

**FIGURE 1. Up-regulation of TRPC6 by  $G\alpha_{13}$  activation.** *A*, RT-PCR analysis of RNA expression of TRPC channels in LacZ- and CA  $G\alpha_{13}$ -expressing cardiac fibroblasts. *Bottom*, real time RT-PCR analysis of RNA expression of TRPC6. *B*, expression of TRPC6 protein in LacZ-expressing and the respective  $G\alpha$ -overexpressing cells. Cells were treated with forskolin (50  $\mu$ M) for 24 h. *C*, effects of TRPC6 siRNAs (C6-1609 and C6-1786) on TRPC6 protein expression. *D*, effects of TRPC6 siRNAs on the basal  $Ca^{2+}$  influx activity in CA  $G\alpha_{13}$ -expressing cells. Four min after  $Ca^{2+}$  measurement in  $Ca^{2+}$ -free solution, basal  $Ca^{2+}$  influx was determined by the addition of 2 mM  $Ca^{2+}$ . *E*, the amplitude of maximum [ $Ca^{2+}$ ]<sub>i</sub> rises ( $\Delta$ Ratio) induced by the addition of 2 mM  $Ca^{2+}$  was calculated. *F*, effects of TRPC6 siRNAs on CA  $G\alpha_{13}$ -induced increase in NFAT activity. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  versus control of LacZ-expressing cells. #,  $p < 0.05$ ; ##,  $p < 0.01$  versus control of CA  $G\alpha_{13}$ -expressing cells.

viously (18). Briefly, total RNA (150 ng) was subjected to real time RT-PCR. Oligonucleotide primers and fluorescence-labeled TaqMan<sup>®</sup> probes were designed with Primer Express software (Applied Biosystems, Foster City, CA). Primers and probe were as follows: for rat TRPC6 mRNA, forward primer (5'-AGCAG-CAGCTCCTCTCCATATG-3'), reverse primer (5'-GAGGAC-CACGAGGAATTCAC-3'), and TaqMan<sup>®</sup> probe (5'-TAT-GAGAACCTCTCTGGTTTACGGCAGCA-3'); for collagen type I, forward primer (5'-GGAGAGTACTGGATCGACCCTA-AC-3'), reverse primer (5'-CTGACCTGTCTCCATGTTGCA-3'), and TaqMan<sup>®</sup> probe (5'-AAGGCTGCAACCTGGATGCC-ATCAA-3'); for collagen type III, forward primer (5'-CCTGCT-TCACCCCTCTTATTT-3'), reverse primer (5'-TCCCAG-TCGCAGACACATAT-3'), and TaqMan<sup>®</sup> probe (5'-TAGAGA-TGCTGGAAGCCAGAACCATGTCA-3'). All reactions were performed in TaqMan<sup>®</sup> One-Step RT-PCR Master Mix Reagents (Applied Biosystems) and the Applied Biosystems 7500 real time PCR system. The rat glyceraldehyde-3-phosphate dehydrogenase mRNA (Applied Biosystems (catalog number 4308313)) was used

as an internal control to normalize the differences in the amount of total RNA in each sample.

**Western Blot Analysis**—Cardiac fibroblasts ( $3 \times 10^5$  cells) plated on 6-well dishes were directly harvested with 200  $\mu$ l of 2 $\times$  SDS sample buffer. The protein samples (20  $\mu$ l) were fractionated by 8% SDS-polyacrylamide gel and then transferred onto polyvinylidene difluoride membrane. The expression of endogenous TRPC proteins was assessed by Western blotting using anti-TRPC antibodies (12, 27). The Src and ERK activities were assessed by using anti-phospho-specific antibodies. The JNK activity was assessed as described previously (23–25). For  $\alpha$ -SMA expression, cells were harvested with lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Igepal CA-630, and protease inhibitor mixture (6). The protein samples were fractionated by 10% SDS-PAGE.

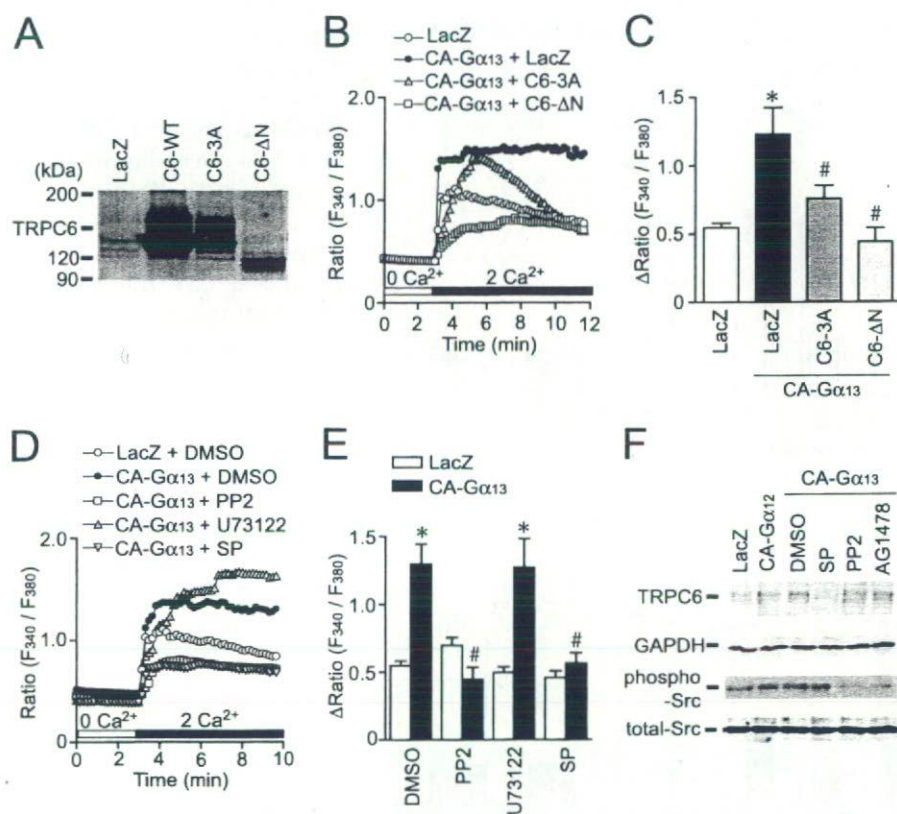
**Measurement of Rac Activity**—Activation of small G proteins was determined by the method of Nishida *et al.* (25). Activated Rac was pulled down with 10  $\mu$ g of glutathione *S*-transferase-fused Rac-interacting domain of p21-activated kinase (PAK-CRIB). Pulled down small G proteins were detected with anti-Rac1 antibody.

**Measurement of Cardiac Myofibroblast Formation**—Transformation of cardiac fibroblasts to myo-

fibroblasts was assessed by the expression of  $\alpha$ -SMA and production of ECM components (6). Briefly, 24 h after infection, cardiomyocytes were stimulated with ET-1 (100 nM) for 48 h. The cells were washed, fixed, incubated with anti- $\alpha$ -SMA antibody, and then stained with Alexa Fluor 546 goat anti-mouse IgG. Morphological changes and  $\alpha$ -SMA expression of cardiac fibroblasts were visualized with confocal microscopy. For the measurement of collagen synthesis, cells were harvested with lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Igepal CA-630, and protease inhibitor mixture (Nacalai, Japan). Production of ECM components was assessed by [<sup>3</sup>H]proline incorporation. After cells were stimulated with ET-1 (100 nM) for 2 h, [<sup>3</sup>H]proline (1  $\mu$ Ci/ml) was added to the culture medium and further incubated for 6 h. The incorporated [<sup>3</sup>H]proline was extracted by 1 N NaOH overnight and measured using a liquid scintillation counter.

**Measurement of Hypertrophic Responses of Cardiomyocytes**—Preparation of rat neonatal cardiomyocytes and measurement of hypertrophic responses were performed as described (12).

## Inhibition of Myofibroblast Formation by TRPC6-NFAT Signaling



**FIGURE 2. Involvement of tyrosine kinase in CA  $G\alpha_{13}$ -induced increase in basal  $Ca^{2+}$  influx activity in cardiac fibroblasts.** *A*, expression of wild type TRPC6 (WT) and DN TRPC6 (C6-3A and C6- $\Delta$ N) proteins in cardiac fibroblasts. One-fourth volume of protein samples (5  $\mu$ l) was subjected to 7% SDS-PAGE. The expression level of endogenous TRPC6 was low, and expression levels of wild type and mutated TRPC6 were very high. Therefore, the band intensity of endogenous TRPC6 is weak under these conditions. *B* and *C*, effects of DN TRPC6 proteins on the CA  $G\alpha_{13}$ -induced increase in basal  $Ca^{2+}$  influx activity. *B*, average time courses of  $Ca^{2+}$  responses induced by the addition of 2 mM  $Ca^{2+}$ . *C*, the amplitude of sustained  $[Ca^{2+}]_i$  increase 4 min after the addition of 2 mM  $Ca^{2+}$ . *D* and *E*, effects of PP2, SP600125 (SP), and U73122 on the CA  $G\alpha_{13}$ -induced increase in basal  $Ca^{2+}$  influx activity. Cells were treated with PP2 (1  $\mu$ M) or U73122 (1  $\mu$ M) for 20 min and SP600125 (1  $\mu$ M) for 48 h prior to  $[Ca^{2+}]_i$  measurement. *D*, average time courses of  $Ca^{2+}$  responses. *E*, peak changes in  $[Ca^{2+}]_i$  induced by the addition of 2 mM  $Ca^{2+}$ . *F*, effects of SP600125, PP2, and AG1478 on CA  $G\alpha_{13}$ -induced increase in TRPC6 protein expression and Src phosphorylation. Cells were treated with SP600125 (1  $\mu$ M) for 48 h and with PP2 (1  $\mu$ M) or AG1478 (1  $\mu$ M) for 30 min prior to cell harvest. \*,  $p < 0.05$  versus LacZ-expressing cells. #,  $p < 0.05$  versus control (LacZ or Me<sub>2</sub>SO (DMSO)) of CA  $G\alpha_{13}$ -expressing cells.

Briefly, 48 h after siRNA treatment, cardiomyocytes were stimulated with ET-1 (100 nM) for 24 h. The cells were stained with Alexa Fluor 594-phalloidin to visualize actin filament. Protein synthesis was measured by [<sup>3</sup>H]leucine incorporation. After cells were stimulated with ET-1 (100 nM) for 2 h, [<sup>3</sup>H]leucine (1  $\mu$ Ci/ml) was added and further incubated for 6 h.

**Statistical Analysis**—The results are presented as mean  $\pm$  S.E. The data were accumulated under each condition from at least three independent experiments. For the measurements of  $[Ca^{2+}]_i$ , representative data of time course experiments were plotted from one of three similar experiments that were performed with more than 30 cells. Mean values were compared with control by Student's *t* test.

## RESULTS

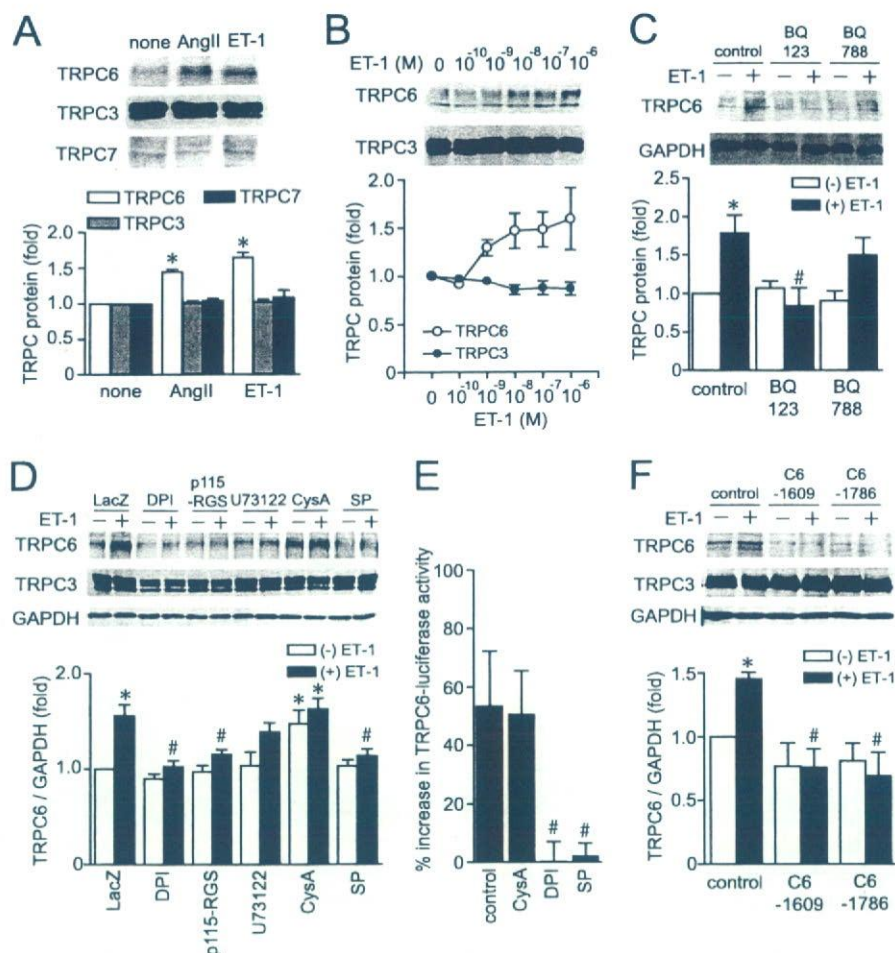
**Up-regulation of TRPC6 Proteins by  $G\alpha_{13}$  Activation**—We previously demonstrated that the expression of GTPase-defective mutants of  $G\alpha_{12}$  (Q229L; CA  $G\alpha_{12}$ ) and  $G\alpha_{13}$  (Q226L; CA  $G\alpha_{13}$ ) increases  $[Ca^{2+}]_i$  and NFAT activity through an SK&F96365-sensitive  $Ca^{2+}$  influx pathway (17, 18). TRPC chan-

nels are thought to be molecular candidates for RACCs. Thus, we examined which TRPC channels are expressed in rat cardiac fibroblasts. Among TRPC1 to -7 channels, TRPC1, TRPC3, TRPC6, and TRPC7 mRNAs were detected (Fig. 1A). Interestingly, only TRPC6 mRNA expression was increased 2.5-fold by the expression of CA  $G\alpha_{13}$ . The expression of TRPC6 proteins was also increased by CA  $G\alpha_{12}$  (Fig. 1B). Expression of CA  $G\alpha_q$  (R183C) and WT  $G\alpha_{12}$  did not increase TRPC6 protein levels, although both mutants significantly increased ERK activity (supplemental Fig. 1). Treatment with forskolin (50  $\mu$ M) rather decreased basal expression of TRPC6 proteins. These results indicate that  $G\alpha_{12/13}$  specifically up-regulate TRPC6 proteins in cardiac fibroblasts. In order to examine whether up-regulation of TRPC6 participates in CA  $G\alpha_{12/13}$ -induced enhancement of  $Ca^{2+}$  responses, knockdown experiments of TRPC6 proteins were performed by using TRPC6 siRNAs (12). Since the extent of NFAT activation induced by CA  $G\alpha_{13}$  was larger than that by CA  $G\alpha_{12}$ , CA  $G\alpha_{13}$ -expressing cells were used to examine the involvement of TRPC6 in  $G\alpha_{12/13}$ -mediated  $Ca^{2+}$  responses. Treatment with TRPC6 siRNAs completely suppressed CA  $G\alpha_{13}$ -induced up-regulation of TRPC6 protein expression

(Fig. 1C). We confirmed that TRPC6 siRNAs did not affect the expression levels of TRPC3 and TRPC7 proteins (data not shown). The basal  $Ca^{2+}$  influx activity was determined by the increase in  $[Ca^{2+}]_i$  induced by the addition of 2 mM extracellular  $Ca^{2+}$  to cells in  $Ca^{2+}$ -free Tyrode's solution (18). This basal  $Ca^{2+}$  influx activity was enhanced by the expression of CA  $G\alpha_{13}$  and CA  $G\alpha_{12}$  (Fig. 1D). The CA  $G\alpha_{13}$ -induced increase in  $[Ca^{2+}]_i$  was completely suppressed by the treatment with TRPC6 siRNAs (Fig. 1, C–E). The sustained increase in  $[Ca^{2+}]_i$  activated NFAT activity, and the treatment with TRPC6 siRNAs inhibited CA  $G\alpha_{13}$ -induced NFAT activation (Fig. 1F). These results suggest that up-regulation of TRPC6 by  $G\alpha_{13}$  activation enhances  $Ca^{2+}$  influx and NFAT activation in cardiac fibroblasts.

