cell adhesion receptors can sense and interact with extracellular matrix (ECM) proteins. Therefore, bi-directional signal transduction between extracellular and intracellular spaces occurs by integrins that cross-talks with transforming growth factor β1 (TGFβ1) or growth factor signaling pathways [Hynes, 2002; Lee and Juliano, 2004].

Transforming growth factor β1 is a multifunctional cytokine to inhibit cell growth and also mediate cell differentiation and metastasis. Typically TGFβ1 signal transduction involves Smad proteins to regulate numerous developmental and homeostatic processes via regulations in gene induction [Akhurst and Derynck, 2001]. An inhibitory Smad7 competes with R-Smads 2 and 3 for phosphorylation of Smad2/3 by activated TGFβ1 receptor on TGFβ1 treatment [Massague, 1998]. TGFβ1 also affects diverse intracellular signaling molecules such as RhoA via Smad-independent manners [Hartsough and Mulder, 1995; Engel et al., 1999; Massague, 2000; Yu et al., 2002; Derynck and Zhang, 2003; Kim et al., 2005].

Phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), are important model stimuli

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that have been shown to modulate diverse cellular events through protein kinase C (PKC) activation [Carter, 2000]. Specifically, PMA acts as an alternative stimulus to activate endogenous PKC, diacylglycerol (DAG). Either DAG or phorbol ester can activate a novel family of the PKC families including PKCδ [Yang and Kazanietz, 2003].

Although the collaborative relationship between integrin- and TGFβ1-mediated signal pathways appears to be important for expression of integrins, ECMs and TGFβ1 themselves [Kumar et al., 1995; Mainiero et al., 2000; Rodriguez-Barbero et al., 2002] that essentially function in diverse cellular functions, mechanistic details underlying their collaboration and signaling network are largely unknown. Meanwhile, we previously reported that a signaling network involving Smad-dependent TGFβ pathway, PKCδ, and integrins is responsible for spreading of SNU16mAd gastric carcinoma cells, based on the observations that TGFβ1 caused induction and activation of integrins and their signal transduction via PKCδ. However, TGFβ1-mediated cell spreading was not supported by a simple linear linkage from Smads to focal adhesion (FA) molecules, since overexpression of PKCδ did not result in efficient spreading on fibronectin (Fn) in the absence of TGFβ1 treatment [Lee et al., 2005]. Therefore, it appeared that TGFβ1 treatment of SNU16mAd cells led to activation of integrins and FA molecules via PKCδ-dependent and -independent manners.

Therefore, in this study, we tried to examine whether PMA could mimic the TGFβ1 effects on activation of intracellular signaling molecules finally leading to cell spreading. We observed that an acute treatment of PMA at a lower dose could also activate integrin-related signaling pathways including RhoA finally leading to cell spreading, as a chronic TGFβ1 treatment did. However, there appear to be subtle differences in both stimuli-mediated cell spreading in terms of actin structures involved in the spreading.

MATERIALS AND METHODS

Cell

SNU16mAd cells [Kim et al., 2004] were cultured at 37°C and 5% CO_2, in RPMI-1640 culture media containing 10% (v/v) fetal bovine serum and 0.2 mg/ml G418.

Cell Lysates Preparation and Western Blots

Replating of SNU16mAd cells on Fn-precoated dishes (10 μg/ml, Chemicon, Temecula, CA) was done as explained previously [Lee et al., 2004]. In certain cases, a pharmacological PKCδ inhibitor (10 μM rottlerin, Calbiochem, San Diego, CA) was pretreated, 30 min prior to the replating without or with TGFβ1 treatment. Upon replating, TGFβ1 (5 ng/ml, Chemicon) was added directly to the replating media (RPMI-1640 plus 1% BSA) and the treatment lasted for 20 h or indicated periods. In case of PMA treatment, cells replated on Fn for 19 h and 20 min were treated with PMA at the indicated concentration alone or together with TGFβ1, for additional 40 min (Fig. 1A). In addition, PMA alone or plus TGFβ1 was also additionally treated for the indicated periods following the 19-h replating incubation (Fig. 1B–D). Phase contrast images of the cells were taken by using a digital camera-equipped microscopy after the incubation. Cell lysates were prepared as described in the previous studies [Lee and Juliano, 2002; Lee et al., 2004]. The lysates were used in Western blots using phospho-Y^{397}FAK, phospho-Y^{416}c-Src, phospho-S^{3}cofilin, cofilin, PKCδ, c-Src, RhoA (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Y^{118}Paxillin, phospho-S^{643}PKCδ (Cell Signaling Technology, Beverly, MA), FLAG (Sigma), integrins α2, α3 (Chemicon), FAK, paxillin, p130Cas, Nck, or α-tubulin (BD Transduction Laboratories, San Jose, CA). In some cases, the membrane was stripped by incubation in a stripping buffer (62.5 mM Tris pH 6.8, 2% SDS, and 100 mM β-mercaptoenthanol) at 65°C for 30 min, washed for 1 h (3 times × 20 min) with Tris-based saline with 0.05% Tween-20 (TBST), reblocked with TBST containing 1% BSA plus 1% skim milk proteins, and then reprobed with another primary antibody.

