Upregulation of Tenascin-C Expression by IL-13 in Human Dermal Fibroblasts via the Phosphoinositide 3-kinase/Akt and the Protein Kinase C Signaling Pathways

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In this study, we examined the genes targeted by IL-13 in human dermal fibroblasts using a cDNA microarray. We focused on the tenascin-C (TN-C) gene, which was identified as one of the genes induced by IL-13. IL-13 induced transcriptional activity of TN-C. IL-13-mediated TN-C expression was inhibited by treatment with wortmannin or LY294002, or Calphostin C. IL-13 induced the phosphorylation of the phosphoinositide 3-kinase (PI3K) regulatory subunit p85, induced tyrosine phosphorylation of Akt, upregulated Akt kinase activity, and activated protein kinase C (PKC)-δ and -ε. The IL-13-induced increase in TN-C protein expression was abrogated by the transfection of a dominant-negative mutant of Akt, PKC-δ, or PKC-ε. In conclusion, we showed that the PI3K/Akt and/or PKC signaling pathways are essential for the IL-13-mediated increase in TN-C. Both serum levels of IL-13 and the expression levels of TN-C in the dermis are increased in patients with systemic sclerosis. Our findings suggest that the expression of TN-C is upregulated in this disease due to IL-13 signaling, and that a blockade of the PI3K or PKC signaling pathway may also have therapeutic value by reducing the amount of TN-C produced during fibrosis.

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INTRODUCTION

IL-13 is a lymphokine of 112 amino acids with a molecular mass of 12.5 kDa produced by activated Type 2 helper cells (Minty *et al.,* 1993). The gene for IL-13 is closely linked to the IL-4 gene (Morgan *et al.,* 1992), and IL-13 protein has been shown to have a 30% identity in amino-acid sequence with IL-4 protein (Zurawski *et al.,* 1993). In addition, the receptor for IL-4 and that for IL-13 may share a common component that appears to function in signal transduction (Zurawski *et al.,* 1993).

Sharing many biological properties with IL-4 as a pleiotropic immune regulatory cytokine, IL-13 induces changes in the cell surface phenotype and has immunomodulatory effects on B cells and human monocytes (McKenzie *et al.,* 1993; Zurawski and de Vries, 1994). In sharp contrast to IL-4, IL-13 has been shown not to affect resting or activated T cells (Punnonen *et al.,* 1993; Zurawski and de Vries, 1994).

On the other hand, there have been quite a few reports that discussed the effects of Il-13 on dermal fibroblasts. High-affinity IL-13 receptors are reported to be expressed in normal skin fibroblasts (Yoshidome *et al.,* 1999). The expression of total collagen and α1(III) procollagen is upregulated in normal skin fibroblasts after stimulation by IL-13 (Oriente *et al.,* 2000). Moreover, IL-13 inhibits production of IL-1β-induced matrix metalloproteinase-1 and -3 and enhances production of tissue inhibitor of metalloproteinase-1 by dermal fibroblasts (Oriente *et al.,* 2000). Thus, in the skin, IL-13 is thought to be associated with the regulation of extracellular matrix synthesis and tissue remodeling.

In this study, we identified the target genes of IL-13 in human dermal fibroblasts using a cDNA microarray. We then focused on the tenascin-C (TN-C) gene, which was identified as one of the genes induced by IL-13 in the microarray analysis. We clarified the mechanisms by which TN-C expression is regulated by IL-13 in dermal fibroblasts, because both IL-13 and TN-C are implicated in fibrotic disorders.

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Abbreviations: PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; TGF, transforming growth factor; TN-C, tenascin-C; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

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RESULTS

Effects of IL-13 on gene expression profiles as measured with the cDNA microarray

The technique of differential hybridization of cDNA expression arrays was used to identify differences in the expression pattern of genes between control and IL-13-treated fibroblasts. Among nearly 630 genes, over 300 showed no significant hybridization signal in either control or IL-13-treated cultures. Results for important genes are shown in Table 1. The expression of extracellular matrix-related genes or cancer-related genes as well as immune-related genes was upregulated by IL-13. TN-C expression increased significantly