**Involvement of Tyrosine Kinase in TRPC6-mediated  $Ca^{2+}$  Influx by  $G\alpha_{12/13}$  Activation**—We next examined whether the channel activity of TRPC6 proteins is actually enhanced by  $G\alpha_{13}$  activation. WT TRPC6 or two DN mutants of TRPC6 (C6-3A and C6- $\Delta$ N) were overexpressed in cardiac fibroblasts (Fig. 2A). The expression of DN TRPC6 significantly sup-

## Inhibition of Myfibroblast Formation by TRPC6-NFAT Signaling



**FIGURE 3. ET-1 up-regulates TRPC6 through the  $G_{\alpha_{13}}$ -ROS-JNK-dependent pathway.** *A*, changes in expression levels of TRPC3, TRPC6, and TRPC7 proteins by the treatment with Ang II (100 nM) and ET-1 (100 nM) for 48 h. *B*, concentration-dependent increase in TRPC6 protein expression by ET-1 treatment. *C*, effects of ET receptor antagonists on ET-1-induced increase in TRPC6 expression. Cells were treated with BQ123 (3  $\mu$ M) or BQ788 (3  $\mu$ M) for 5 min prior to ET-1 stimulation. *D*, involvement of  $G_{\alpha_{12/13}}$ , ROS, and JNK in the ET-1-induced increase in TRPC6 expression. Twenty-four h after infection with LacZ or p115-RGS, cells were treated with ET-1 (100 nM) for 48 h. Cells were treated with DPI (1  $\mu$ M), U73122 (1  $\mu$ M), CysA (0.5  $\mu$ g/ml), and SP600125 (SP; 1  $\mu$ M) for 20 min prior to ET-1 stimulation. *E*, effects of CysA, DPI, and SP600125 on ET-1-induced increase in the TRPC6-luciferase activity. Cells were treated with ET-1 (100 nM) for 6 h. *F*, effects of TRPC6 siRNAs on TRPC3 and TRPC6 protein expressions. \*,  $p < 0.05$  versus nontreatment (no ET-1) of control or LacZ-expressing cells. #,  $p < 0.05$  versus ET-1 treatment of control or LacZ-expressing cells.

pressed the sustained  $[Ca^{2+}]_i$  increase of CA  $G_{\alpha_{13}}$ -expressing cells induced by the addition of 2 mM extracellular  $Ca^{2+}$  (Fig. 2, *B* and *C*), indicating that the increase in  $[Ca^{2+}]_i$  of CA  $G_{\alpha_{13}}$ -expressing cells is enhanced by  $Ca^{2+}$  influx through TRPC6 channels. It has been reported that TRPC6 activity is regulated by DAG (28) and tyrosine phosphorylation (29), and activated  $G_{\alpha_{13}}$  induces activation of tyrosine kinase (30, 31) and PLC (32). We examined the involvement of TRPC6 in CA  $G_{\alpha_{13}}$ -induced  $[Ca^{2+}]_i$  increase by determining the requirement of tyrosine phosphorylation or PLC for TRPC6 activation. Treatment with PP2, a Src tyrosine kinase inhibitor, and SP600125, a JNK inhibitor, but not U73122, a PLC inhibitor, significantly suppressed the increase in  $[Ca^{2+}]_i$  of CA  $G_{\alpha_{13}}$ -expressing cells induced by the addition of 2 mM  $Ca^{2+}$  (Fig. 2, *D* and *E*). Consistent with the involvement of Src, the expression of CA  $G_{\alpha_{12}}$  and CA  $G_{\alpha_{13}}$  increased Src activity about 2.5-fold (Fig. 2*F*). CA  $G_{\alpha_{13}}$ -induced Src activation was suppressed by treatment with AG1478, an EGFR kinase inhibitor (Fig. 2*F*), suggesting that

EGFR kinase mediates the CA  $G_{\alpha_{13}}$ -induced Src activation. Treatment with SP600125, but not PP2 and AG1478, suppressed CA  $G_{\alpha_{13}}$ -induced up-regulation of TRPC6 protein, whereas treatment with PP2 and AG1478, but not SP600125, suppressed CA  $G_{\alpha_{13}}$ -induced Src activation (Fig. 2*F*). These results suggest that EGFR- and PP2-sensitive tyrosine kinase(s) participate in  $G_{\alpha_{13}}$ -mediated enhancement of  $Ca^{2+}$  influx independently of TRPC6 up-regulation. These findings are consistent with the report that EGFR participates in  $G_{\alpha_{13}}$ -mediated responses (33).

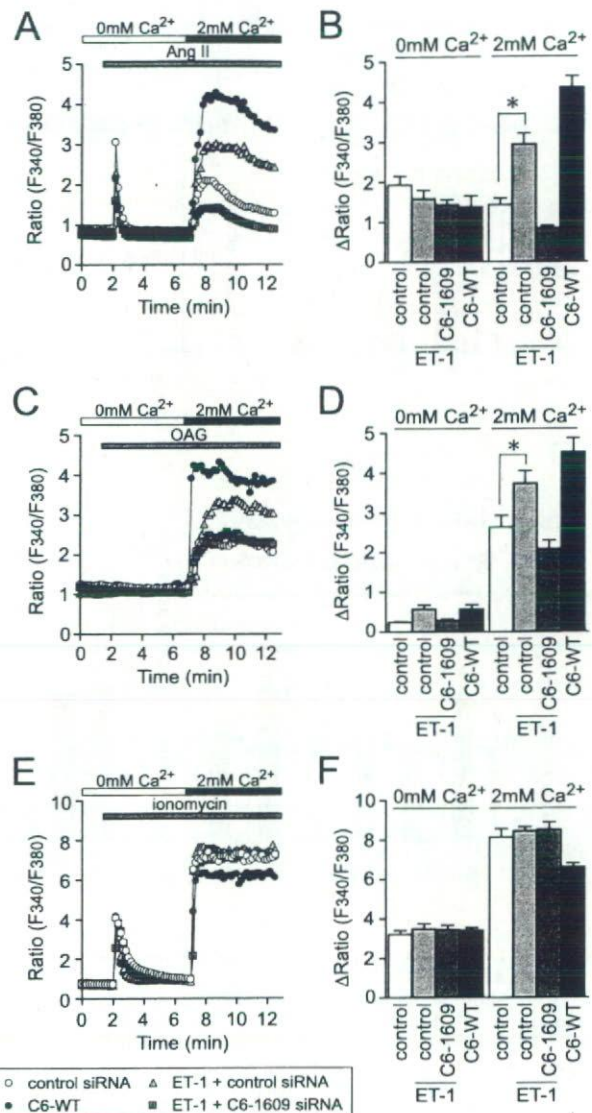
**Up-regulation of TRPC6 Proteins by ET-1 and Ang II Stimulation**—We next examined whether up-regulation of TRPC6 is actually induced by  $G_{13}$ -coupled receptor stimulation. We have previously demonstrated that  $G_{\alpha_{12/13}}$  mediate Ang II- and ET-1-induced activation of JNK and hypertrophic responses in rat neonatal cardiomyocytes (24, 25). The TRPC6 protein expression levels were significantly increased by Ang II treatment and more potently by ET-1 treatment, in a concentration-dependent manner (Fig. 3, *A* and *B*). Treatment with ET-1 actually increased TRPC6 mRNA levels (supplemental Fig. 2*A*). The up-regulation of TRPC6 protein was detected at 6 h after ET-1 stimulation and the expression of TRPC6 proteins reached a peak at 48 h (data

not shown). In contrast to TRPC6, Ang II and ET-1 treatment did not affect the expression levels of TRPC3 and TRPC7 proteins (Fig. 3*A*). These results indicate that ET-1 treatment selectively up-regulates TRPC6 proteins in cardiac fibroblasts. Since the ET-1-induced increase in TRPC6 protein expression levels was completely suppressed by BQ123, a selective ET<sub>A</sub> receptor antagonist, but not by BQ788, a selective ET<sub>B</sub> receptor antagonist (Fig. 3*C*), the ET<sub>A</sub> receptor predominantly mediates ET-1-induced TRPC6 up-regulation. It has been reported that the activation of JNK and STAT3 is required for up-regulation of TRPC6 proteins induced by ET-1 and PDGF stimulation in pulmonary vascular smooth muscle cells (34, 35). We previously demonstrated that  $G_{\alpha_{12/13}}$  mediate Ang II-induced Rac activation, ROS production, and JNK activation in cardiac myocytes and fibroblasts (17, 25). Thus, we examined whether ROS and JNK are involved in ET-1-induced TRPC6 up-regulation. Expression of p115-RGS, a selective inhibitory polypeptide of  $G_{\alpha_{12/13}}$ , completely suppressed ET-1-induced increase in

## Inhibition of Myofibroblast Formation by TRPC6-NFAT Signaling

TRPC6 proteins, as expected (Fig. 3D). The ET-1-induced TRPC6 up-regulation was also suppressed by DPI and SP600125 but not by a PLC inhibitor, U73122, and CysA. Since DPI is a selective inhibitor of ROS production by NADPH oxidase complex and SP600125 selectively inhibits JNK activation, these results suggest that the  $G_{\alpha_{12/13}}$ -ROS-JNK pathway participates in ET-1-induced TRPC6 up-regulation. NFAT is activated through its dephosphorylation by calcineurin that is inhibited by CysA. Since the treatment with CysA increased TRPC6 mRNA levels and the expression of CA NFAT decreased TRPC6 mRNA levels (supplemental Fig. 2B), NFAT may negatively regulate ET-1-induced TRPC6 expression in cardiac fibroblasts. Furthermore, the ET-1-induced increase in TRPC6 promoter-dependent luciferase activity was completely suppressed by DPI and SP600125 but not by CysA (Fig. 3E). These results suggest that the  $G_{\alpha_{12/13}}$ -ROS-JNK pathway participates in the transcriptional activation of the TRPC6 gene. ET-1-stimulated up-regulation of TRPC6 protein was completely suppressed by siRNAs against TRPC6 (Fig. 3F), suggesting the utility of these siRNAs for future analysis.

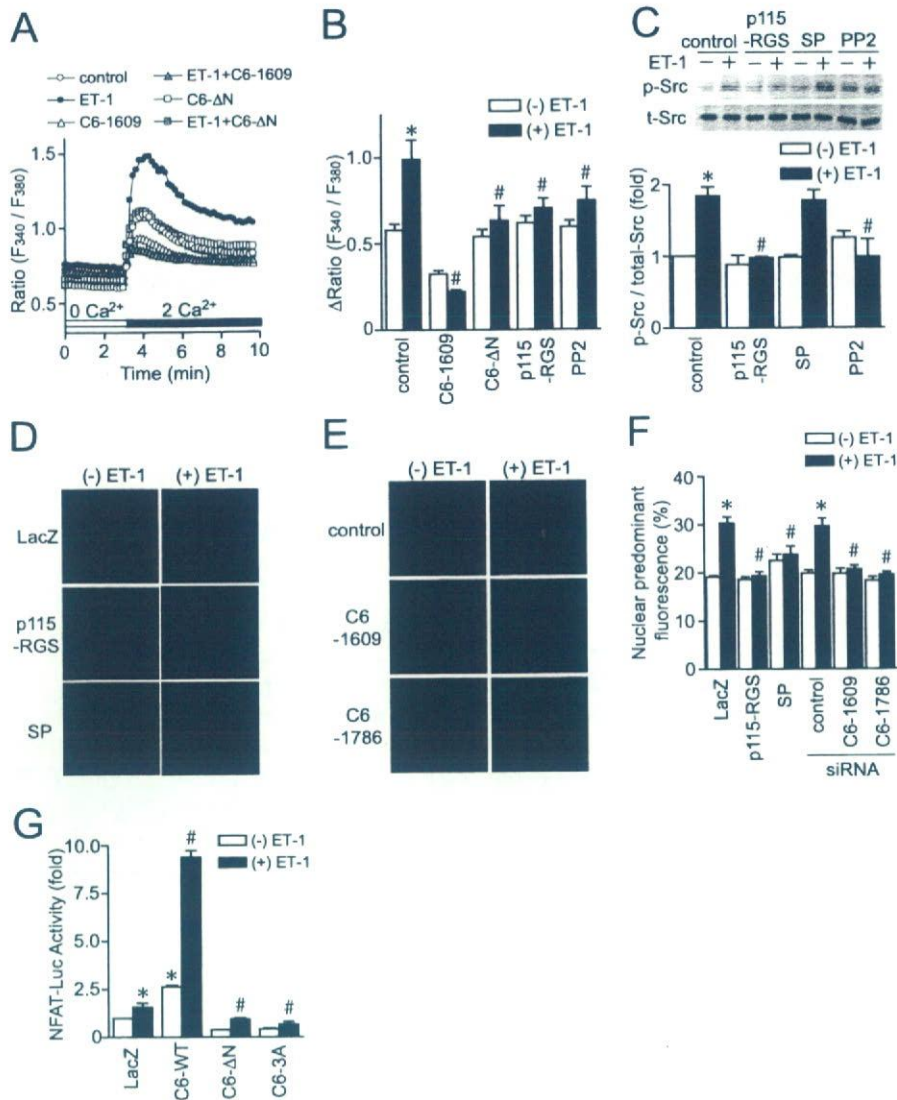
**Up-regulation of TRPC6 Enhances Receptor-activated  $Ca^{2+}$  Responses but Not Store-operated  $Ca^{2+}$  Responses**—TRPC proteins have been emerged as a candidate subunit of RACCs and SOCs (19). To examine whether up-regulation of TRPC6 enhances receptor-activated or store-operated  $Ca^{2+}$  entry, we have individually determined store-operated and receptor-activated  $Ca^{2+}$  entry. The increase in  $[Ca^{2+}]_i$  through receptor-activated  $Ca^{2+}$  entry is determined by the addition of  $Ca^{2+}$  after receptor stimulation in the absence of extracellular  $Ca^{2+}$ . The increase in  $[Ca^{2+}]_i$  through store-operated  $Ca^{2+}$  entry is determined by the addition of  $Ca^{2+}$  after the treatment of cells with  $Ca^{2+}$  ionophore ionomycin in the absence of extracellular  $Ca^{2+}$ . Since the cells were treated with ET-1 for long period of time, we used Ang II as a receptor stimulant to avoid the underestimation of ET-1-stimulated increase in  $[Ca^{2+}]_i$  through receptor-activated  $Ca^{2+}$  entry due to desensitization. The expression of TRPC6 did not affect the Ang II-induced increase in  $[Ca^{2+}]_i$  in the absence of extracellular  $Ca^{2+}$  but enhanced the increase in  $[Ca^{2+}]_i$  by the addition of extracellular  $Ca^{2+}$  (Fig. 4, A and B). The treatment with TRPC6 siRNA (C6-1609) did not affect the initial increase in  $[Ca^{2+}]_i$  of ET-1-treated cells (Fig. 4, A and B). However, TRPC6 siRNA (C6-1609) completely inhibited the increase in  $[Ca^{2+}]_i$  induced by the addition of  $Ca^{2+}$ . These results are consistent with the findings that the prolonged treatment of cells with ET-1 increases TRPC6 expression. It has been known that TRPC6-mediated  $Ca^{2+}$  influx is enhanced by DAG derivative OAG (19). We examined whether OAG-stimulated increases in  $[Ca^{2+}]_i$  are suppressed by TRPC6 siRNA. Stimulation with OAG enhanced the increase in  $[Ca^{2+}]_i$  induced by the addition of extracellular  $Ca^{2+}$  of WT TRPC6-expressing or ET-1-treated cells (Fig. 4, C and D). TRPC6 siRNA (C6-1609) completely suppressed the OAG-induced increase in  $[Ca^{2+}]_i$  of WT TRPC6-expressing or ET-1-treated cells. Although compensatory up-regulation of TRPC3 mRNA expression is reported in TRPC6-deficient mice (36), TRPC6 siRNAs did not affect TRPC3 protein expression (Fig. 3F). These results suggest that up-regulation of TRPC6 enhances receptor-activated  $Ca^{2+}$  entry through a DAG-de-



**FIGURE 4. Up-regulation of TRPC6 enhances  $Ca^{2+}$  responses induced by  $Ca^{2+}$  entry upon receptor stimulation.** A, average time courses of  $Ca^{2+}$  responses upon Ang receptor stimulation with Ang II (100 nM).  $Ca^{2+}$  release was first evoked in  $Ca^{2+}$ -free solution, and  $Ca^{2+}$  entry-mediated  $Ca^{2+}$  responses were induced by the addition of 2 mM  $Ca^{2+}$ . Forty-eight h after the transfection with siRNA (control or C6-1609), cells were treated with ET-1 (100 nM) for 48 h, and then  $[Ca^{2+}]_i$  levels were measured. B, peak Ang II-induced increase in  $[Ca^{2+}]_i$  in  $Ca^{2+}$ -free solution and after the addition of  $Ca^{2+}$ . C, average time courses of  $Ca^{2+}$  responses induced by OAG (25 μM). D, peak OAG-induced  $[Ca^{2+}]_i$  increase observed after the addition of  $Ca^{2+}$  to  $Ca^{2+}$ -free solution. E, average time courses of  $Ca^{2+}$  responses induced by ionomycin (1 μM). F, peak ionomycin-induced  $[Ca^{2+}]_i$  increase observed after the addition of  $Ca^{2+}$  to  $Ca^{2+}$ -free solution. \*,  $p < 0.05$  versus nontreatment (no ET-1) of control cells.

pendent mechanism. In contrast to the involvement of TRPC6 in receptor-activated  $Ca^{2+}$  entry, overexpression of TRPC6 did not affect  $Ca^{2+}$  entry-dependent increase in  $[Ca^{2+}]_i$  induced by ionomycin treatment that completely depletes intracellular  $Ca^{2+}$  stores (27) (Fig. 4, E and F). Up-regulation of TRPC6 by ET-1 treatment did not affect the increase in  $[Ca^{2+}]_i$  induced by  $Ca^{2+}$  entry after ionomycin treatment. These results suggest that the increased expression of TRPC6 does not contribute to the increase in  $[Ca^{2+}]_i$  through SOC-mediated  $Ca^{2+}$  entry in cardiac fibroblasts. Since the Ang II-induced  $Ca^{2+}$  release and

## Inhibition of Myofibroblast Formation by TRPC6-NFAT Signaling



**FIGURE 5. Involvement of TRPC6 up-regulation in ET-1-induced sustained NFAT activation.** *A*, effects of TRPC6 siRNA and DN TRPC6 (C6-ΔN) on the basal  $Ca^{2+}$  influx activity in ET-1-treated cells. Twenty-four h after the infection with adenovirus coding C6-ΔN or the transfection with siRNA (C6-1609), cells were treated with ET-1 (100 nM) for 48 h, and then basal  $Ca^{2+}$  influx activity was determined. *B*, peak amplitudes of  $[Ca^{2+}]_i$  increase, induced by the addition of 2 mM  $Ca^{2+}$ . Cells were first treated with ET-1 for 48 h and then treated with SP600125 (SP; 1  $\mu$ M) or PP2 (1  $\mu$ M) for 20 min prior to  $[Ca^{2+}]_i$  measurement. *C*, effects of p115-RGS, PP2, and SP600125 on ET-1-induced Src phosphorylation. Cells were first treated with PP2 and SP600125 for 20 min and then treated with ET-1 for 10 min. *D*, effects of p115-RGS and SP600125 on ET-1-induced nuclear localization of GFP-NFAT4 proteins. *E*, effects of TRPC6 siRNAs on ET-1-induced NFAT translocation. Twenty-four h after the transfection with siRNAs, cells were treated with ET-1 for 48 h, and the localization of NFAT was determined with confocal microscopy. *F*, quantification of nuclear predominant fluorescence of GFP-NFAT4. *G*, effects of TRPC6 WT, C6-ΔN, and C6-3A on the ET-1-induced increase in NFAT-luciferase activity. Twenty-four h after infection, cells were treated with ET-1 for 48 h. \*,  $p < 0.05$  versus nontreatment (no ET-1) of control or LacZ-expressing cells. #,  $p < 0.05$  versus ET-1 treatment of control or LacZ-expressing cells.

ionomycin-induced  $Ca^{2+}$  release were not affected by ET-1 treatment or TRPC6 siRNA treatment, the changes in TRPC6 proteins may not influence  $Ca^{2+}$  content of intracellular  $Ca^{2+}$  stores or  $IP_3$ -mediated functions.