Immunofluorescence Microscopy

Cells were first cotransfected with pcDNA3-GFP and C3 exotoxin (pRC/CMV-C3), 24 h before replating on 10 μg/ml Fn-precoated glass coverslips and TGFβ1 treatment and incubation for 20 h at 37°C. Alternatively, SNU16mAd cells were replated on 10 μg/ml Fn-precoated coverslips with or without TGFβ1 treatment, as explained above. In cases, cells were pretreated with 10 μM rottlerin or 12.5 μM GF-109203×, 30 min before the replating. In case of PMA

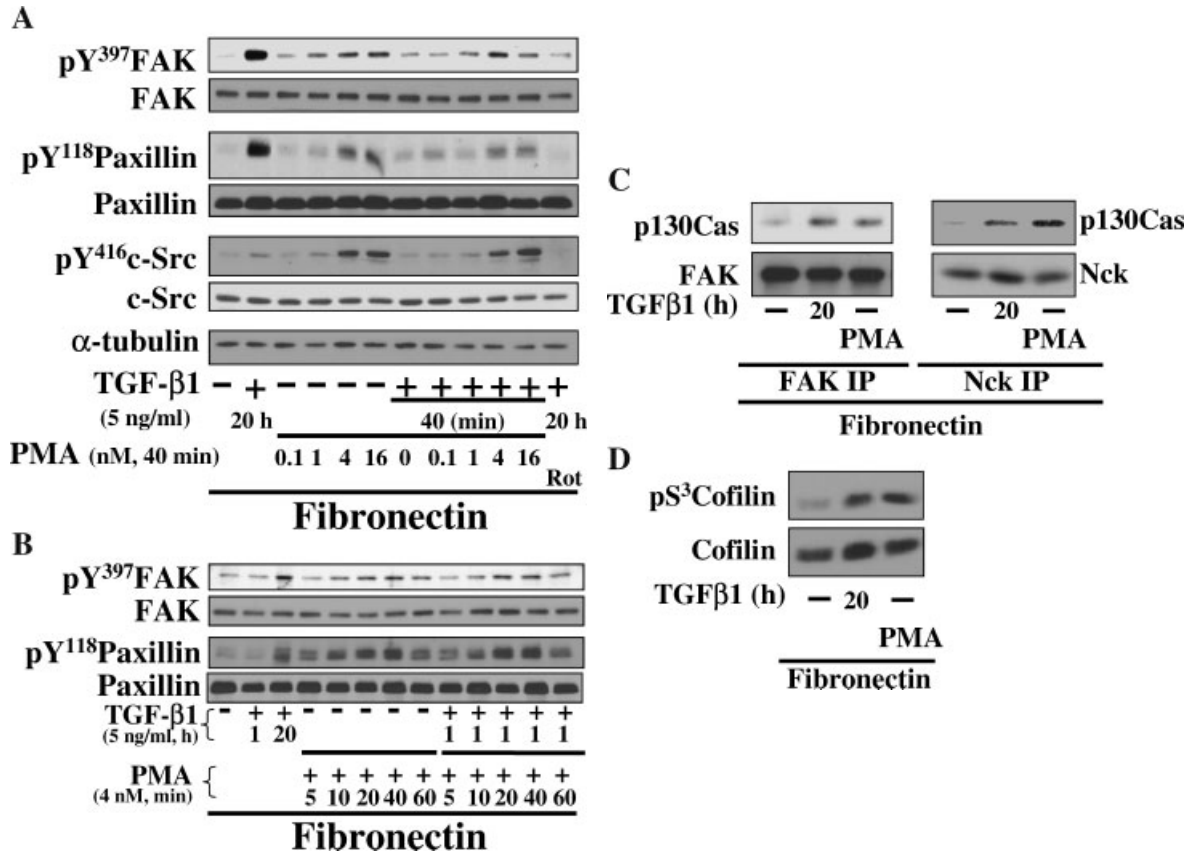

Fig. 1. An acute PMA treatment also regulates integrin signal transduction. **A**: Cells were replated on fibronectin (Fn)-precoated dishes. In cases, the cells were pretreated with rottlerin (10 μM, Rot), 30 min prior to cells replating on Fn and TGFβ1 treatment for 20 h. PMA treatments for 40 min at the indicated concentrations were done in the absence or presence of TGFβ1 treatment for 40 min following 19 h and 20 min TGFβ1-free incubation on Fn. That is, 40-min treatment indicates that TGFβ1 was directly added to the replating media for the cells already replated for 19 h and 20 min on Fn. After the incubations, the whole cell lysates were prepared and proteins at an equal amount were analyzed by the standard Western blots for the indicated molecules. **B**: Cells were replated on Fn-precoated dishes without or with TGFβ1 treatment for 1 h or 20 h. For TGFβ1 treatment for 20 h or 1 h, TGFβ1 was added to the replating media on replating or following 19 h incubation on Fn, respectively. PMA treatment was done at 4 nM for the indicated minutes by adding PMA at the end of the 20-h incubation period without or with TGFβ1 treatment (for the last 1 h). Cell lysates were prepared after the incubations and used in standard Western blots for the indicated molecules. **C**, **D**: PMA at 4 nM was treated for the last 1 h of the total 20-h incubation period on Fn. Data shown are representative of at least three independent experiments. C: Whole cell lysates from cells under the diverse conditions were subjected to immunoprecipitation with mouse monoclonal anti-FAK or Nck antibodies, and the immunoprecipitates were used in immunoblots for the indicated molecules, as explained in Materials and Methods. D: Whole cell lysates from cells under the diverse conditions were subjected to standard Western blots for phospho-S^3cofilin and cofilin.