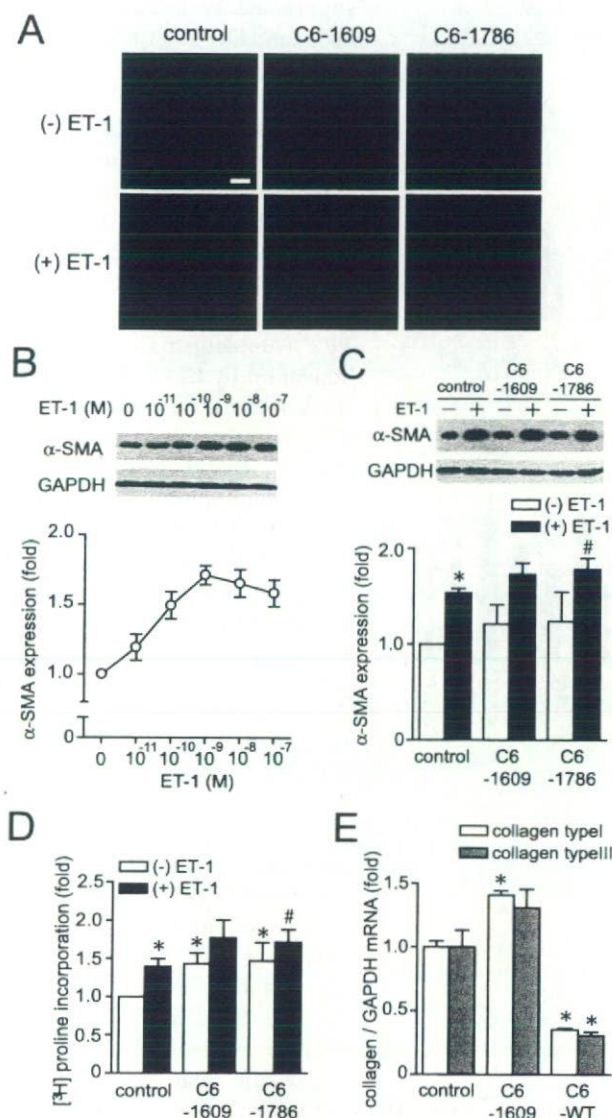
**Requirement of TRPC6 for ET-1-induced NFAT Activation**—Since CA  $G\alpha_{13}$ -induced TRPC6 up-regulation enhances basal  $Ca^{2+}$  influx activity, we examined whether ET-1 treatment enhances basal  $Ca^{2+}$  influx activity through  $G\alpha_{12/13}$ -mediated TRPC6 up-regulation. Treatment with ET-1 for 48 h enhanced the increase in  $[Ca^{2+}]_i$  induced by the addition of 2 mM  $Ca^{2+}$  to ET-1-containing  $Ca^{2+}$ -free solution, which was completely

suppressed by TRPC6 siRNA (C6-1609) and DN TRPC6 (C6-ΔN) (Fig. 5, *A* and *B*). The ET-1-induced enhancement of  $Ca^{2+}$  influx was suppressed by p115-RGS and PP2. Treatment with ET-1 increased Src activity, which was completely suppressed by p115-RGS and PP2 but not by SP600125 (Fig. 5C). We confirmed that ET-1-induced TRPC6 up-regulation was not affected by PP2 (supplemental Fig. 2C). Src activation by ET-1 stimulation was mediated by  $ET_A$  receptor, since  $ET_A$  receptor-selective (BQ123) but not  $ET_B$  receptor-selective (BQ788) blockers inhibited ET-1-stimulated Src activation (supplemental Fig. 2D). These results suggest that  $G\alpha_{12/13}$ -mediated activation of Src tyrosine kinase participate in ET-1-induced increase in basal  $Ca^{2+}$  influx activity apart from TRPC6 up-regulation. On the other hand, treatment with ET-1 for 48 h induced nuclear localization of GFP-NFAT4 proteins, which were inhibited by p115-RGS and SP600125 (Fig. 5, *D* and *F*). Treatment with SP600125 (1  $\mu$ M) significantly inhibited the ET-1 treatment-induced increase in basal  $Ca^{2+}$  influx activity ( $\Delta$ Ratio =  $0.70 \pm 0.05$ ,  $n = 67$  cells) and TRPC6 protein levels (Fig. 3D), suggesting that  $G\alpha_{12/13}$ -mediated activation of JNK participates in ET-1-induced NFAT translocation through TRPC6 up-regulation. The ET-1 treatment-induced NFAT translocation was also suppressed by TRPC6 siRNAs (Fig. 5, *E* and *F*). The expression of WT TRPC6 increased basal NFAT activity 2.5-fold, and it also enhanced NFAT activity induced by ET-1 treatment (Fig. 5G). In contrast, the expression

of DN TRPC6 mutants completely suppressed the ET-1 treatment-induced increase in NFAT activity. These results suggest that  $G\alpha_{12/13}$ -mediated up-regulation and activation of TRPC6 channels participate in sustained activation of NFAT induced by ET-1 treatment in cardiac fibroblasts.

**Inhibition of Cardiac Myofibroblast Formation by TRPC6 Activation**—Transformation of cardiac fibroblasts to myofibroblasts is characterized by expression of  $\alpha$ -SMA and production of ECM components that are a key event in connective tissue remodeling (6). Since NFAT is activated by the sustained increase in  $[Ca^{2+}]_i$  and the calcineurin-NFAT

## Inhibition of Myofibroblast Formation by TRPC6-NFAT Signaling



**FIGURE 6. Knockdown of TRPC6 enhances cardiac myofibroblast formation.** A–C, effects of TRPC6 siRNAs on the ET-1-induced increase in  $\alpha$ -SMA expression. Forty-eight h after transfection with siRNAs, cells were treated with ET-1 (100 nM) for 24 h. A, immunohistochemistry of cardiac fibroblasts stained with anti- $\alpha$ -SMA antibody. Scale bar, 20  $\mu$ m. B and C, concentration-dependent increase in  $\alpha$ -SMA expression by ET-1 treatment (B) and changes in  $\alpha$ -SMA expression by TRPC6 knockdown (C) were determined by Western blotting. D, effects of TRPC6 siRNAs on [ $^3$ H]proline incorporation in cardiac fibroblasts with or without ET-1 treatment. E, effects of TRPC6 siRNA and WT TRPC6 on the mRNA expression of collagen type I and III. \*,  $p < 0.05$  versus nontreatment (no ET-1) of control cells. #,  $p < 0.05$  versus ET-1 treatment of control cells.

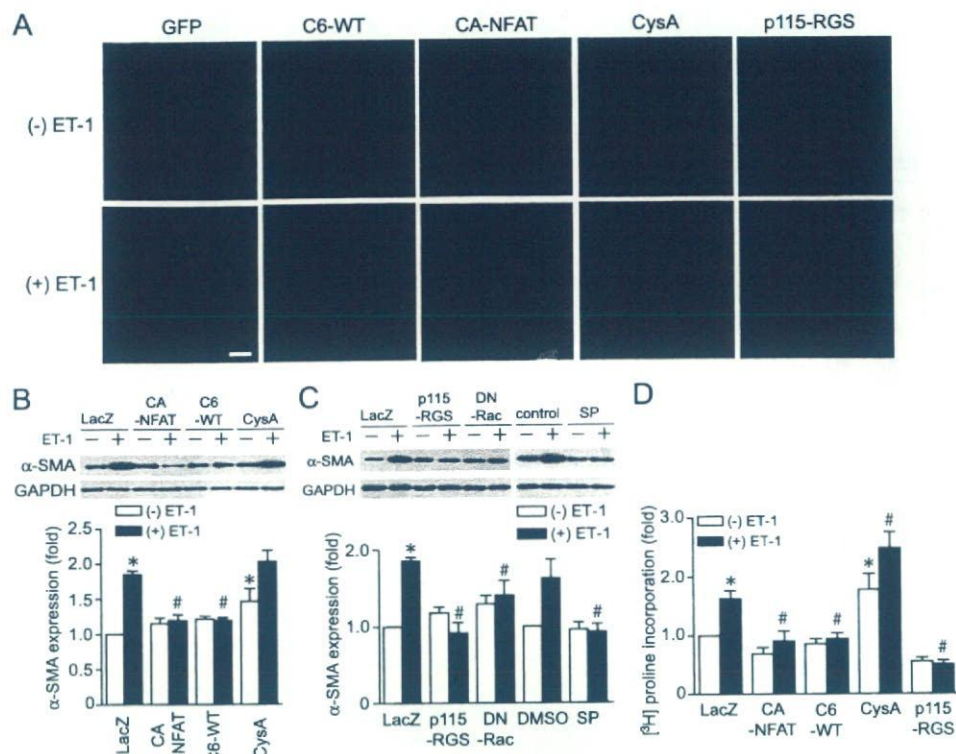
pathway plays a critical role in the pathogenesis of heart failure, we examined whether TRPC6-mediated NFAT activation participates in ET-1 treatment-induced transformation of cardiac fibroblasts. Treatment with ET-1 increased  $\alpha$ -SMA expression in a concentration-dependent manner (Fig. 6, A and B). Treatment with ET-1 (1 nM) caused maximal  $\alpha$ -SMA expression, whereas ET-1 treatment (>10 nM) slightly decreased  $\alpha$ -SMA expression. Knockdown of TRPC6 did not affect basal  $\alpha$ -SMA protein expression levels. However, it weakly but significantly enhanced ET-1-induced  $\alpha$ -SMA expression (Fig. 6, A and C). Furthermore, TRPC6 siRNAs significantly enhanced the basal incorporation of [ $^3$ H]proline, an

index of synthesis of ECM proteins (Fig. 6D). TRPC6 siRNAs also enhanced ET-1-induced [ $^3$ H]proline incorporation. Collagen expression is another index of transformation of cardiac fibroblasts to myofibroblasts. TRPC6 siRNA increased mRNAs of collagen type I and III, whereas overexpression of WT TRPC6 significantly suppressed the basal expression of collagen mRNAs (Fig. 6E). These results suggest that TRPC6 negatively regulates ET-1-induced transformation of cardiac fibroblasts to myofibroblasts and synthesis of ECM components.

**Inhibition of Cardiac Myofibroblast Formation by NFAT Activation**—The expression of TRPC6 WT inhibited the collagen mRNA expression and enhanced NFAT activation by ET-1 treatment (Figs. 5G and 6E). In addition to cardiac fibroblasts, we have previously demonstrated in cardiac myocytes that TRPC6 regulates NFAT activation (12). Then we examined whether TRPC6-mediated NFAT activation negatively regulates ET-1-induced transformation of cardiac fibroblasts to myofibroblasts. The expression of CA NFAT increased basal NFAT activity ( $8.3 \pm 0.9$ -fold) and suppressed the increase in  $\alpha$ -SMA expression by ET-1 treatment (Fig. 7, A and B). Expression of  $\alpha$ -SMA by ET-1 treatment was also suppressed by expression of TRPC6 WT. In contrast, treatment with CysA increased the basal  $\alpha$ -SMA expression levels and [ $^3$ H]proline incorporation, which was further enhanced by ET-1 treatment (Fig. 7, B and D). These results suggest that TRPC6-mediated NFAT activation inhibits ET-1-induced  $\alpha$ -SMA expression in cardiac fibroblasts. Furthermore, the ET-1-induced increase in  $\alpha$ -SMA expression and [ $^3$ H]proline incorporation was suppressed by the expression of p115-RGS and DN Rac and treatment with SP600125 (Fig. 7, C and D). These results suggest that the  $G_{\alpha_{12/13}}$ -mediated signaling pathway ( $G_{\alpha_{12/13}}$ -Rac-JNK) mediates ET-1-induced myofibroblast formation in cardiac fibroblasts. However,  $G_{\alpha_{12/13}}$ -mediated NFAT activation negatively regulates ET-1-induced cardiac myofibroblast formation.

**Inhibition of ET-1-induced JNK Activation by TRPC6-mediated NFAT Activation**—In order to examine how NFAT attenuates myofibroblast formation by ET-1 treatment, we next examined the effects of NFAT on ET-1-induced mitogen-activated protein kinase activation. Treatment with ET-1 increased JNK activity, which was suppressed by the expression of CA NFAT and WT TRPC6 but was potentiated by CysA (Fig. 8A). However, the ET-1-induced ERK activation was not affected by CA NFAT, TRPC6 WT, and CysA (Fig. 8B). This is consistent with the result that ERK activation is not involved in the increased expression of TRPC6 by ET-1 treatment (supplemental Fig. 2C). These results suggest that NFAT activation through TRPC6 up-regulation specifically inhibits ET-1-induced JNK activation in cardiac fibroblasts. The ET-1-induced ROS production and Rac activation, both of which work upstream of JNK, were suppressed by TRPC6 overexpression and NFAT activation (Fig. 8, C and D). These results suggest that NFAT inhibits JNK activation through inhibition of Rac activation and ROS production.

**Requirement of TRPC6 Up-regulation in ET-1-induced Hypertrophic Responses of Cardiomyocytes**—It has been reported that up-regulation of TRPC6 amplifies calcineurin



**FIGURE 7. Inhibition of cardiac myofibroblast formation by NFAT activation.** A and B, effects of TRPC6 WT, CA NFAT, CysA, and p115-RGS on the ET-1-induced increase in  $\alpha$ -SMA expression. Cells were infected with adenovirus coding one of TRPC6 WT, p115-RGS, and CA NFAT at a multiplicity of infection of 100 for 24 h and then treated with ET-1 (100 nM) for 48 h. Cells were treated with CysA (0.5  $\mu$ g/ml) for 20 min prior to ET-1 treatment. A, immunohistochemistry of cardiac fibroblasts stained with anti- $\alpha$ -SMA antibody. Scale bar, 20  $\mu$ m. B, changes in  $\alpha$ -SMA expression determined by Western blotting. C, effects of p115-RGS, DN Rac, and SP600125 on the ET-1-induced increase in  $\alpha$ -SMA expression. Twenty-four h after infection with adenovirus coding one of LacZ, p115-RGS, and DN Rac, cells were treated with ET-1 for 48 h. Cells were treated with SP600125 (SP; 1  $\mu$ M) for 20 min prior to ET-1 treatment. D, effects of CA NFAT, TRPC6 WT, CysA, and p115-RGS on [ $^3$ H]proline incorporation in cardiac fibroblasts with or without ET-1 treatment. \*,  $p < 0.05$  versus nontreatment (no ET-1) of LacZ-expressing cells. #,  $p < 0.05$  versus ET-1 treatment of LacZ-expressing cells.

signaling, leading to pathologic cardiac remodeling in mice (37). We have previously reported that  $G\alpha_{12/13}$ -mediated JNK activation participates in ET-1-induced hypertrophic responses of cardiomyocytes (24). We examined whether TRPC6 up-regulation is involved in ET-1-induced hypertrophic growth of rat neonatal cardiomyocytes. Treatment with ET-1 increased TRPC6 protein levels in a concentration-dependent manner, and the  $EC_{50}$  value was about 3 nM (Fig. 9A). The ET-1-induced increase in TRPC6 protein expression was completely suppressed by p115-RGS (Fig. 9B), suggesting that  $G\alpha_{12/13}$  mediate ET-1-induced TRPC6 up-regulation. Treatment with ET-1 increased BNP-luciferase activity, a marker for cardiac hypertrophy, and the  $EC_{50}$  value was about 5 nM (Fig. 9C). ET-1-stimulated BNP-luciferase activity was blocked by expression of p115-RGS, an inhibitory polypeptide of  $G\alpha_{12/13}$ . This result suggests that  $G\alpha_{12/13}$  mediate ET-1-stimulated hypertrophic response. Although we could not reliably determine the effects of TRPC6 siRNAs on ET-1-stimulated BNP-luciferase activity, the ET-1-induced NFAT activation and hypertrophic responses (actin reorganization and protein synthesis) were significantly suppressed by TRPC6 siRNAs (Fig. 9, D–F). These results suggest that  $G\alpha_{12/13}$  also play a role in ET-1-induced increase in TRPC6 protein expression in car-

diomyocytes. However, in the case of cardiomyocytes, the up-regulated TRPC6 may positively regulate hypertrophic responses induced by ET-1.

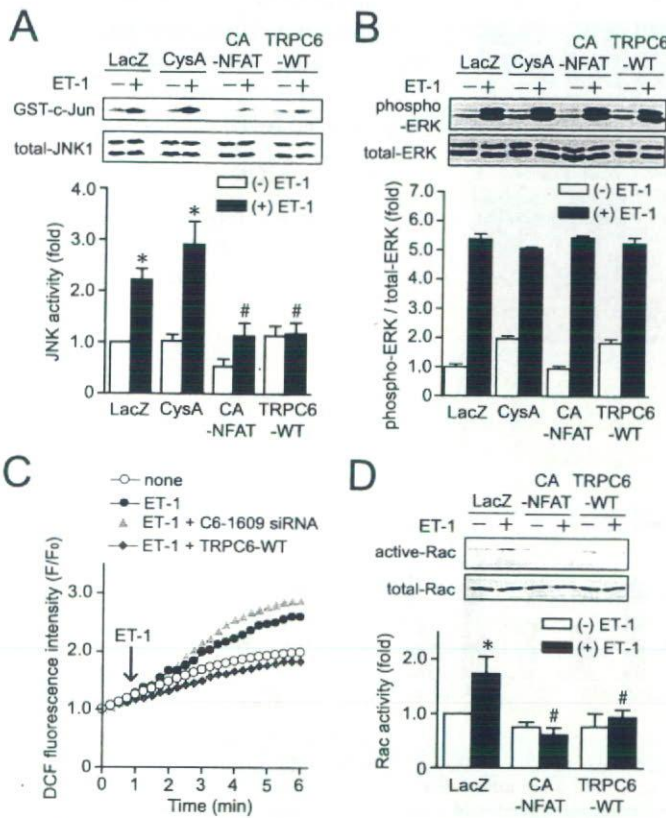
## DISCUSSION

In this study, we demonstrated that TRPC6 proteins are up-regulated by ET-1 treatment in cardiac fibroblasts. Furthermore, we demonstrated that the ET-1-induced increase in TRPC6 expression is critical for NFAT activation, which negatively regulates myofibroblast formation. Previous reports have shown that TRPC6 proteins are up-regulated by ET-1 and platelet-derived growth factor in pulmonary vascular smooth muscle cells (34, 35). These reports are consistent with the present results. We first demonstrated that  $G\alpha_{12/13}$  mediate ET-1-induced up-regulation of TRPC6 mRNAs and proteins in cardiac fibroblasts. We previously reported that  $G\alpha_{12/13}$  mediate Ang II-induced JNK activation through Rac-dependent ROS production (17, 25). Since it was reported that c-Jun, one of the target molecules of JNK, is required for agonist-induced up-regulation of TRPC6 proteins in pulmonary vascular smooth muscle

cells (36),  $G\alpha_{12/13}$ -mediated JNK activation may participate in the increased expression of TRPC6 in cardiac fibroblasts. Furthermore, we clearly demonstrated that  $G\alpha_{12/13}$  mediate ET-1-induced myofibroblast formation, since p115-RGS completely suppressed the increase in  $\alpha$ -SMA expression and [ $^3$ H]proline incorporation by ET-1 treatment (Fig. 6). Since ET-1-induced myofibroblast formation was also suppressed by DN Rac and SP600125 (Fig. 6C), the  $G\alpha_{12/13}$ -Rac-JNK signaling pathway may be involved in ET-1-induced myofibroblast formation. However, ET-1-induced fibrotic responses were inhibited by up-regulation of TRPC6 expression and resultant NFAT activation through the  $G\alpha_{12/13}$ -mediated pathway (Fig. 6). Therefore, ET-1 treatment caused fibrotic responses through the Rac-JNK pathway, and at the same time ET-1 treatment negatively regulated fibrotic responses through NFAT activation by the increased expression of TRPC6 (Fig. 10). The concentration of ET-1 to induce maximal  $\alpha$ -SMA expression was about 1 nM, which was about 10 times lower than that of ET-1 to induce maximal expression of TRPC6 proteins (Figs. 3B and 6B). Thus, cardiac fibroblasts may turn on their own protective system against excess progression of fibrotic responses when the cells are exposed to a high concentration of ET-1.

Although we cannot explain in detail the mechanism of how NFAT inhibits JNK activation (Fig. 8A) and TRPC6 expression

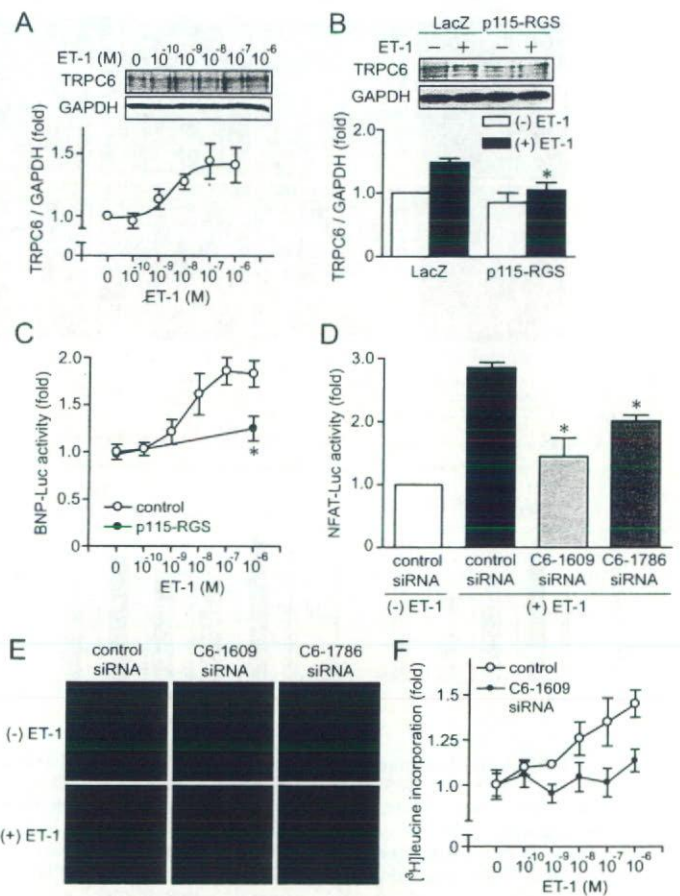
## Inhibition of Myofibroblast Formation by TRPC6-NFAT Signaling



**FIGURE 8. Inhibition of ET-1-induced Rac activation, ROS production, and JNK activation by TRPC6-mediated NFAT activation.** *A* and *B*, effects of CA NFAT, CysA, and TRPC6 WT on ET-induced JNK activation (*A*) and ERK activation (*B*). Forty-eight h after infection with adenovirus coding one of LacZ, CA NFAT, and TRPC6 WT or 24 h after treatment with CysA (0.5  $\mu$ g/ml), cells were treated with ET-1 (100 nM) for 20 min. *C*, effects of TRPC6 WT and TRPC6 siRNA (1609) on ET-1-induced ROS production. *D*, effects of CA NFAT and TRPC6 WT on ET-1-induced Rac activation. Cells were treated with ET-1 (100 nM) for 2 min. \*,  $p < 0.05$  versus nontreatment (no ET-1) of control or LacZ-expressing cells. #,  $p < 0.05$  versus ET-1 treatment of control or LacZ-expressing cells.

(supplemental Fig. 2) induced by ET-1 stimulation, there are a couple of possibilities. Since DPI and SP600125 suppressed TRPC6 promoter activity by ET-1 treatment, the ROS-JNK pathway plays an important role in TRPC6 expression (Fig. 3E). Expression of CA NFAT suppressed ET-1-stimulated Rac activation, and we previously reported that Rac mediates JNK activation (17, 25). Therefore, NFAT inhibits TRPC6 expression through inhibition of the Rac-JNK pathway. We can exclude down-regulation of ET receptors as a mechanism of NFAT-mediated inhibition of TRPC6 expression, since the ET-1-induced ERK activation was not affected by CA NFAT and TRPC6 WT (Fig. 8B). Since NFAT acts as a transcriptional factor, one possibility is that some factor(s) produced by NFAT activation negatively regulates JNK activity through inhibition of Rac activation in cardiac fibroblasts. For example, NFAT is reported to regulate the expression levels of BMP2 (bone morphogenetic protein 2), an antifibrotic factor (38). Further studies are required for understanding the mechanism of NFAT-dependent inhibition of JNK activation.

It has been reported that TRPC6 channel activity is positively regulated by various stimuli, such as DAG (28), tyrosine phosphorylation (29), and mechanical stress (39). We found that the increase in  $[Ca^{2+}]_i$  of CA  $G_{\alpha_{13}}$ -expressing cells was completely



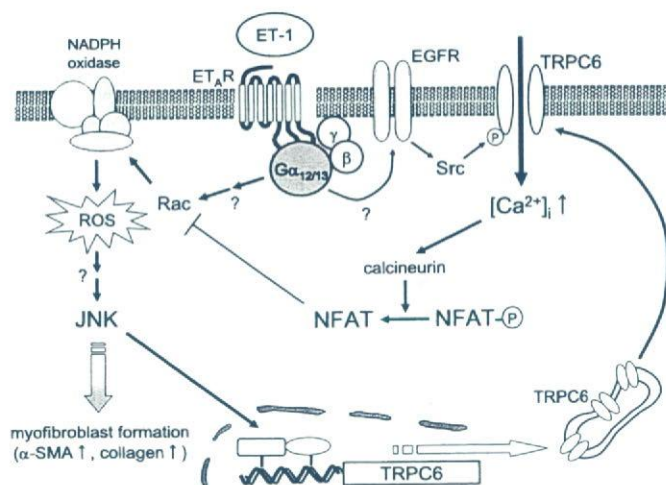
**FIGURE 9. Involvement of TRPC6 up-regulation in ET-1-induced hypertrophic responses of cardiomyocytes.** *A*, concentration-dependent increase in TRPC6 protein expression by ET-1 stimulation. Cells were treated with ET-1 for 48 h. TRPC6 proteins were detected with Western blot. *B*, effects of p115-RGS on ET-1-induced increase in TRPC6 protein expression. Twenty-four h after infection, cells were treated with ET-1 (100 nM) for 48 h. *C*, concentration-dependent increase in BNP-luciferase activity by ET-1 stimulation. Cells were treated with ET-1 for 24 h, and luciferase activity was determined. *D–F*, effects of TRPC6 siRNAs on ET-1-induced NFAT activation (*D*), actin reorganization (*E*), and protein synthesis (*F*). Forty-eight h after transfection with siRNAs, cells were treated with ET-1 for 24 h (*B* and *C*) or 8 h (*D*). \*,  $p < 0.05$  versus ET-1 treatment of control or LacZ-expressing cells.

inhibited by PP2 but not by U73122 (Fig. 2D), suggesting that Src tyrosine kinase but not PLC-generated DAG is involved in CA  $G_{\alpha_{13}}$ -mediated  $Ca^{2+}$  influx. It also suggests that CA  $G_{\alpha_{13}}$  does not activate PLC in cardiac fibroblasts. We also found that AG1478 inhibits CA  $G_{\alpha_{13}}$ -induced Src activation (Fig. 2F), suggesting the involvement of EGFR kinase in  $G_{\alpha_{13}}$ -mediated Src activation. Our results suggest that CA  $G_{\alpha_{13}}$ -mediated Src activation is involved in CA  $G_{\alpha_{13}}$ -induced  $Ca^{2+}$  influx.

We also demonstrated that  $Ca^{2+}$  influx-induced increase in  $[Ca^{2+}]_i$  evoked by ionomycin is not enhanced by TRPC6 over-expression (Fig. 4C). This result suggests that TRPC6 does not participate in store-operated  $Ca^{2+}$  entry in cardiac fibroblasts. TRPC1 is often referred to as a SOC, but it is unknown whether TRPC1 works as a true SOC, because this can also be activated by DAG and membrane stretch (19, 40). In addition to TRPC1, it has been recently reported that STIM1, located predominantly in the endoplasmic reticulum, acts as a  $Ca^{2+}$  sensor for store-operated  $Ca^{2+}$  entry (41, 42) and that Orai1/CRACM1 is identified as the  $Ca^{2+}$  release-activated  $Ca^{2+}$  channel gene (43,



## Inhibition of Myofibroblast Formation by TRPC6-NFAT Signaling



**FIGURE 10. Scheme for two opposite roles of  $G\alpha_{12/13}$  in cardiac fibroblasts.** Stimulation of  $ET_A R$  by ET-1 treatment activates  $G\alpha_{12/13}$ , which mediate ET-1-induced TRPC6 up-regulation and myofibroblast formation through Rac-dependent ROS production and JNK activation. On the other hand,  $G\alpha_{12/13}$ -mediated  $Ca^{2+}$  influx through Src-dependent activation of up-regulated TRPC6 induces NFAT activation. The activated NFAT inhibits ET-1-induced Rac activation, ROS production, and JNK activation through unknown mechanism(s). NFAT may work as a negative feedback regulator against ET-1-induced myofibroblast formation in cardiac fibroblasts.

44). Since TRPC1, STIM1, and Orai1/CRACM1 are ubiquitously expressed, these molecules may participate in store-operated  $Ca^{2+}$  entry in cardiac fibroblasts.

A variety of evidence has implicated that the sustained increase in  $[Ca^{2+}]_i$  is involved in the pathogenesis of heart failure (45). For example, up-regulation of TRPC proteins are associated with the reduction of sarcoplasmic reticulum  $Ca^{2+}$ -ATPase (20), suggesting the importance of TRPC in remodeling of the  $Ca^{2+}$  signaling mechanism in cardiac myocytes. Furthermore, it has been recently reported that diverse signals for cardiac hypertrophy stimulate the expression of TRPC channels and increase NFAT activity (21). Other groups demonstrated that the cardiac myocytes-specific expression of TRPC6 or CA calcineurin (upstream activator of NFAT) induces cardiac hypertrophy, and NFAT is activated in pathological but not physiological hypertrophy (46–48). We found in this study that  $G\alpha_{12/13}$ -mediated up-regulation of TRPC6 protein participates in ET-1-induced cardiomyocyte hypertrophy (Fig. 9). Thus, the TRPC-NFAT signaling pathway in myocytes plays an essential role in cardiac hypertrophy. In contrast to cardiac myocytes, we demonstrated in cardiac fibroblasts that up-regulation of TRPC6 expression and resultant activation of NFAT inhibit myofibroblast formation and synthesis of ECM components induced by ET-1 treatment. Therefore, we suggest that TRPC6 and TRPC6-mediated NFAT activation in cardiac fibroblasts work as negative regulators against cardiac fibrosis. Many lines of evidence have indicated that TRPC channels represent novel pharmacologic targets, since NFAT is activated by  $Ca^{2+}$  influx through TRPC channels in pathological hypertrophy. However, the role of TRPC channels in cardiac fibroblasts and cardiac fibrosis does not support this idea, since the TRPC6-NFAT signaling pathway works as a negative regulator of fibrotic responses. Inhibitor of TRPC channels may worsen the fibrotic process accompanied with hypertrophy. Since cardiac fibrosis

leads to myocardial stiffness and diastolic dysfunction, future *in vivo* work will be required for understanding the role of TRPC proteins in the cardiac function and development of cardiac fibrosis. The present findings also indicate that activators of TRPC channel-NFAT signaling or downstream signaling components are novel anti-fibrotic drugs.

In summary, we have demonstrated a signal transduction pathway of ET-1-induced up-regulation of TRPC6 expression and NFAT activation. As the sustained increase in  $[Ca^{2+}]_i$  through TRPC6 activates NFAT, the  $G\alpha_{12/13}$ -mediated TRPC6 expression and NFAT activation may act as a negative feedback regulator against ET-1-mediated fibrotic responses.

**Acknowledgments**—We thank Yuichi Nagamatsu for real time RT-PCR measurement during the early stage of this study and Yusuke Narita for production of CA NFAT adenovirus.

## REFERENCES

- Cohn, J. N., Ferrari, R., and Sharpe, N. (2000) *J. Am. Coll. Cardiol.* **35**, 569–582
- Manabe, I., Shindo, T., and Nagai, R. (2002) *Circ. Res.* **91**, 1103–1113
- Brown, R. D., Ambler, S. K., Mitchell, M. D., and Long, C. S. (2005) *Annu. Rev. Pharmacol. Toxicol.* **45**, 657–687
- Braudino, T. A., Carver, W., Giles, W., and Borg, T. K. (2006) *Am. J. Physiol.* **291**, H1015–H1026
- Tomasek, J. J., Gabbiani, G., Hinz, B., Chaponnier, C., and Brown, R. A. (2002) *Nat. Rev. Mol. Cell Biol.* **3**, 349–363
- Swaney, J. S., Roth, D. M., Olson, E. R., Naugle, J. E., Meszaros, J. G., and Insel, P. A. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102**, 437–442
- Powell, D. W., Mifflin, R. C., Valentich, J. D., Crowe, S. E., Saada, J. I., and West, A. B. (1999) *Am. J. Physiol.* **277**, C1–C9
- Serini, G., and Gabbiani, G. (1999) *Exp. Cell Res.* **250**, 273–283
- Kawano, H., Do, Y. S., Kawano, Y., Starnes, V., Barr, M., Law, R. E., and Hsueh, W. A. (2000) *Circulation* **101**, 1130–1137
- Katwa, L. C. (2003) *Am. J. Physiol.* **285**, H1132–H1139
- Wilkins, B. J., and Molkenkin, J. D. (2004) *Biochem. Biophys. Res. Commun.* **322**, 1178–1191
- Onohara, N., Nishida, M., Inoue, R., Kobayashi, H., Sumimoto, H., Sato, Y., Mori, Y., Nagao, T., and Kurose, H. (2006) *EMBO J.* **25**, 5305–5316
- Shimoyama, M., Hayashi, D., Takimoto, E., Zou, Y., Oka, T., Uozumi, H., Kudoh, S., Shibasaki, F., Yazaki, Y., Nagai, R., and Komuro, I. (1999) *Circulation* **100**, 2449–2454
- Øie, E., Bjørnerheim, R., Clausen, O. P. F., and Attramadal, H. (2000) *Am. J. Physiol.* **278**, H2115–H2123
- Kolar, F., Papousek, F., MacNaughton, C., Pelouch, V., Milerova, M., and Korecky, B. (1996) *Mol. Cell Biochem.* **163**, 253–260
- Yang, G., Meguro, T., Hong, C., Asai, K., Takagi, G., Karoor, V. L., Sadoshima, J., Vatner, D. E., Bishop, S. P., and Vatner, S. F. (2001) *J. Mol. Cell Cardiol.* **33**, 1505–1514
- Fujii, T., Onohara, N., Maruyama, Y., Tanabe, S., Kobayashi, H., Fukutomi, M., Nagamatsu, Y., Nishihara, N., Inoue, R., Sumimoto, H., Shibasaki, F., Nagao, T., Nishida, M., and Kurose, H. (2005) *J. Biol. Chem.* **280**, 23041–23047
- Nagamatsu, Y., Nishida, M., Onohara, N., Fukutomi, M., Maruyama, Y., Kobayashi, H., Sato, Y., and Kurose, H. (2006) *J. Pharmacol. Sci.* **101**, 144–150
- Clapham, D. E. (2003) *Nature* **426**, 517–524
- Seth, M., Sumbilla, C., Mullen, S. P., Lewis, D., Klein, M. G., Hussain, A., Soboloff, J., Gill, D. L., and Inesi, G. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 16683–16688
- Bush, E. W., Hood, D. B., Papst, P. J., Chapo, J. A., Minobe, W., Bristow, M. R., Olson, E. N., and McKinsey, T. A. (2006) *J. Biol. Chem.* **281**, 33487–33496
- Nishida, M., Maruyama, Y., Tanaka, R., Kontani, K., Nagao, T., and Ku-