treatment, cells replated on Fn for 19 h and 20 min were treated with 4 nM PMA for an additional 40 min. Cells were then fixed with 3.7% formaldehyde in PBS for 5 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature, and washed three times with PBS. The cells were then incubated with anti-phospho-Y^{397}FAK antibody for 1 h and washed three times with PBS (3 times × 10 min). Cells were then incubated with anti-rabbit IgG-conjugated FITC (Chemicon) in a dark and humidified chamber for 1 h. In case for actin staining, cells were then stained with phalloidin-conjugated rhodamine (Molecular Probes, Eugene, OR) for 1 h before washing three times with PBS and mounting with a mounting solution (DakoCytomation, Germany). Mounted samples were visualized by a fluorescent microscopy.

Immunoprecipitation

Cells were replated on Fn under diverse conditions as explained above. After the 20-h incubation, cells were washed with cold PBS and immediately lysed with an immunoprecipitation buffer (10 mM Tris-HCl pH 7.4, 150 mM

NaCl, 0.5 mM EGTA, 0.2 mM Na_3VO_4, 0.2 mM PMSF, 1% Triton X-100, and 0.5% NP-40) on ice. The lysates were cleared by a centrifugation at 13,000 rpm for 30 min at 4°C. An equal amount of proteins was mixed with an equal amount of anti-FAK or Nck antibody (500 μg proteins/0.5 μg antibody) and incubated for 2 h or overnight at 4°C with rotation. After addition of 30 μl of 50% slurry of protein A/G sepharose beads (Upstate, New York), an additional incubation for 2 h at 4°C with rotation was done. Immunoprecipitates were collected by a centrifugation at 13,000 rpm for 3 min at 4°C and washed twice with ice-cold lysis buffer and twice with cold PBS. The immunoprecipitates were then eluted with 2 × SDS–PAGE sample buffer, and proteins were analyzed by SDS–PAGE.

RhoA Activity Assay

RhoA activity was measured based on the method described previously [Arthur and Burridge, 2001]. Briefly, cells under diverse conditions were lysed in 1 ml of 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 1 μg/ml aprotinin and 10 μM leupeptin, 1 μM pepstatin A, and 1 mM PMSF. Lysates were cleared at 13,000 rpm for 10 min, and the supernatant (1.0 mg total protein) was rotated for 30 min with 30 μg of GST-RBD [GST fusion protein containing the RhoA-binding domain (amino acids 7–89) of Rhotekin, a kind gift from Dr. Keith Burridge, University of North Carolina, Chapel Hill, NC] bound to glutathione-sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden). Samples were washed three times with a buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, 1% Triton X-100, and protease inhibitors. Then protein complexes were eluted by SDS–PAGE sample buffer and immunoblotted with RhoA monoclonal antibodies. Whole cell lysates were also immunoblotted for RhoA as input controls.

RESULTS

To understand how PMA affects integrin-related signaling, we analyzed effects of PMA on integrin-related signaling pathway in a dose- or time-dependent manner. PMA at different concentrations for 1 h or PMA at 4 nM for 40 min was treated without or with a concomitant TGFβ1 treatment following 19 h or 19 h and 20 min, respectively, incubation on Fn. Collectively, treatment of PMA (e.g., 4 or 16 nM) alone or together with TGFβ1 for 40 min of cells replated on Fn already for 19 h and 20 min could activate FAK and paxillin (Fig. 1). Interestingly, the cotreatment of PMA with TGFβ1 for 40 min or 1 h resulted in the effects as much as PMA-alone treatment. However, the cotreatment appears to result in the enhanced effects, when compared to treatment of TGFβ1 alone for such a short period (Fig. 1B, the 2nd lane vs. the last 4 lanes). The activations appeared to depend on PKCδ activity since they were blocked by PKCδ inhibition via rottlerin treatment (Fig. 1A). The acute PMA treatment also stabilized complex formation of FA molecules including FAK, p130Cas, and Nck (Fig. 1C). In addition, the acute PMA treatment increased phosphorylation of cofilin Ser3, just as TGFβ1 did (Fig. 1D), indicating that the acute treatment of PMA alone could mimic the 20-h TGFβ1 treatment, and that either treatment caused actin polymerization dynamically involved in the cell spreading processes. Furthermore, TGFβ1 treatment for 20 h caused cell spreading on Fn in a PKCδ activity-dependent manner (Fig. 2 upper panel). Being consistent, an acute PMA treatment at 4 nM for 40 min at the end of the 20-h incubation on Fn alone or with TGFβ1 could cause the cell spreading, although treatment of TGFβ1 alone for 40 min did not. (Fig. 2 lower panel). These observations on the signaling activity and cell spreading indicate that the PMA treatment can supplement the short TGFβ1 treatment, leading to effects presumably similar to a long (i.e., 20 h) TGFβ1 treatment. Although such a lower dose of PMA resulted in cell spreading similar to TGFβ1-mediated spreading, treatments at higher than 32 nM for 40 min caused transformation with losing cell boundaries (data not shown).

RhoA small GTPases are responsible for actin reorganization and morphological changes by integrin signaling [Arthur and Burridge, 2001]. Therefore, we examined which GTPase might be involved in the actin organization and morphological change during the TGFβ1- or PMA-mediated cell spreading. First, we analyzed activities of the GTPases under diverse experimental conditions to see their correlation with the TGFβ1- or PMA-mediated cell spreading on Fn. RhoA-GTP levels in cells on Fn were increased by TGFβ1 treatment in a PKCδ activity-dependent manner, whereas Rac1-GTP