## Inhibition of Myofibroblast Formation by TRPC6-NFAT Signaling

- rose, H. (2000) *Nature* **408**, 492–495
23. Maruyama, Y., Nishida, M., Sugimoto, Y., Tanabe, S., Turner, J. H., Kozasa, T., Wada, T., Nagao, T., and Kurose, H. (2002) *Circ. Res.* **91**, 961–969
  24. Arai, K., Maruyama, Y., Nishida, M., Tanabe, S., Takagahara, S., Kozasa, T., Mori, Y., Nagao, T., and Kurose, H. (2003) *Mol. Pharmacol.* **63**, 478–488
  25. Nishida, M., Tanabe, S., Maruyama, Y., Mangmool, S., Urayama, K., Nagamatsu, Y., Takagahara, S., Turner, J. H., Kozasa, T., Kobayashi, H., Sato, Y., Kawanishi, T., Inoue, R., Nagao, T., and Kurose, H. (2005) *J. Biol. Chem.* **280**, 18434–18441
  26. Inoue, R., Jensen, L. J., Shi, J., Morita, H., Nishida, M., Honda, A., and Ito, Y. (2006) *Circ. Res.* **99**, 119–131
  27. Nishida, M., Sugimoto, K., Hara, Y., Mori, E., Morii, T., Kurosaki, T., and Mori, Y. (2003) *EMBO J.* **22**, 4677–4688
  28. Hofmann, T., Obukhov, A. G., Schaefer, M., Harteneck, C., Gudermann, T., and Schultz, G. (1999) *Nature* **397**, 259–263
  29. Hisatsune, C., Kuroda, Y., Nakamura, K., Inoue, T., Nakamura, T., Michikawa, T., Mizutani, A., and Mikoshiba, K. (2004) *J. Biol. Chem.* **279**, 18887–18894
  30. Shi, C.-S., Sinnarajah, S., Cho, H., Kozasa, T., and Kehrl, J. H. (2000) *J. Biol. Chem.* **274**, 24470–24476
  31. Kim, M., Nozu, F., Kusama, K., and Imawari, M. (2006) *Biochem. Biophys. Res. Commun.* **339**, 271–276
  32. Lopez, I., Mak, E. C., Ding, J., Hamm, H. E., and Lomasney, J. W. (2001) *J. Biol. Chem.* **276**, 2758–2765
  33. Gohla, A., Harhammer, R., and Schultz, G. (1998) *J. Biol. Chem.* **273**, 4653–4659
  34. Yu, Y., Sweeney, M., Zhang, S., Platoshyn, O., Landsberg, J., Rothman, A., and Yuan, J. X.-J. (2003) *Am. J. Physiol.* **284**, C316–C330
  35. Kunichika, N., Landsberg, J. W., Yu, Y., Kunichika, H., Thistlethwaite, P. A., Rubin, L. J., and Yuan, J. X. (2004) *Am. J. Respir. Crit. Care Med.* **170**, 1101–1107
  36. Dietrich, A., Mederos y Schnizler, M., Gollasch, M., Gross, V., Storch, U., Dubrovskaja, G., Obst, M., Yildirim, E., Salanova, B., Kalwa, H., Essin, K., Pinkenburg, O. C., Luft, F., Gudermann, T., and Birnbaumer, L. (2005) *Mol. Cell Biol.* **25**, 6980–6989
  37. Kuwahara, K., Wang, Y., McAnally, J., Richardson, J. A., Bassel-Duby, R., Hill, J. A., and Olson, E. N. (2006) *J. Clin. Invest.* **116**, 3114–3126
  38. Koga, T., Matus, Y., Asagiri, M., Kodama, T., de Crombrughe, B., Nakashima, K., and Takayanagi, H. (2005) *Nat. Med.* **11**, 880–885
  39. Spassova, M. A., Hewavitharana, T., Xu, W., Soboloff, J., and Gill, D. L. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103**, 16586–16591
  40. Lewis, R. S. (2007) *Nature* **446**, 284–287
  41. Liou, J., Kim, M. L., Heo, W. D., Jones, J. T., Myers, J. W., Ferrell, J. E. Jr., and Meyer, T. (2005) *Curr. Biol.* **15**, 1235–1241
  42. Roos, J., DiGregorio, P. J., Yeromin, A. V., Ohlsen, K., Lioudyno, M., Zhang, S., Safrina, O., Kozak, J. A., Wagner, S. L., Cahalan, M. D., Veli-celebi, G., and Stauderman, K. A. (2005) *J. Cell Biol.* **169**, 435–445
  43. Feske, S., Gwack, Y., Prakriya, M., Srikanth, S., Puppel, S. H., Tanasa, B., Hogan, P. G., Lewis, R. S., Daly, M., and Rao, A. (2006) *Nature* **441**, 179–185
  44. Vig, M., Peinelt, C., Beck, A., Koomoa, D. L., Rabah, D., Koblan-Huberson, M., Kraft, S., Turner, H., Fleig, A., Penner, R., and Kinet, J. P. (2006) *Science* **312**, 1220–1223
  45. Molkenkin, J. D., Lu, J.-R., Antos, C. L., Markham, B., Richardson, J., Robbins, J., Grant, S. R., and Olson, E. N. (1998) *Cell* **93**, 215–228
  46. Nakayama, H., Wilkin, B. J., Bodi, I., and Molkenkin, J. D. (2006) *FASEB J.* **20**, 1660–1670
  47. Wilkins, B. J., Dai, Y.-S., Bueno, O. F., Parsons, S. A., Xu, J., Plank, D. M., Jones, F., Kimball, T. R., Jeffery, D., and Molkenkin, J. D. (2004) *Circ. Res.* **94**, 110–118
  48. Frey, N., McKinsey, T. A., and Olson, E. N. (2000) *Nat. Med.* **6**, 1221–1227

# Granulocyte Colony-Stimulating Factor Promotes the Translocation of Protein Kinase C $\zeta$ in Neutrophilic Differentiation Cells

TOSHIE KANAYASU-TOYODA,<sup>1</sup> TAKAYOSHI SUZUKI,<sup>1</sup> TADASHI OSHIZAWA,<sup>1</sup>  
ERIKO UCHIDA,<sup>2</sup> TAKAO HAYAKAWA,<sup>2</sup> AND TERUHIDE YAMAGUCHI<sup>1\*</sup>

<sup>1</sup>Division of Cellular and Gene Therapy Products, National Institute of Health Sciences, Tokyo, Japan

<sup>2</sup>National Institute of Health Sciences, Tokyo, Japan

Previously, we suggested that the phosphatidylinositol 3-kinase (PI3K)-p70 S6 kinase (p70 S6K) pathway plays an important role in granulocyte colony-stimulating factor (G-CSF)-dependent enhancement of the neutrophilic differentiation and proliferation of HL-60 cells. While atypical protein kinase C (PKC) has been reported to be a regulator of p70 S6K, abundant expression of PKC $\zeta$  was observed in myeloid and lymphoid cells. Therefore, we analyzed the participation of PKC $\zeta$  in G-CSF-dependent proliferation. The maximum stimulation of PKC $\zeta$  was observed from 15 to 30 min after the addition of G-CSF. From 5 to 15 min into this lag time, PKC $\zeta$  was found to translocate from the nucleus to the membrane. At 30 min it re-translocated to the cytosol. This dynamic translocation of PKC $\zeta$  was also observed in G-CSF-stimulated myeloperoxidase-positive cells differentiated from cord blood cells. Small interfering RNA for PKC $\zeta$  inhibited G-CSF-induced proliferation and the promotion of neutrophilic differentiation of HL-60 cells. These data indicate that the G-CSF-induced dynamic translocation and activation processes of PKC $\zeta$  are important to neutrophilic proliferation.

J. Cell. Physiol. 211: 189–196, 2007. © 2006 Wiley-Liss, Inc.

Hematopoietic cell differentiation is regulated by a complex network of growth and differentiation factors (Tenen et al., 1997; Ward et al., 2000). Granulocyte colony-stimulating factor (G-CSF) and its receptors are pivotal to the differentiation of myeloid precursors into mature granulocytes. In previous studies (Kanayasu-Toyoda et al., 2002) on the neutrophilic differentiation of HL-60 cells treated with either dimethyl sulfoxide (DMSO) or retinoic acid (RA), heterogeneous transferrin receptor (Trf-R) populations—transferrin receptor-positive (Trf-R<sup>+</sup>) cells and transferrin receptor-negative (Trf-R<sup>-</sup>) cells—appeared 2 days after the addition of DMSO or RA. The Trf-R<sup>+</sup> cells were proliferative-type cells that had higher enzyme activity of phosphatidylinositol 3-kinase (PI3K) and protein 70 S6 kinase (p70 S6K), whereas the Trf-R<sup>-</sup> cells were differentiation-type cells of which Tyr705 in STAT3 was much more phosphorylated by G-CSF. Inhibition of either PI3K by wortmannin or p70 S6K by rapamycin was found to eliminate the difference in differentiation and proliferation abilities between Trf-R<sup>+</sup> and Trf-R<sup>-</sup> cells in the presence of G-CSF (Kanayasu-Toyoda et al., 2002). From these results, we concluded that proteins PI3K and p70 S6K play important roles in the growth of HL-60 cells and negatively regulate neutrophilic differentiation. On the other hand, the maximum kinase activity of PI3K was observed at 5 min after the addition of G-CSF (Kanayasu-Toyoda et al., 2002) and that of p70 S6K was observed between 30 and 60 min after, indicating a lag time between PI3K and p70 S6K activation. It is conceivable that any signal molecule(s) must transduce the G-CSF signal during the time lag between PI3K and p70 S6K. Chung et al. (1994) also showed a lag time between PI3K and p70 S6K activation on HepG2 cells stimulated by platelet-derived growth factor (PDGF), suggesting that some signaling molecules also may transduce between PI3K and p70S6K.

Protein kinase C (PKC) is a family of Ser/Thr kinases involved in the signal transduction pathways that are triggered by numerous extracellular and intracellular stimuli. The PKC

family has been shown to play an essential role in cellular functions, including mitogenic signaling, cytoskeleton rearrangement, glucose metabolism, differentiation, and the regulation of cell survival and apoptosis. Eleven different members of the PKC family have been identified so far. Based on their structural similarities and cofactor requirements, they have been grouped into three subfamilies: (1) the classical or conventional PKCs (cPKC $\alpha$ ,  $\beta_1$ ,  $\beta_2$ , and  $\gamma$ ), activated by Ca<sup>2+</sup>, diacylglycerol, and phosphatidyl-serine; (2) the novel PKCs (nPKC $\delta$ ,  $\epsilon$ ,  $\eta$ , and  $\theta$ ), which are independent of Ca<sup>2+</sup> but still responsive to diacylglycerol; and (3) the atypical PKCs (aPKC $\zeta$  and  $\iota/\lambda$ ), where PKC $\lambda$  is the homologue of human PKC $\zeta$ . Atypical PKCs differ significantly from all other PKC family

**Abbreviations:** DMSO, dimethyl sulfoxide; fMLP-R, formyl-Met-Leu-Phe receptor; RA, retinoic acid; G-CSF, granulocyte colony-stimulating factor; Trf-R, transferrin receptor; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase; p70 S6K, protein 70 S6 kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; siRNA, small interfering RNA; PMN, polymorphonuclear leukocyte.

Contract grant sponsor: Ministry of Health, Labor, and Welfare of Japan.

Contract grant sponsor: Ministry of Education, Culture, Sports, Science, and Technology of Japan.

\*Correspondence to: Teruhide Yamaguchi, Division of Cellular and Gene Therapy Products, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan.  
E-mail: yamaguch@nihs.go.jp

Received 31 May 2006; Accepted 22 September 2006

Published online in Wiley InterScience  
(www.interscience.wiley.com.), 28 November 2006.  
DOI: 10.1002/jcp.20930

members in their regulatory domains, in that they lack both the calcium-binding domain and one of the two zinc finger motifs required for diacylglycerol binding (Liu and Heckman, 1998). Romanelli et al. (1999) reported that p70 S6K is regulated by PKC $\zeta$  and participates in a PI3K-regulated signaling complex. On the other hand, Selbie et al. (1993) reported that the tissue distribution of PKC $\zeta$  is different from that of PKC $\iota/\lambda$ , and that PKC $\iota/\lambda$  appears to be widely expressed. If the p70 S6K could be activated by aPKC, the regulation of p70 S6K activation would seem to depend on the tissue-specific expression of PKC $\iota$  and/or PKC $\zeta$ . In neutrophilic lineage cells, the question is which aPKC participates in the regulation of p70 S6K on G-CSF signaling.

In this study, we show that G-CSF activated PKC $\iota$ , promoting its translocation from the nucleus to the cell surface membrane and subsequently to the cytosol in DMSO-treated HL-60 cells. We also show the translocation of PKC $\iota$  using myeloperoxidase-positive neutrophilic lineage differentiated from cord blood, which is a rich source of immature myeloid cells (Fritsch et al., 1993; Rappold et al., 1997; Huang et al., 1999; Debili et al., 2001; Hao et al., 2001). We concluded that PKC $\iota$  translocation and activation by G-CSF are needed for neutrophilic proliferation.

## Materials and Methods

### Reagents

Anti-p70 S6K polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PKC $\iota$  polyclonal antibody and monoclonal antibody were purchased from Santa Cruz Biotechnology and from Transduction Laboratories (Lexington, KY), respectively. Anti-PKC $\zeta$  polyclonal antibody was purchased from Cell Signaling Technology (Beverly, MA). Anti-myeloperoxidase antibody was purchased from Serotec Ltd. (Oxford, UK). GF 109203X, and Gö 6983 were obtained from Calbiochem-Novabiochem (San Diego, CA). Wortmannin was obtained from Sigma Chemical (St. Louis, MO). Anti-Histon-H1 antibody, anti-Fc $\gamma$  receptor IIa (CD32) antibody, and anti-lactate dehydrogenase antibody were from Upstate Cell Signaling Solutions (Lake Placid, NY), Lab Vision Corp. (Fremont, CA), and Chemicon International, Inc. (Temecula, CA), respectively.

### Cell culture

HL-60, Jurkat, K562, U937, and THP-1 cells were kindly supplied by the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Cells were maintained in RPMI 1640 medium containing 10% heat-inactivated FBS and 30 mg/L kanamycin sulfate at 37°C in moisturized air containing 5% CO $_2$ . The HL-60 cells, which were at a density of  $2.5 \times 10^5$  cells/ml, were differentiated by 1.25% DMSO. Two days after the addition of DMSO, the G-CSF-induced signal transduction was analyzed using either magnetically sorted cells or non-sorted cells.

### Magnetic cell sorting

To prepare Trf-R $^-$  and Trf-R $^+$  cells, magnetic cell sorting was performed as previously reported (Kanayasu-Toyoda et al., 2002), using an automatic cell sorter (AUTO MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). After cell sorting, both cell types were used for Western blotting and PKC $\iota$  enzyme activity analyses.

### Preparation of cell lysates and immunoblotting

For analysis of PKC $\iota$  and PKC $\zeta$  expression, a PVDF membrane blotted with 50  $\mu$ g of various tissues per lane was purchased from BioChain Institute (Hayward, CA). Both a polymorphonuclear leukocytes (PMNs) fraction and a fraction containing lymphocytes and monocytes were isolated by centrifugation (400g, 25 min) using a Mono-poly resolving medium (Dai-Nippon Pharmaceutical, Osaka, Japan) from human whole blood, which was obtained from a healthy volunteer with informed consent. T-lymphocytes were further isolated from the mixture fraction using the Pan T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's protocol. T-lymphocytes, PMNs, HL-60 cells, Jurkat cells, K562 cells, and U937 cells ( $1 \times 10^7$ ) were

collected and lysed in lysis buffer containing 1% Triton X-100, 10 mM K $_2$ HPO $_4$ /KH $_2$ PO $_4$  (pH 7.5), 1 mM EDTA, 5 mM EGTA, 10 mM MgCl $_2$ , and 50 mM  $\beta$ -glycerophosphate, along with 1/100 (v/v) protease inhibitor cocktail (Sigma Chemical) and 1/100 (v/v) phosphatase inhibitor cocktail (Sigma Chemical). The cellular lysate of  $10^6$  cells per lane was subjected to Western blotting analysis. Human cord blood was kindly supplied from the Metro Tokyo Red Cross Cord Blood Bank (Tokyo, Japan) with informed consent. Mononuclear cells, isolated with the Lymphoprep<sup>TM</sup> Tube (Axis-Shield PoC AS, Oslo, Norway), were cultured in RPMI 1640 medium containing 10% FBS in the presence of G-CSF for 3 days. Cultured cells were collected, and the cell lysate was subjected to Western blotting analysis.

A fraction of the plasma membrane, cytosol, and nucleus of the DMSO-treated HL-60 cells was prepared by differential centrifugation after the addition of G-CSF, as described previously (Yamaguchi et al., 1999). After the cells that had been suspended in 250 mM sucrose/10 mM Tris-HCl (pH 7.4) containing 1/100 (v/v) protease inhibitor cocktail (Sigma Chemical) were gently disrupted by freezing and thawing, they were centrifuged at 800g, 4°C for 10 min. The precipitation was suspended in 10 mM Tris-HCl (pH 6.7) supplemented with 1% SDS. It was then digested by benzonuclease at 4°C for 1 h and used as a sample of the nuclear fraction. After the post-nucleus supernatant was re-centrifuged at 100,000 rpm (452,000g) at a temperature of 4°C for 40 min, the precipitate was used as a crude membrane fraction and the supernatant as a cytosol fraction. Western blotting analysis was then performed as described previously (Kanayasu-Toyoda et al., 2002). The bands that appeared on x-ray films were scanned, and the density of each band was quantitated by Scion Image (Scion, Frederick, MD) using the data from three separate experiments.

### Kinase assay

The activity of PKC $\iota$  was determined by phosphorous incorporation into the fluorescence-labeled pseudosubstrate (Pierce Biotechnology, Rockford, IL). The cell lysates were prepared as described above and immunoprecipitated with the anti-PKC $\iota$  antibody. Kinase activity was measured according to the manufacturer's protocol. In the analysis of inhibitors effects, cells were pretreated with a PI3K inhibitor, wortmannin (100 nM), or PKC inhibitors, GF109207X (10  $\mu$ M) and Gö6983 (10  $\mu$ M) for 30 min, and then stimulated by G-CSF for 15 min.

### Observation of confocal laser-scanning microscopy

Upon the addition of G-CSF, PKC $\iota$  localization in the DMSO-treated HL-60 cells for 2 days was examined by confocal laser-activated microscopy (LSM 510, Carl Zeiss, Oberkochen, Germany). The cells were treated with 50 ng/ml G-CSF for the indicated periods and then fixed with an equal volume of 4.0% paraformaldehyde in PBS(-). After treatment with ethanol, the fixed cells were labeled with anti-PKC $\iota$  antibody and with secondary antibody conjugated with horseradish peroxidase. They were then visualized with TSA<sup>TM</sup> Fluorescence Systems (PerkinElmer, Boston, MA).

Mononuclear cells prepared from cord blood cells were cultured in RPMI 1640 medium containing 10% FBS in the presence of G-CSF for 7 days. Then, for serum and G-CSF starvation, cells were cultured in RPMI 1640 medium containing 1% BSA for 11 h. After stimulation by 50 ng/ml G-CSF, the cells were fixed, stained with both anti-PKC $\iota$  polyclonal antibody and anti-myeloperoxidase monoclonal antibody, and finally visualized with rhodamine-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG, respectively.

### RNA interference

Two pairs of siRNAs were chemically synthesized: annealed (Dharmacon RNA Technologies, Lafayette, CO) and transfected into HL-60 cells using Nucleofector<sup>TM</sup> (Amaxa, Cologne, Germany). The sequences of sense siRNAs were as follows: PKC $\iota$ , GAAGAAGCCUUUAGACUUUA; p70 S6K, GCAAGGAGUCUAUCCAUGAUU. As a control, the sequence ACUCUAUCGCCAGCGUGACUU was used. Forty-eight hours after treatment with siRNA, the cells were lysed for Western blot analysis. For proliferation and differentiation assay, cells were transfected with siRNA on the first day, treated with DMSO on the second day, and supplemented with G-CSF on the third day. After cells were subsequently cultured for 5 days, cell numbers and formyl-Met-Leu-Phe receptor (fMLP-R) expression were determined.

**fMLP-R expression**

The differentiated cells were collected and incubated with FITC-conjugated fMLP; then, labeled cells were subjected to flow cytometric analysis (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ).

**Statistical analysis**

Statistical analysis was performed using unpaired Student's *t*-test. Values of  $P < 0.05$  were considered to indicate statistical significance. Each experiment was repeated at least three times and representative data were indicated.

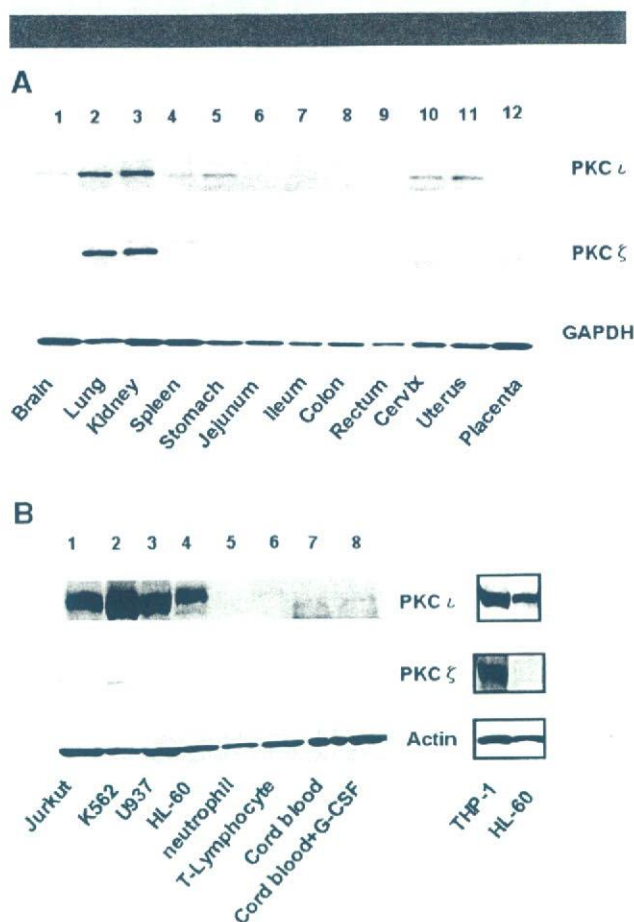
**Results****The distribution of atypical PKC in various tissues and cells**

Previously, we reported that the PI3K-p70 S6K-cMyc pathway plays an important role in the G-CSF-induced proliferation of DMSO-treated HL-60 cells, not only by enhancing the activity of both PI3K and p70 S6K but also by inducing the c-Myc protein (Kanayasu-Toyoda et al., 2002, 2003). We also reported that G-CSF did not stimulate Erk1, Erk2, or 4E-binding protein 1. The maximum kinase activity of PI3K was observed 5 min after the addition of G-CSF, and that of p70 S6K was observed between 30 and 60 min after. It is conceivable that any signal molecule(s) must transduce the G-CSF signal during the time lag between PI3K and p70 S6K. Romanelli et al. (1999) suggested that the activation of p70 S6K is regulated by PKC $\xi$  and participates in the PI3K-regulated signaling complex. To examine the role of atypical PKC in the G-CSF-dependent activation and the relationship between atypical PKC and p70 S6K, the protein expression of PKC $\zeta$  and PKC $\iota$  in various human tissues and cells was analyzed by Western blotting. As shown in Figure 1A, both of the atypical PKCs were markedly expressed in lung and kidney but were weakly expressed in spleen, stomach, and placenta. In brain, cervix, and uterus, the expression of only PKC $\iota$  was observed. Selbie et al. (1993) have reported observing the expression of PKC $\zeta$  not in protein levels but in RNA levels, in the kidney, brain, lung, and testis, and that of PKC $\iota$  in the kidney, brain, and lung. In this study, the protein expression of PKC $\iota$  in the kidney, brain, and lung was consistent with the RNA expression of PKC $\iota$ . Despite the strong expression of PKC $\zeta$  RNA in brain (Selbie et al., 1993), PKC $\zeta$  protein was scarcely observed. Although PKC $\iota$  proteins were scarcely expressed in neutrophils and T-lymphocytes in peripheral blood, they were abundantly expressed in immature blood cell lines, that is, Jurkut, K562, U937, and HL-60 cells (Fig. 1B), in contrast with the very low expression of PKC $\zeta$  proteins. In mononuclear cells isolated from umbilical cord blood, which contains large numbers of immature myeloid cells and has a high proliferation ability, the expression of PKC $\iota$  proteins was also observed. Since Nguyen and Dessauer (2005) have reported observing abundant PKC $\zeta$  proteins in THP-1 cells, as a positive control for PKC $\zeta$ , we also performed a Western blot of THP-1 cells (Fig. 1B, right part). While PKC $\iota$  was markedly expressed in both THP-1 and HL-60 cells, PKC $\zeta$  was observed only in THP-1 cells.

These data suggested that PKC $\zeta$  and PKC $\iota$  were distributed differently in various tissues and cells, and that mainly PKC $\iota$  proteins were expressed in proliferating blood cells.

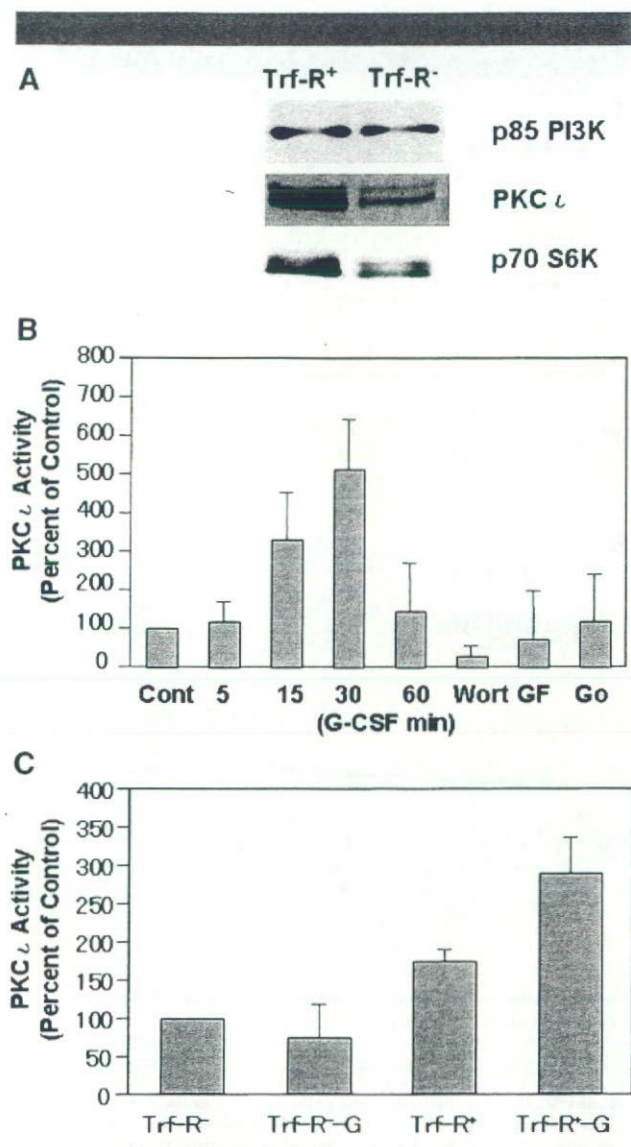
**Stimulation of PKC $\iota$  activity by G-CSF**

Among the 11 different members of the PKC family, the  $\alpha$ PKCs ( $\zeta$  and  $\iota$ ) have been reported to activate p70 S6K activity and to be regulated by PI3K (Akimoto et al., 1998; Romanelli et al., 1999). As shown in Figure 1, although the PKC $\zeta$  proteins were not detected by Western blotting in HL-60 cells or mononuclear cells isolated from cord blood cells, it is possible that PKC $\iota$  could functionally regulate p70 S6K as an upstream



**Fig. 1.** Different distributions of PKC $\zeta$  and PKC $\iota$ . The protein expression of PKC $\iota$  appears in the upper part and that of PKC $\zeta$  in the middle part in various tissues and cells. **A:** 1, brain; 2, lung; 3, kidney; 4, spleen; 5, stomach; 6, jejunum; 7, ileum; 8, colon; 9, rectum; 10, cervix; 11, uterus; 12, placenta. Anti-GAPDH blot is a control for various tissues. **B:** 1, jurkut cells; 2, K562 cells; 3, U937 cells; 4, HL-60 cells; 5, neutrophils; 6, T-lymphocytes; 7, mononuclear cells from cord blood in the absence of G-CSF; 8, mononuclear cells from cord blood in the presence of G-CSF. Anti-actin blot is a control. The right part shows immunoblots of PKC $\iota$ , PKC $\zeta$ , and actin of THP-1 cells as a positive control for PKC $\zeta$ . The cell numbers of THP-1 and HL-60 cells were adjusted in relation to other cells on the left parts.

regulator in these cells. Therefore, we focused on the role of PKC $\iota$  as the possible upstream regulator of p70 S6K in neutrophil lineage cells. First, we compared the expression of PKC $\iota$  in both Trf-R $^{+}$  and Trf-R $^{-}$  cells. PKC $\iota$  proteins were expressed more abundantly in Trf-R $^{+}$  cells than in Trf-R $^{-}$  cells (Fig. 2A, middle part), as with the p70 S6K proteins. A time course study of PKC $\iota$  activity upon the addition of G-CSF revealed the maximum stimulation at 15 min, lasting until 30 min. The G-CSF-dependent activation of PKC $\iota$  was inhibited by the PKC inhibitors wortmannin, GF 109203X, and Gö 6983. Considering the marked inhibitory effect of wortmannin on PKC $\iota$  and evidence that the maximum stimulation of PI3K was observed at 5 min after the addition of G-CSF, PI3K was determined to be the upstream regulator of PKC $\iota$  in the G-CSF signal transduction of HL-60 cells. The basal activity of PKC $\iota$  in Trf-R $^{+}$  cells was higher than that in Trf-R $^{-}$  cells, and G-CSF was more augmented. In Trf-R $^{-}$  cells, PKC $\iota$  activity was scarcely stimulated by G-CSF. This tendency of PKC $\iota$  to be activated by G-CSF was similar to that of PI3K (Kanayasu-Toyoda et al., 2002).



**Fig. 2.** Expression of PKC $\iota$  in Trf-R<sup>+</sup> and Trf-R<sup>-</sup> cells and effects of G-CSF on PKC $\iota$  activity. **A:** The expression of PKC $\iota$  in Trf-R<sup>+</sup> and Trf-R<sup>-</sup> cells was subjected to Western blot analysis after magnetic cell sorting. **B:** The G-CSF-dependent PKC $\iota$  activation of the DMSO-treated HL-60 cells was measured. The x-axis represents the time lapse (min) after the G-CSF stimulation and the y-axis percent of control that was not stimulated by G-CSF. Columns and bars represent the mean  $\pm$  SD, using data from three separate experiments. **Wort:** wortmannin (100 nM), **GF:** GF 109207X (10  $\mu$ M), **Gö:** Gö6983 (10  $\mu$ M). Cells were pretreated with each inhibitor and then stimulated by G-CSF for 15 min. **C:** The PKC $\iota$  activity in the Trf-R<sup>+</sup> and Trf-R<sup>-</sup> cells 30 min after the addition of G-CSF. The y-axis represents the percentage of control that was non-stimulated Trf-R<sup>-</sup> cells. Columns and bars represent the mean  $\pm$  SD, using data from three separate experiments.

#### Effects of G-CSF on PKC $\iota$ translocation

Muscella et al. (2003) demonstrated that the translocation of PKC $\zeta$  from the cytosol to the nucleus or membrane is required for c-Fos synthesis induced by angiotensin II in MCF-7 cells. It was also reported that high glucose induced the translocation of PKC $\iota$  (Chuang et al., 2003). These results suggest that the translocation of aPKC plays an important role in its signaling. To clarify the translocation of PKC $\iota$ , immuno-histochemical staining (Fig. 3) and biochemical fractionation (Fig. 4) in

DMSO-induced HL-60 cells were performed after the addition of G-CSF. In a non-stimulated condition, PKC $\iota$  in the HL-60 cells treated with DMSO for 2 days (Fig. 3, control) was detected mainly in the nucleus. Analysis of Western blotting (Fig. 4, left parts) and quantification of the bands (Fig. 4, right columns) also revealed that PKC $\iota$  was localized and observed mainly in the nuclear fraction (Fig. 4A). During the 5–15 min period after the addition of G-CSF, PKC $\iota$  was found to translocate (Figs. 3 and 4B) into the membrane fraction, after which it re-translocated into the cytosol fraction (Fig. 4C). In the presence of wortmannin, the G-CSF-induced translocation of PKC $\iota$  into the plasma membrane failed, but PKC $\iota$  was found to localize in the cytosolic fraction (Figs. 3 and 4B). Myeloperoxidase is thought to be expressed in stage from promyelocytes to mature neutrophils (Manz et al., 2002). In human cord blood cells (Fig. 3), PKC $\iota$  in the cells co-stained with anti-myeloperoxidase antibody was also localized in the nucleus after serum depletion (Fig. 3B top parts). Ten minutes after the addition of G-CSF, PKC $\iota$  was found to translocate into the membrane, and then into the cytosol at 30 min after the addition of G-CSF. In the presence of wortmannin, the G-CSF-induced translocation of PKC $\iota$  into the plasma membrane failed but PKC $\iota$  was found to localize in the cytosol. This suggested that the dynamic translocation of PKC $\iota$  induced by G-CSF is a universal phenomenon in neutrophilic lineage cells. Taken together, these data support the possibility that PI3K plays not only an important role upstream of PKC $\iota$  but also triggers the translocation from nucleus to membrane upon the addition of G-CSF.

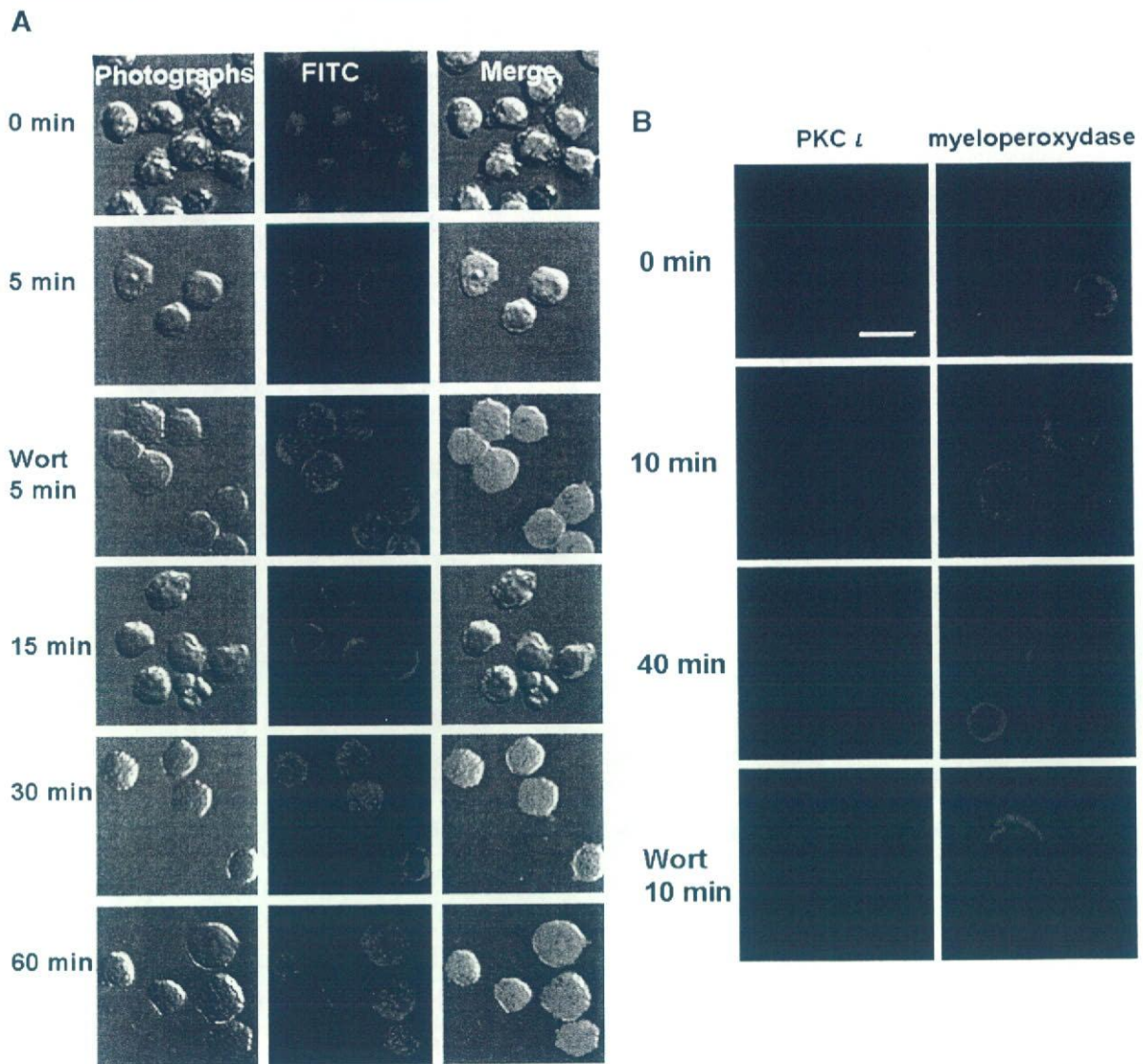
In order to assess the purity of each cellular fraction, antibodies against specific markers were blotted. As specific markers, Histone-H1, Fc $\gamma$  receptor IIa (CD32), and lactate dehydrogenase (LDH) were used for the nuclear, membrane, and cytosolic fractions, respectively. The purities of the nuclear, membrane, and cytosolic fractions were 82.0, 78.5, and 72.2%, respectively (Fig. 4D).

#### Effects of siRNA for PKC $\iota$ on proliferation and differentiation

To determine the role of PKC $\iota$  in neutrophilic proliferation and differentiation, PKC $\iota$  was knocked down by siRNA. When the protein level of PKC $\iota$  was specifically downregulated by siRNA for PKC $\iota$  (Fig. 5A), G-CSF failed to enhance proliferation of the cells during 5 days' cultivation (Fig. 5B). The effect of siRNA for PKC $\iota$  on neutrophilic differentiation in terms of fMLP-R expression was also determined. As shown in Figure 5C, fMLP-R expression was promoted by siRNA for PKC $\iota$  in either the presence (lower part) or absence (upper part) of G-CSF. These data indicate that PKC $\iota$  positively regulates G-CSF-induced proliferation and negatively regulates the differentiation of DMSO-treated HL-60 cells.