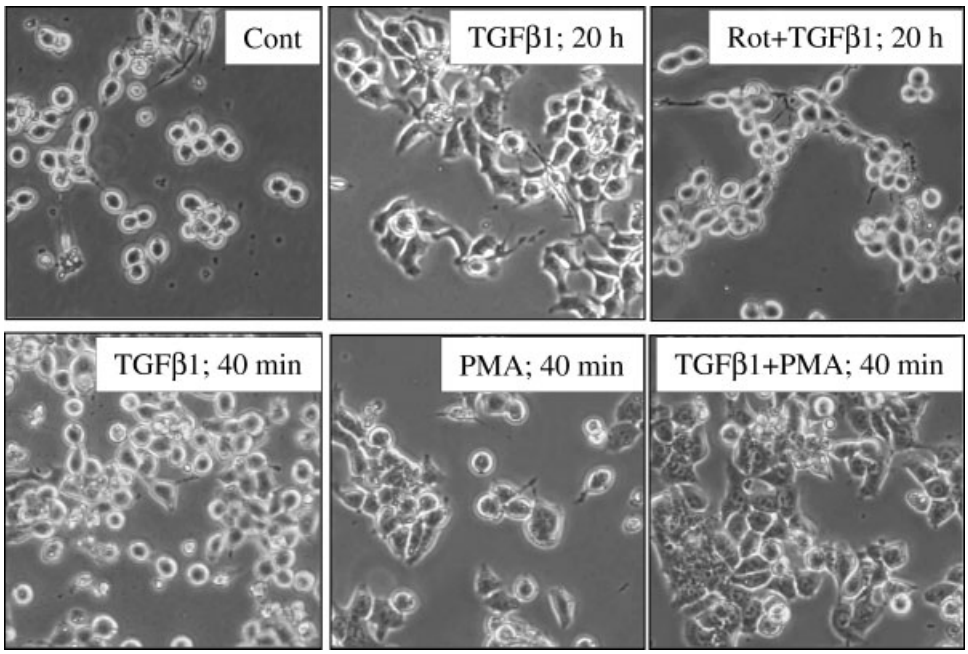

Fig. 2. An acute PMA treatment also leads to the cell spreading on Fn. SNU16mAd cells were replated on Fn-precoated dishes, as explained above, without or with rottlerin (Rot) pretreatment in the absence or presence of TGFβ1 treatment for 20 h (**upper panel**). TGFβ1 treatment for 40 min (left in **lower panel**) was also performed by TGFβ1 addition to cells that had been replated for 19 h and 20 min on Fn. PMA at 4 nM was treated with together or without TGFβ1 to cells which were already replated and incubated for 19 h and 20 min on Fn in the absence of TGFβ1 treatment (lower panel). After the 20-h total incubation, cell images were taken by using a phase-contrast microscopy.

level did not change under any conditions we tested (Fig. 3A and data not shown). Furthermore, cells transfected with a RhoA inhibitor, C3 exotoxin, did not spread nor formed stress fibers even in the presence of TGFβ1 treatment, unlike untransfected neighbor cells (Fig. 3B). These observations indicate an involvement of RhoA signaling in the spreading of SNU16mAd cells. Cells were next stained with phalloidin to visualize actin or immunostained for Tyr397-phosphorylated FAK to visualize FAs. Stress fiber and FA formation were obvious in the TGFβ1-treated cells on Fn, depending on the PKCδ activity (Fig. 3C,D, respectively). Furthermore, an acute PMA treatment also resulted in a certain degree of RhoA activation and the cell spreading as well (Figs. 2, 3A–D). These observations suggest that TGFβ1- or PMA-mediated cell spreading on Fn involves RhoA pathway. Meanwhile, PMA-treated cells showed mostly cortical actin organization, compared to TGFβ1-treated cells with prominent stress fiber formation (Fig. 3C). However, FAs were formed similarly in cells spread by either TGFβ1- or PMA-treatment (Fig. 3D). Therefore, although there are certain different aspects in actin organization in cells treated with either TGFβ1 or PMA, an acute PMA treatment could activate integrin signaling pathway necessary for the actin organization and cell spreading, as a chronic TGFβ1 treatment did.

DISCUSSION

In this study, an acute PMA treatment could activate integrin signal transduction leading to actin rearrangement, FA formation, and finally cell spreading, as a chronic TGFβ1 treatment did. Furthermore, the acute PMA treatment supplemented the integrin signal transduction mediated by a short TGFβ1 treatment, so that a cotreatment of PMA with TGFβ1 for 40 min could lead to the cell spreading, although treatment of TGFβ1 alone for such a short period did not. It was previously reported that a signaling network for cell spreading from Smad-dependent TGFβ1 signaling to integrin-mediated signaling was shown to include induction and activation of PKCδ and presumably PKCδ-independent biochemical events, since PKCδ overexpression alone did not result in the spreading [Lee et al., 2005]. Therefore, this study suggests additional evidences beyond the