#### Discussion

We previously reported that PI3K/p70 S6K plays an important role in the regulation of the neutrophilic differentiation and proliferation of HL-60 cells. Akimoto et al. (1998) and Romanelli et al. (1999) reported that p70 S6K is regulated by aPKC and aPKC $\lambda$ /PKC $\zeta$ , respectively. At first, we showed that the distribution of PKC $\zeta$  and PKC $\iota$  proteins in various human tissues and cells was not similar (Fig. 1A), and that PKC $\iota$  are more abundantly expressed in proliferating blood cells: Jurkat, K562, U937, and HL-60 cells (Fig. 1B). Moreover, PKC $\iota$  proteins were also observed in cultured mononuclear cells of cord blood, in which the myeloid progenitors were enriched in the presence or absence of G-CSF (Fig. 1B). The myeloperoxidase-positive cells as neutrophilic lineage cells, a myeloid marker, were also stained with the antibody of PKC $\iota$  (Fig. 3B). Although PKC $\zeta$  proteins are barely detected in



**Fig. 3.** Translocation of PKC $\zeta$  after the activation of G-CSF. **A:** 2 days after the addition of DMSO, HL-60 cells stimulated by G-CSF were fixed, incubated with anti-PKC $\zeta$  antibody, and visualized as described above. The photographs can be seen at the left part of the figure, the fluorescent photographs in the middle of the figure, and the merged images at the right. **B:** G-CSF-stimulated mononuclear cells from cord blood were stained with anti-PKC $\zeta$  antibody (red, left part) and anti-myeloperoxidase antibody (green, right part) after serum depletion. Under no stimulation, PKC $\zeta$  was observed in the nucleus. G-CSF promoted the translocation of PKC $\zeta$  to the membrane within 5–15 min, and then to the cytosol. Wort: wortmannin. White bar: 10  $\mu$ m.

neutrophilic HL-60 cells, PKC $\zeta$  proteins were markedly expressed in these cells (Fig. 1B). This study showed, for the first time, the stimulation of PKC $\zeta$  activity in G-CSF-treated HL-60 cells (Fig. 2B) at 15–30 min after the addition of G-CSF. Maximum activation from the addition of NGF in PC12 cells was also observed at 15 min (Wooten et al., 2001). Atypical PKCs are lipid-regulated kinases that need to be localized to the membrane in order to be activated. PKC $\zeta$  is directly activated by phosphatidylinositol 3,4,5-trisphosphate, a product of PI3K (Nakanishi et al., 1993). We previously reported that the maximum activation of PI3K was observed in HL-60 cells 5 min after the addition of G-CSF (Kanayasu-Toyoda et al., 2002). Most investigators have reported the translocation of aPKC in either muscle cells or adipocytes stimulated by insulin (Andjelkovic et al., 1997; Goransson et al.,

1998; Galetic et al., 1999; Standaert et al., 1999; Braiman et al., 2001; Chen et al., 2003; Kanzaki et al., 2004; Sasaoka et al., 2004; Herr et al., 2005). In response to insulin stimulation, aPKC $\zeta/\lambda$  is translocated to the plasma membrane (Standaert et al., 1999; Braiman et al., 2001), where aPKC $\zeta/\lambda$  is believed to be activated (Galetic et al., 1999; Kanzaki et al., 2004). In the present study, the addition of G-CSF induced PKC $\zeta$  to translocate to the membrane from the nucleus within 5–15 min (Figs. 3 and 4), and this translocation to the plasma membrane accompanied the full activation of PKC $\zeta$  (Fig. 2B). Previously we reported also that the maximum activation of p70 S6K in HL-60 cells was observed from 30 to 60 min after the addition of G-CSF (Kanayasu-Toyoda et al., 1999, 2002), suggesting that there was a time lag between the activation of PI3K and p70 S6K upon the addition of G-CSF in HL-60 cells. In the present study, PKC $\zeta$  was

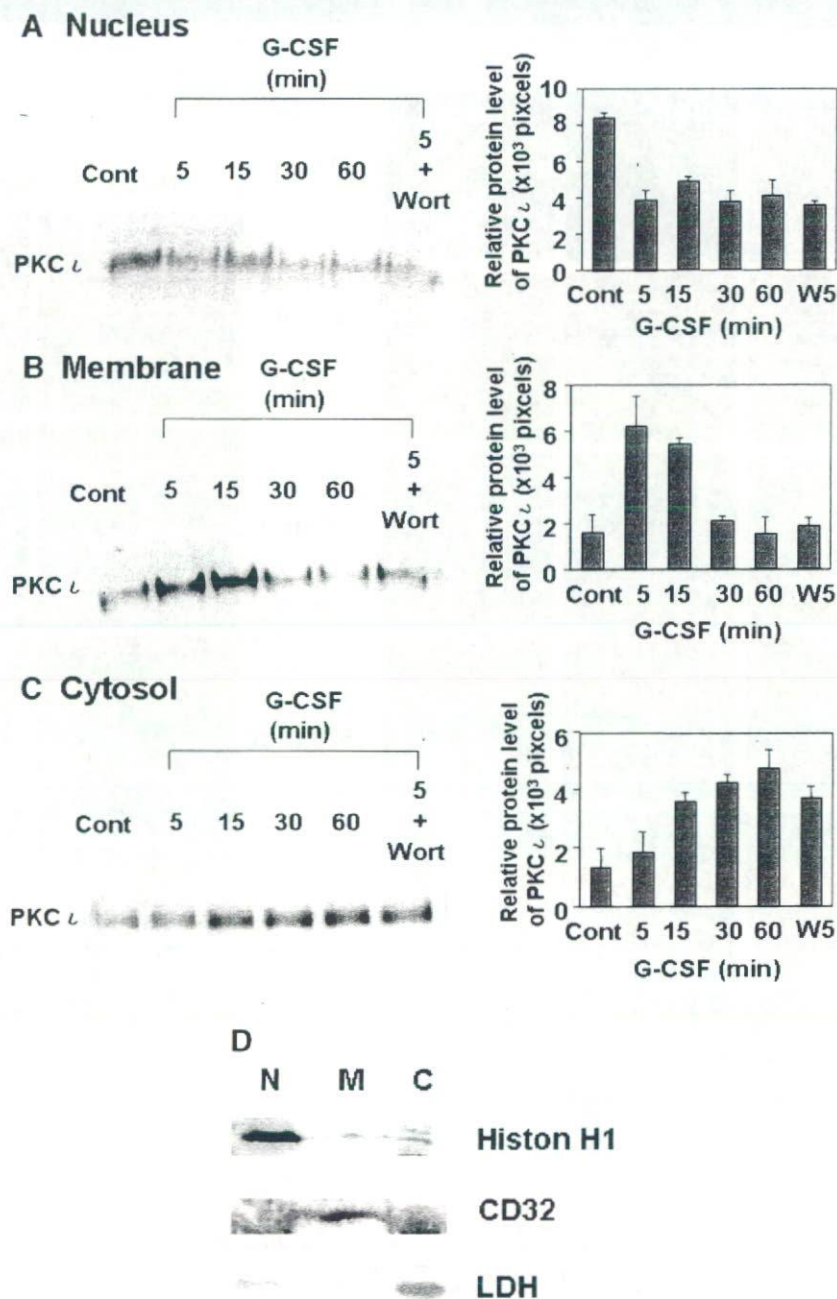


Fig. 4. Translocation of PKC $\zeta$  after activation by G-CSF on biochemical fractionation. The cells were differentiated as described in the Figure 3 legend. After stimulation by G-CSF, the amounts of PKC $\zeta$  proteins in the nucleus (A), plasma membrane (B), and cytosol (C), as fractionated by differential centrifugation, were analyzed by Western blotting (left parts). The right parts show the quantitation of the bands of PKC $\zeta$  proteins. Wort or W: wortmannin. PKC $\zeta$  protein was quantitated using data from three separate experiments. Columns and bars represent the mean  $\pm$  SD. D: Each cell fraction was immunoblotted with antibodies of specific marker. Histone-H1, Fc $\gamma$  receptor IIa (CD32), and lactate dehydrogenase (LDH) are specific markers for nuclear (N), membrane (M), and cytosolic (C) fractions, respectively.

found to re-translocate from the plasma membrane to the cytosol (Figs. 3 and 4C). In the presence of wortmannin, an inhibitor of PI3K, PKC $\zeta$  failed to translocate into the plasma membrane, but instead translocated to cytosol directly from the nucleus upon the addition of G-CSF (Figs. 3 and 4B). PKC $\zeta$  translocation was also observed in myeloperoxidase-positive cells derived from human cord blood (Fig. 3B), indicating that G-CSF-induced dynamic translocation of PKC $\zeta$  occurred in not

only a limited cell line but also neutrophilic lineage cells. These data suggest that PI3K plays an important role in the activation and translocation of PKC $\zeta$  during the G-CSF-induced activation of myeloid cells. Furthermore, the translocation to the plasma membrane in response to G-CSF is wortmannin sensitive, but the translocation from the nucleus upon G-CSF stimulation is not affected by wortmannin, suggesting that the initial signal of G-CSF-induced PKC $\zeta$  translocation from the nucleus may be



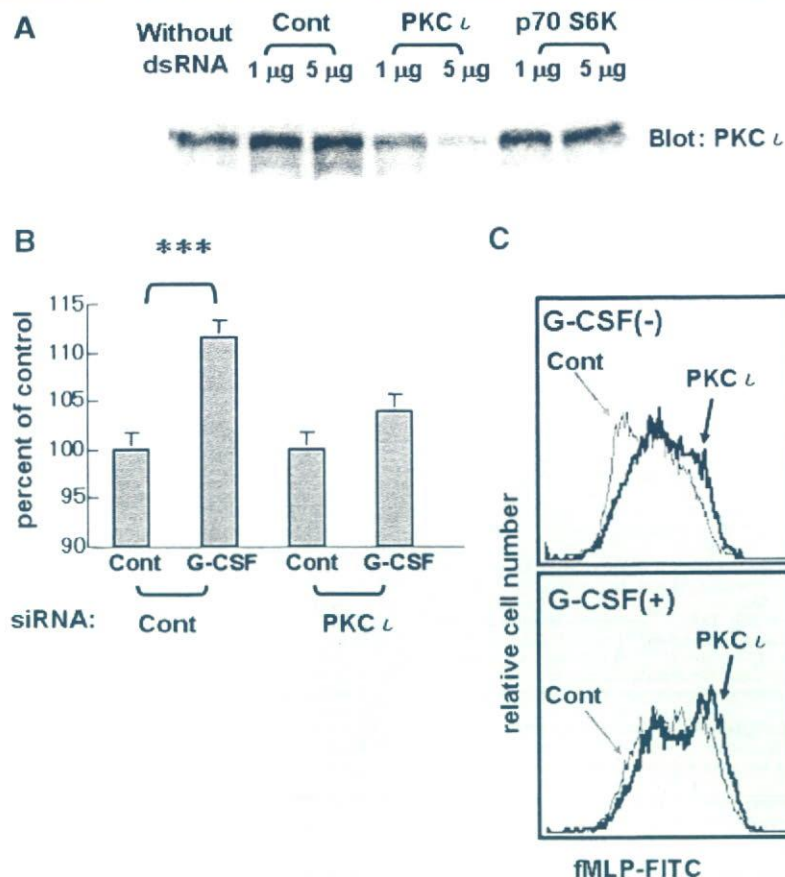


Fig. 5. Effects of siRNA of PKC $\zeta$  on proliferation, differentiation, and phosphorylation at various sites of p70 S6K. **A:** Forty-eight hours after transfection with siRNA of PKC $\zeta$  or p70 S6K, protein levels of PKC $\zeta$  were compared. **B:** Proliferation of the cells transfected with siRNA of PKC $\zeta$  or control (Cont) was measured 5 days after the addition of G-CSF. Columns and bars represent the mean  $\pm$  SD of triplicate wells (\*\* $P < 0.001$ ). **C:** fMLP-R expression was analyzed by flow cytometry 5 days after the addition of G-CSF. The gray arrow indicates cells transfected with the control sequence of double-stranded RNA (Cont, gray lines), and the black arrow the cells transfected with siRNA for PKC $\zeta$  (black lines) in the presence (lower part) or absence (upper part) of G-CSF.

PI3K-independent, but association of PKC $\zeta$  with the plasma membrane could be mediated through a PI3K-dependent signal. Cord blood is an important material of blood transplantation for leukemia (Bradstock et al., 2006; Ooi, 2006; Yamada et al., 2006) or for congenital neutropenia (Mino et al., 2004; Nakazawa et al., 2004) because it contains many hematopoietic stem cells such as CD34-positive cells or CD133-positive cells, and also contains immature granulocytes. The neutrophilic differentiation and proliferation are necessary processes after transplantation.

Formyl-Met-Leu-Phe peptide evokes the migration, superoxide production, and phagocytosis of neutrophils through fMLP-R, a suitable marker for neutrophilic differentiation. In this study, the reduction of PKC $\zeta$  by siRNA inhibited G-CSF-induced proliferation (Fig. 5B) and promoted neutrophilic differentiation (Fig. 5C) in terms of fMLP-R expression. These data, however, suggest that PKC $\zeta$  promoted G-CSF-induced proliferation and blocked differentiation at the same time. The substrates of aPKC have recently been reported: namely, the cytoskeletal protein Lethal giant larvae (Lgl) was phosphorylated by *Drosophila* aPKC (Betschinger et al., 2003) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was phosphorylated by PKC $\zeta$  (Tisdale, 2002) directly in both cases. While the direct phosphorylation of p70 S6K by aPKC was not observed (Akimoto et al., 1998; Romanelli et al.,

1999), the enzyme activity of p70 S6K was markedly enhanced by co-transfection with aPKC and PDK-1, the latter of which is recruited to the membrane due to the binding of phosphatidylinositol-3,4,5-trisphosphate to its PH domain (Anderson et al., 1998). The addition of G-CSF induced PKC $\zeta$  to increase phosphorylation at Thr-389, which is the site most closely related to enzyme activity among the multi-phosphorylation sites of p70 S6K (Weng et al., 1998). However, the mammalian target of rapamycin (mTOR), an upstream regulator, also phosphorylates Thr-389 of p70 S6K and markedly stimulates p70 S6K activity under coexistence with PDK-1 (Isotani et al., 1999). We could not rule out the possibility that other PKC isoforms can contribute to the activation of p70 S6K. We postulated that in G-CSF-stimulated HL-60 cells, PKC $\zeta$  contributes to p70 S6K activation as an upstream regulator.

Atypical PKC isoforms are reported to play an important role in the activation of I $\kappa$ B kinase  $\beta$  (Lallena et al., 1999). In PKC $\zeta$ -deficient mice, impaired signaling through the B-cell receptor resulted in the inhibition of cell proliferation and survival while also causing defects in the activation of ERK and the transcription of NF- $\kappa$ B-dependent genes (Martin et al., 2002). Moreover, Lafuente et al. (2003) demonstrated that the loss of Par-4, that is, the genetic inactivation of the aPKC inhibitor, led to an increased proliferative response of

peripheral T cells when challenged through the T-cell receptor. However, it has been reported that PKC $\lambda$ -deficient mice have a lethal phenotype at the early embryonic stage (Soloff et al., 2004). Based on the present results and those of previous reports (Kanayasu-Toyoda et al., 1999, 2002), we postulate that PKC $\zeta$  plays an important role in regulating G-CSF-induced proliferation in neutrophilic lineage cells.

### Acknowledgments

We thank the Metro Tokyo Red Cross Cord Blood Bank (Tokyo, Japan) for their kind cooperation. This work was supported in part by a grand-in-aid for health and labor science research (H17-SAISEI-021) from the Japanese Ministry of Health, Labor and Welfare, and in part by a grand-in-aid for Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation.