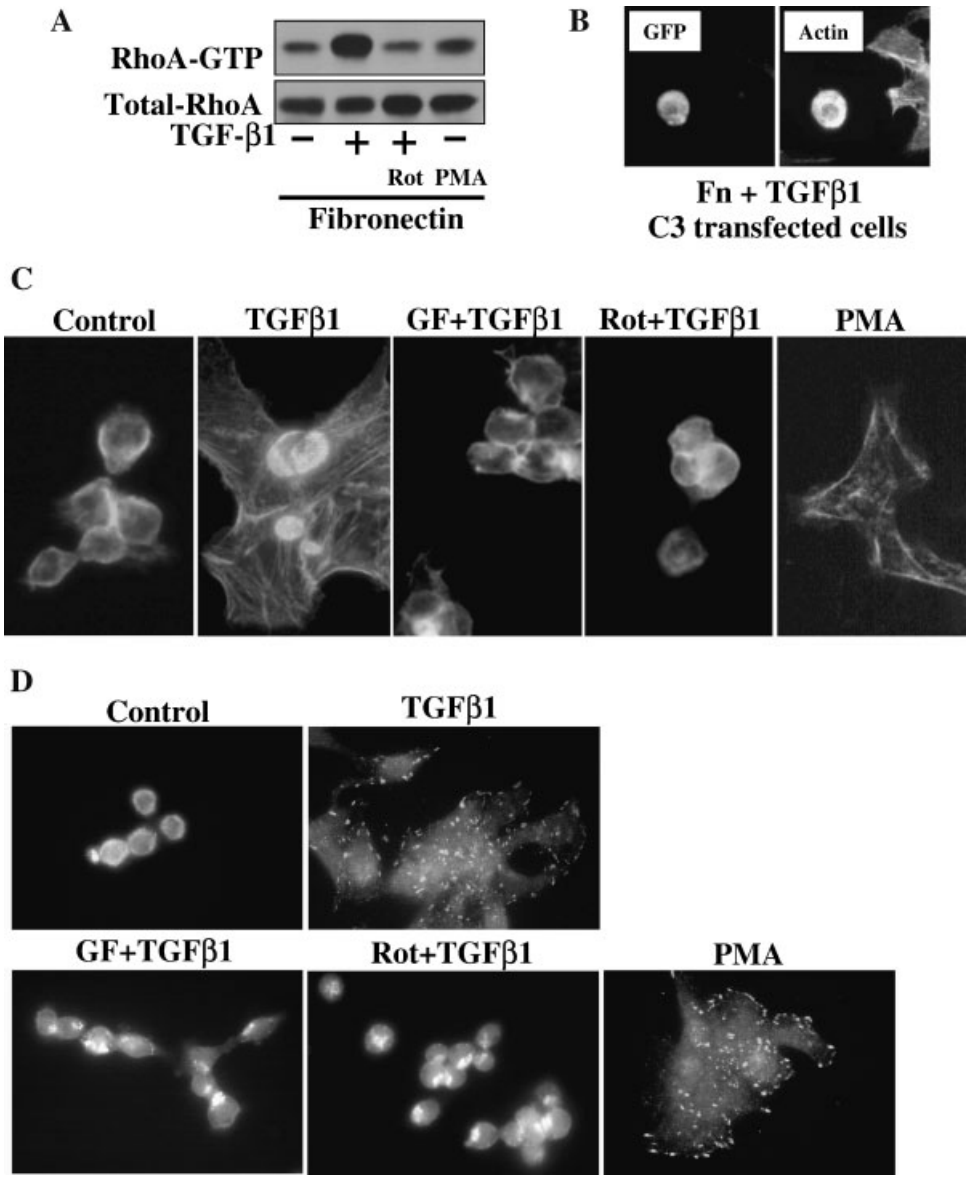

Fig. 3. TGFβ1- or PMA-mediated cell spreading involves RhoA pathway. **A**: Manipulation of cells, preparation of cell lysates, and GTPase pull-down assays were done as explained in Materials and Methods. Data shown represent three independent experiments. **B**: Cells were cotransfected with pRc/CMV-C3 and pcDNA3-GFP constructs. Twenty-four hours later, cells were replated onto Fn-precoated coverslips with a concomitant TGFβ1 treatment and incubated for 20 h before processing for actin staining as explained in Materials and Methods. **C**, **D**: Replating and TGFβ1- or PMA-treatment was done as explained in Materials and Methods. Actin staining with phalloidin-conjugated rhodamine (C) or with anti-pY^{397}FAK and then anti-rabbit IgG-FITC (D) were performed as explained in the Materials and Methods.

previous study by showing that an acute PMA treatment could mimic the effects of chronic TGFβ1 treatment or supplement a short TGFβ1 treatment, leading to the efficient activation of the FA molecules and spreading of SNU16-mAd cells, and that both the PMA- and TGFβ1-mediated spreading involved RhoA signaling. As shown in the previous report that the TGFβ1- and integrin-mediated spreading of the gastric carcinoma cells resulted in more spindle-typed morphology and increased migration [Lee et al., 2005], such a PMA-mediated spreading may also lead to regulation of migration through RhoA signaling and actin organization, like the TGFβ1- (and integrin-) mediated spreading.

Evidences were being accumulated that TGFβ1 activates diverse intracellular signaling molecules that are also regulated by integrin-mediated cell adhesion [Massague, 2000;

Derynck and Zhang, 2003]. TGFβ1-mediated Smad signaling was known to be downregulated by PKC, through PKC-mediated phosphorylation of MH1 domain of Smad3 [Yakymovych et al., 2001]. However, it is also reported that TGFβ1 induced growth arrest via an activation of PKCα [Sakaguchi et al., 2004]. Therefore, the regulatory relationship between TGFβ1 and PKC pathways may be differential depending on the systems. However, it was found in this study that the TGFβ1 effects could be blocked by PKCδ inhibition and mimicked by an acute PMA treatment. An acute treatment of PMA at a lower dose (less than 16 nM) for 40 min could lead to spreading of the SNU16mAd gastric carcinoma cells. Furthermore, the PMA treatment also caused increased phosphorylation (i.e., activation) and complex formation of the FA molecules and activation of RhoA. Because PKCδ (but not α, θ, etc. [Lee et al., 2005]) was shown to be involved in the spreading system, the effects by PMA might include activation of PKCδ although we cannot rule out the possibility of the general PMA effects on PKC isoforms which may be involved in the spreading upon PMA treatment. Furthermore, the observation that the cotreatment of TGFβ1 with PMA resulted in comparable phosphorylations of the FA molecules, compared to PMA-alone treatment, but enhanced phosphorylations, compared with TGFβ1 alone treatment for 1 h. Therefore, this may indicate that activation of PKC(δ) plays major roles downstream of TGFβ1 in the spreading. Since PKC(δ) is a Ser/Thr kinase [Carter, 2000] and integrin-activated signaling molecules are phosphorylated at Tyr residues and activated during cell adhesion/spreading and migration [Hynes, 2002; Lee and Juliano, 2004], it may be likely that the TGFβ1 and/or PMA-mediated PKC(δ) activity may regulate the phosphorylation and complex formation of the FA molecules indirectly through other mediator(s). Alternatively, as shown in the previous reports [Lee et al., 2005], the activity of PKC(δ) could increase the expression of integrin and/or ECM molecules that lead to activation of the integrin-mediated intracellular signaling molecules, such as FA molecules and RhoA.

On the other hand, there are a few differences between the TGFβ1- and the PMA-induced cell spreading on Fn. That is, cell spreading by TGFβ1 treatment required new protein synthesis of PKCδ and integrins α2 and α3, whereas the PMA-induced cell spreading would not, just as expected from the difference in treatment duration. Therefore, probably PMA-mediated activation and complex stabilization of the FA molecules might be sufficient for the spreading although there were no enhancements of PKCδ and integrin expression levels during PMA-mediated spreading. In addition, the effects of TGFβ1 or PMA on RhoA pathway were different: (1) RhoA-GTPases level was a little higher preferentially by TGFβ1 treatment, compared to that by the PMA treatment at a dose that showed the most efficient spreading, and (2) stress fiber formation was more prominent by the TGFβ1 treatment, whereas cortical actin arrangement was by the PMA treatment, although both the treatments caused similar FA formation. Therefore, we can suggest that mechanisms of the PMA-induced cell spreading may be partially different from those of the TGFβ1-mediated spreading, although both the stimuli commonly caused activation of the FA molecules and RhoA.

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