### Literature Cited

- Akimoto K, Nakaya M, Yamanaka T, Tanaka J, Matsuda S, Weng QP, Avruch J, Ohno S. 1998. Atypical protein kinase C $\lambda$  binds and regulates p70 S6 kinase. *Biochem J* 335:417–424.
- Anderson KE, Coadwell J, Stephens LR, Hawkins PT. 1998. Translocation of PDK-1 to the plasma membrane is important in allowing PDK-1 to activate protein kinase B. *Curr Biol* 8:684–691.
- Andjelkovic M, Alessi DR, Meier R, Fernandez A, Lamb NJ, Frech M, Cron P, Cohen P, Lucocq JM, Hemmings BA. 1997. Role of translocation in the activation and function of protein kinase B. *J Biol Chem* 272:31515–31524.
- Betschinger J, Mechtler K, Knoblich JA. 2003. The Par complex directs asymmetric cell division by phosphorylating the cytoskeletal protein Lgl. *Nature* 422:326–330.
- Bradstock KF, Hertzberg MS, Kerridge IH, Svensson J, McGurgan M, Huang G, Antonenas V, Gottlieb DJ. 2006. Unrelated umbilical cord blood transplantation for adults with haematological malignancies: Results from a single Australian centre. *Intern Med J* 36:355–361.
- Braiman L, Alt A, Kuroki T, Ohba M, Bak A, Tennenbaum T, Sampson SR. 2001. Activation of protein kinase C zeta induces serine phosphorylation of VAMP2 in the GLUT4 compartment and increases glucose transport in skeletal muscle. *Mol Cell Biol* 21:7852–7861.
- Chen X, Al-Hasani H, Olausson T, Wentz AM, Smith U, Cushman SW. 2003. Activity, phosphorylation state and subcellular distribution of GLUT4-targeted Akt2 in rat adipose cells. *J Cell Sci* 116:3511–3518.
- Chuang LY, Guh JY, Liu SF, Hung MY, Liao TN, Chiang TA, Huang JS, Huang YL, Lin CF, Yang YL. 2003. Regulation of type II transforming-growth-factor-beta receptors by protein kinase C  $\iota$ . *Biochem J* 375:385–393.
- Chung J, Grammer TC, Lemon KP, Kazlauskas A, Blenis J. 1994. PDGF- and insulin-dependent p70 S6K activation mediated by phosphatidylinositol-3-OH kinase. *Nature* 370:71–75.
- Debili N, Robin C, Schiavon V, Letestu R, Pflumio F, Mitjavila-Garcia MT, Coulombel L, Vainchenker W. 2001. Different expression of CD41 on human lymphoid and myeloid progenitors from adults and neonates. *Blood* 97:2023–2030.
- Fritsch G, Buchinger P, Printz D, Fink FM, Mann G, Peters C, Wagner T, Adler A, Gadner H. 1993. Rapid discrimination of early CD34+ myeloid progenitors using CD45-RA analysis. *Blood* 81:2301–2309.
- Galetic I, Andjelkovic M, Meier R, Brodbeck D, Park J, Hemmings BA. 1999. Mechanism of protein kinase B activation by insulin/insulin-like growth factor-I revealed by specific inhibitors of phosphoinositide 3-kinase—Significance for diabetes and cancer. *Pharmacol Ther* 82:409–425.
- Goransson O, Wijkander J, Manganiello V, Degerman E. 1998. Insulin-induced translocation of protein kinase B to the plasma membrane in rat adipocytes. *Biochem Biophys Res Commun* 246:249–254.
- Hao QL, Zhu J, Price MA, Payne KJ, Barsky LW, Crooks GM. 2001. Identification of a novel, human multilymphoid progenitor in cord blood. *Blood* 97:3683–3690.
- Herr HJ, Bernard JR, Reeder DV, Rivas DA, Limon JJ, Yaspelkis BB3rd. 2005. Insulin-stimulated plasma membrane association and activation of Akt2, aPKC zeta and aPKC lambda in high fat fed rodent skeletal muscle. *J Physiol* 565:627–636.
- Huang S, Chen Z, Yu JF, Young D, Bashey A, Ho AD, Law P. 1999. Correlation between IL-3 receptor expression and growth potential of human CD34+ hematopoietic cells from different tissues. *Stem Cells* 17:265–272.
- Isotani S, Hara K, Tokunaga C, Inoue H, Avruch J, Yonezawa K. 1999. Immunopurified mammalian target of rapamycin phosphorylates and activates p70 S6 kinase alpha in vitro. *J Biol Chem* 274:34493–34498.
- Kanayasu-Toyoda T, Yamaguchi T, Uchida E, Hayakawa T. 1999. Commitment of neutrophilic differentiation and proliferation of HL-60 cells coincides with expression of transferrin receptor. Effect of granulocyte colony stimulating factor on differentiation and proliferation. *J Biol Chem* 274:25471–25480.
- Kanayasu-Toyoda T, Yamaguchi T, Oshizawa T, Kogi M, Uchida E, Hayakawa T. 2002. Role of the p70 S6 kinase cascade in neutrophilic differentiation and proliferation of HL-60 cells—a study of transferrin receptor-positive and -negative cells obtained from dimethyl sulfoxide- or retinoic acid-treated HL-60 cells. *Arch Biochem Biophys* 405:21–31.
- Kanayasu-Toyoda T, Yamaguchi T, Oshizawa T, Uchida E, Hayakawa T. 2003. The role of c-Myc on granulocyte colony-stimulating factor-dependent neutrophilic proliferation and differentiation of HL-60 cells. *Biochem Pharmacol* 66:133–140.
- Kanzaki M, Mora S, Hwang JB, Saltiel AR, Pessin JE. 2004. Atypical protein kinase C (PKCzeta/lambda) is a convergent downstream target of the insulin-stimulated phosphatidylinositol 3-kinase and TC10 signaling pathways. *J Cell Biol* 164:279–290.
- Lafuente MJ, Martin P, Garcia-Cao I, Diaz-Meco MT, Serrano M, Moscat J. 2003. Regulation of mature T lymphocyte proliferation and differentiation by Par-4. *Embo J* 22:4689–4698.
- Lallena MJ, Diaz-Meco MT, Bren G, Pava CV, Moscat J. 1999. Activation of IkappaB kinase beta by protein kinase C isoforms. *Mol Cell Biol* 19:2180–2188.
- Liu WS, Heckman CA. 1998. The sevenfold way of PKC regulation. *Cell Signal* 10:529–542.
- Manz MG, Miyamoto T, Akashi K, Weissman IL. 2002. Prospective isolation of human clonogenic common myeloid progenitors. *Proc Natl Acad Sci USA* 99:11872–11877.
- Martin P, Duran A, Minguet S, Gaspar ML, Diaz-Meco MT, Rennert P, Leitges M, Moscat J. 2002. Role of zeta PKC in B-cell signaling and function. *Embo J* 21:4049–4057.
- Mino E, Kobayashi R, Yoshida M, Suzuki Y, Yamada M, Kobayashi K. 2004. Umbilical cord blood stem cell transplantation from unrelated HLA-matched donor in an infant with severe congenital neutropenia. *Bone Marrow Transplant* 33:969–971.
- Muscella A, Greco S, Elia MG, Storelli C, Marsigliante S. 2003. PKC-zeta is required for angiotensin II-induced activation of ERK and synthesis of C-FOS in MCF-7 cells. *J Cell Physiol* 197:61–68.
- Nakanishi H, Brewer KA, Exton JH. 1993. Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 268:13–16.
- Nakazawa Y, Sakashita K, Kinoshita M, Saida K, Shigemura T, Yanagisawa R, Shikama N, Kamijo T, Koike K. 2004. Successful unrelated cord blood transplantation using a reduced-intensity conditioning regimen in a 6-month-old infant with congenital neutropenia complicated by severe pneumonia. *Int J Hematol* 80:287–290.
- Nguyen BT, Dessauer CW. 2005. Relaxin stimulates protein kinase C zeta translocation: Requirement for cyclic adenosine 3',5'-monophosphate production. *Mol Endocrinol* 19:1012–1023.
- Ooi J. 2006. The efficacy of unrelated cord blood transplantation for adult myelodysplastic syndrome. *Leuk Lymphoma* 47:599–602.
- Rappold I, Ziegler BL, Kohler I, Marchetto S, Rosnet O, Birnbaum D, Simmons PJ, Zannettino AC, Hill B, Neu S, Knapp W, Alitalo K, Ullrich A, Kanz L, Buhning HJ. 1997. Functional and phenotypic characterization of cord blood and bone marrow subsets expressing FLT3 (CD135) receptor tyrosine kinase. *Blood* 90:1111–1125.
- Romanelli A, Martin KA, Tokar A, Blenis J. 1999. p70 S6 kinase is regulated by protein kinase C $\zeta$  and participates in a phosphoinositide 3-kinase-regulated signalling complex. *Mol Cell Biol* 19:2921–2928.
- Sasaoka T, Wada T, Fukui K, Murakami S, Ishihara H, Suzuki R, Tobe K, Kadowaki T, Kobayashi M. 2004. SH2-containing inositol phosphatase 2 predominantly regulates Akt2, and not Akt1, phosphorylation at the plasma membrane in response to insulin in 3T3-L1 adipocytes. *J Biol Chem* 279:14835–14843.
- Selbie LA, Schmitz-Peiffer C, Sheng Y, Biden TJ. 1993. Molecular cloning and characterization of PKC  $\iota$ , an atypical isoform of protein kinase C derived from insulin-secreting cells. *J Biol Chem* 268:24296–24302.
- Soloff RS, Katayama C, Lin MY, Feramisco JR, Hedrick SM. 2004. Targeted deletion of protein kinase C lambda reveals a distribution of functions between the two atypical protein kinase C isoforms. *J Immunol* 173:3250–3260.
- Standaert ML, Bandyopadhyay G, Perez L, Price D, Galloway L, Poklepovic A, Sajan MP, Cenni V, Sirri A, Moscat J, Tokar A, Farese RV. 1999. Insulin activates protein kinases C-zeta and C-lambda by an autophosphorylation-dependent mechanism and stimulates their translocation to GLUT4 vesicles and other membrane fractions in rat adipocytes. *J Biol Chem* 274:25308–25316.
- Tenen DG, Hromas R, Licht JD, Zhang DE. 1997. Transcription factors, normal myeloid development, and leukemia. *Blood* 90:489–519.
- Tisdale EJ. 2002. Glyceroldehyde-3-phosphate dehydrogenase is phosphorylated by protein kinase C $\iota$ /lambda and plays a role in microtubule dynamics in the early secretory pathway. *J Biol Chem* 277:3334–3341.
- Ward AC, Loeb DM, Soede-Bobok AA, Touw IP, Friedman AD. 2000. Regulation of granulopoiesis by transcription factors and cytokine signals. *Leukemia* 14:973–990.
- Weng QP, Kozlowski M, Belham C, Zhang A, Comb MJ, Avruch J. 1998. Regulation of the p70 S6 kinase by phosphorylation in vivo. Analysis using site-specific anti-phosphopeptide antibodies. *J Biol Chem* 273:16621–16629.
- Wooten MW, Vandenplas ML, Seibenheuer ML, Geetha T, Diaz-Meco MT. 2001. Nerve growth factor stimulates multisite tyrosine phosphorylation and activation of the atypical protein kinase C's via a src kinase pathway. *Mol Cell Biol* 21:8414–8427.
- Yamada K, Mizusawa M, Harima A, Kajiwara K, Hamaki T, Hoshi K, Kozai Y, Kodo H. 2006. Induction of remission of relapsed acute myeloid leukemia after unrelated donor cord blood transplantation by concomitant low-dose cytarabine and calcitriol in adults. *Eur J Haematol* 77:345–348.
- Yamaguchi T, Mukasa T, Uchida E, Kanayasu-Toyoda T, Hayakawa T. 1999. The role of STAT3 in granulocyte colony-stimulating factor-induced enhancement of neutrophilic differentiation of Me2SO-treated HL-60 cells. GM-CSF inhibits the nuclear translocation of tyrosine-phosphorylated STAT3. *J Biol Chem* 274:15575–15581.



## Study on the quality control of cell therapy products Determination of *N*-glycolylneuraminic acid incorporated into human cells by nano-flow liquid chromatography/Fourier transformation ion cyclotron mass spectrometry

Noritaka Hashii<sup>a,b</sup>, Nana Kawasaki<sup>a,b,\*</sup>, Yukari Nakajima<sup>a,b</sup>, Masashi Toyoda<sup>c</sup>,  
Yoko Katagiri<sup>c</sup>, Satsuki Itoh<sup>a</sup>, Akira Harazono<sup>a</sup>,  
Akihiro Umezawa<sup>c</sup>, Teruhide Yamaguchi<sup>a</sup>

<sup>a</sup> Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

<sup>b</sup> Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Agency (JST),  
4-1-8 Hon-cho, Kawaguchi, Saitama 332-0012, Japan

<sup>c</sup> National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan

Received 6 February 2007; received in revised form 16 May 2007; accepted 21 May 2007

Available online 25 May 2007

### Abstract

*N*-Glycolylneuraminic acid (NeuGc), an acidic nine-carbon sugar, is produced in several animals, such as cattle and mice. Since human cells cannot synthesize NeuGc, it is considered to be immunogenic in humans. Recently, NeuGc contamination was reported in human embryonic stem cells cultured with xenogeneic serum and cells, suggesting that possibly NeuGc may harm the efficacy and safety of cell therapy products. Sialic acids have been determined by derivatization with 1,2-diamino-4,5-methylenedioxybenzene (DMB) followed by liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography/tandem mass spectrometry (LC/MS/MS); however, the limited availability of cell therapy products requires more sensitive and specific methods for the quality test. Here we studied the use of nano-flow liquid chromatography/Fourier transformation ion cyclotron resonance mass spectrometry (nanoLC/FTMS) and nanoLC/MS/MS for NeuGc-specific determination at a low femtomole level. Using our method, we found NeuGc contamination of the human cell line (HL-60RG cells) cultured with human serum. Our method needs only  $2.5 \times 10^3$  cells for one injection and would be applicable to the determination of NeuGc in cell therapy products.

© 2007 Elsevier B.V. All rights reserved.

**Keywords:** *N*-Glycolylneuraminic acid; Nano-flow liquid chromatography; Fourier transformation ion cyclotron mass spectrometry; Cell therapy products

### 1. Introduction

Sialic acids are a family of acidic nine-carbon sugars found in the non-reducing terminal of *N*-linked and *O*-linked oligosaccharides of glycoproteins and glycolipids [1,2]. There are more than 30 members with different substitutions on the amino group at carbon 5 and on hydroxyl groups at carbons 4, 7, 8 and 9 [2–8]. *N*-Glycolylneuraminic acid (NeuGc), a 5-*N*-glycolylated sialic acid, is produced in several animals, such as cattle, horses, mice and rats [9]. Since human cells cannot

synthesize NeuGc due to mutation of the cytidine monophospho (CMP)-*N*-acetylneuraminic acid (NeuAc) hydroxylase gene [10,11], NeuGc is considered to be antigenic and to induce immunoreaction in humans [4,12,13].

Advances in biotechnology and cell culture techniques make it possible to administer human and animal cells directly to patients as cell therapy products. In cell therapy and tissue engineering, human embryonic stem (ES) cells are expected to be useful for the treatment of many diseases. Recently, it was reported that NeuGc is incorporated into ES cells from human and mouse feeder cells and cultivation media containing xenogeneic serum, such as fetal calf serum (FCS) [14,15]. Since NeuGc is a foreign component in humans, it is feared that NeuGc may harm the efficacy and safety of cell therapy products. To

\* Corresponding author. Tel.: +81 3 3700 9074; fax: +81 3 3700 9084.  
E-mail address: [nana@nihs.go.jp](mailto:nana@nihs.go.jp) (N. Kawasaki).

assess the adverse effects of NeuGc, it is necessary to quantify NeuGc in cell therapy products.

Sialic acids have been determined by labeling with 1,2-diamino-4,5-methylenedioxybenzene (DMB) followed by conventional high-performance liquid chromatography (HPLC) with fluorescent detection [16–20]. The femtomole level of sialic acid can be determined by fluorescent detection [19]. The use of liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography/tandem mass spectrometry (LC/MS/MS) has more advantage in the identification of sialic acid species [18,20–22]. The derivatization of sialic acids with DMB has advantages of good separation of NeuGc from NeuAc in chromatography and enhancement of ionization efficiency in MS. However, more sensitive and specific methods are desired for the quality control of cell therapy products, since in many case only a low number of cell products, approximately  $1 \times 10^6$  to  $1 \times 10^8$ , should be available for quality tests.

In this study, we studied the use of nano-flow liquid chromatography/Fourier transformation ion cyclotron resonance mass spectrometry (nanoLC/FTMS) and LC/MS/MS to achieve the sensitive and specific determination of NeuGc. The potential of the method for quality testing of cell therapy products was evaluated using substrain of human promyelocytic leukemia HL-60 cells (HL-60RG cells) as model cells. Using this method, we determined NeuGc in membrane fractions from HL-60RG cells cultured with FCS, human serum and serum-free medium.

## 2. Experimental

### 2.1. Materials

NeuGc and NeuAc were purchased from Nacalai Tesque (Kyoto, Japan). FCS and normal human serum were purchased from Dainippon Sumitomo Pharma (Osaka, Japan). RPMI1640 medium and ASF104 medium were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Ajinomoto (Tokyo, Japan), respectively.

### 2.2. Cell culture

Substrain of human promyelocytic leukemia HL-60 cells (HL-60RG cells, JCRB Cellbank, Osaka, Japan) was cultured in RPMI1640 medium supplemented with 10% FCS, 100 unit/ml of penicillin and 100  $\mu$ g/ml of streptomycin under a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37 °C. HL-60RG cells were replaced at  $2 \times 10^5$  cells/100 mm dish in RPMI1640 medium supplemented with 10% FCS or 10% normal human serum, and in serum-free ASF104 medium. The media were replaced four times, and semi-confluent growth cells were harvested.

### 2.3. Fractionation of the membrane fraction

The cells were washed in phosphate buffer saline (PBS) supplemented with protease inhibitors (protease inhibitor mix

solution, Wako, Tokyo, Japan) three times. The washed cells ( $1 \times 10^6$ ) were suspended in 100  $\mu$ l of 0.25 M sucrose/10 mM Tris-HCl buffer (pH 7.4) containing protease inhibitors, and sonicated at 4 °C for 30 s, two times (40W, Bioruptor UCW-201, Tosyoudenki, Kanagawa, Japan). After the nuclei were removed by centrifugation at 4 °C,  $450 \times g$  for 10 min, the mitochondria and lysosome fractions were removed by re-centrifugation at 4 °C,  $20,000 \times g$  for 10 min. The membrane fractions were precipitated by ultracentrifugation at 4 °C,  $100,000 \times g$  for 60 min. The membrane fractions were washed in 100  $\mu$ l of 150 mM ammonium acetate buffer (pH 7.4) and recovered by re-ultracentrifugation.

### 2.4. Derivatization of NeuGc and NeuAc with DMB reagent

The membrane fractions were sonicated in 250  $\mu$ l of H<sub>2</sub>O and then incubated with 250  $\mu$ l of 4 M acetic acid (final concentration, 2 M) at 80 °C for 3 h. The released sialic acids were passed through a solid-phase extraction cartridge (SepPak C-18, Waters, Milford, MA, USA) with 2 ml of H<sub>2</sub>O, dried under vacuum, and resolved in 50  $\mu$ l of H<sub>2</sub>O. The solution was incubated with DMB according to the manufacturer's instruction (Takara, Tokyo, Japan), and the reaction mixture was applied on a solid-phase extraction cartridge (Envi-Carb C, Supelco, Bellefonte, PA, USA). After washing the cartridge with 2.5 ml of 5 mM ammonium acetate (pH 9.6) for desalting, the DMB-labeled sialic acids were eluted with 3 ml of 45% acetonitrile/5 mM ammonium acetate (pH 9.6). The collected fraction was freeze dried.

### 2.5. nanoLC/FTMS

DMB-labeled sialic acids were separated by HPLC using Paradigm MS4 (Michrom BioResource, Auburn, CA, USA) equipped with a reversed-phase C18 column (Magic C18, 50 mm  $\times$  0.1 mm, 3  $\mu$ m, Michrom BioResource, Auburn, CA, USA). Elution was achieved using 0.1% formic acid/2% ace-

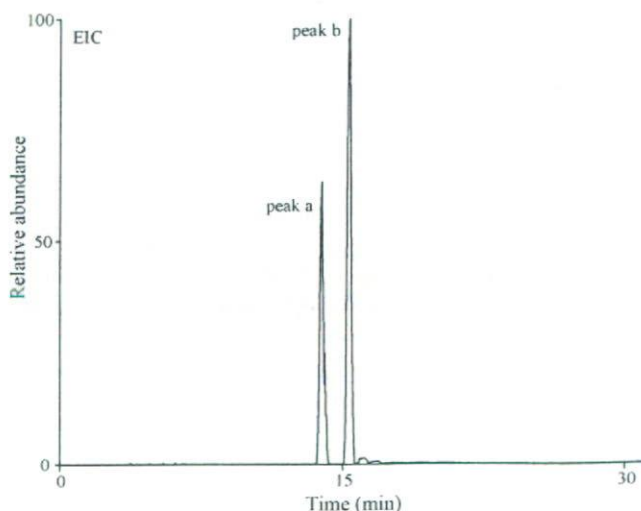


Fig. 1. EIC at  $m/z$  426.13–426.17 and  $m/z$  442.12–442.16 obtained by SIM ( $m/z$  400–450) of DMB-NeuGc and DMB-NeuAc in the positive ion mode.