Table 1. Summary of Il-13-regulated genes identified by microarray analysis

	Gene accession number	Gene name	Expression ratio
Extracelluar matrix-related genes	NM_004994	Matrix metalloproteinase 9 (gelatinase B, 92 kDa gelatinase, 92 kD type IV collagenase)	0.20
	NM_002427	Matrix metalloproteinase 13 (collagenase 3)	0.36
	NM_003247	Thrombospondin 2	0.37
	NM_002345	Lumican	0.43
	U16306	Chondroitin sulfate proteoglycan 2 (versican)	0.59
	NM_002290	Laminin, alpha 4	0.63
	X02761	Fibronectin 1	0.84
	NM_002428	Matrix metalloproteinase 15 (membrane-inserted)	1.73
	NM_002160	Tenascin-C	3.24
Immune response-related genes	NM_006152	Lymphoid-restricted membrane protein	0.17
	NM_005601	Natural killer cell group 7 sequence	0.27
	NM_002209	Integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide)	0.28
	NM_001771	CD22 antigen	0.30
	NM_000576	Interleukin 1, beta	0.36
	NM_004931	CD8 antigen, beta polypeptide 1 (p37)	0.36
	L36531	Integrin, alpha 8	0.40
	X68742	Integrin, alpha 1	0.41
	Y14737	Immunoglobulin heavy constant gamma 3 (G3m marker)	0.46
	NM_001773	CD34 antigen	0.50
	NM_006889	CD86 antigen (CD28 antigen ligand 2, B7-2 antigen)	0.55
	NM_001769	CD9 antigen (p24)	1.96
	NM_005356	Lymphocyte-specific protein tyrosine kinase	4.73
Cancer-related genes	X16354	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	0.12
	NM_000059	Breast cancer 2, early onset	0.22
	NM_003810	Tumor necrosis factor (ligand) superfamily, member 10	0.25
	NM_004619	TNF receptor-associated factor 5	0.39
	NM_006910	retinoblastoma-binding protein 6	0.40
	AF016266	Tumor necrosis factor receptor superfamily, member 10b	0.40
	NM_000594	Tumor necrosis factor (TNF superfamily, member 2)	0.46
	NM_004833	Absent in melanoma 2	0.47
	NM_007295	Breast cancer 1, early onset	0.48
	NM_005426	Tumor protein p53-binding protein, 2	0.51
	NM_006850	Suppression of tumorigenicity 16 (melanoma differentiation)	0.54
	NM_000043	Tumor necrosis factor receptor superfamily, member 6	0.55
	NM_000575	Interleukin 1, alpha	0.56

Table 1 continued on following page

Table 1. continued

	Gene accession number	Gene name	Expression ratio
	NM_000584	Interleukin 8	0.56
	NM_005657	Tumor protein p53-binding protein, 1	0.61
	NM_005610	Retinoblastoma-binding protein 4	0.62
	NM_004620	TNF receptor-associated factor 6	0.67
	NM_002892	Retinoblastoma-binding protein 1	2.10
	NM_007115	Tumor necrosis factor, alpha-induced protein 6	61.15
Transforming growth factor-β-related Genes	NM_000660	Transforming growth factor-β1	1.01
	NM_003238	Transforming growth factor-β2	0.34
	NM_003239	Transforming growth factor-β3	0.42
	NM_003243	Transforming growth factor-β receptor III	0.78
	D50683	Transforming growth factor-β receptor II	0.62
Others	NM_000372	Tyrosinase (oculocutaneous albinism IA)	0.26
	NM_002274	Keratin 13	0.26
	NM_005922	Mitogen-activated protein kinase kinase kinase 4	0.32
	NM_001992	Coagulation factor II (thrombin) receptor	0.34
	NM_003238	Transforming growth factor, beta 2	0.34
	X07695	Keratin 4	0.37
	NM_002753	Mitogen-activated protein kinase 10	0.38
	NM_003239	Transforming growth factor, beta 3	0.42
	NM_001723	Bullous pemphigoid antigen 1 (230/240 kDa)	0.44
	NM_004360	Cadherin 1, type 1, E-cadherin (epithelial)	0.45
	NM_006282	Serine/threonine kinase 4	0.52
	NM_006564	G protein-coupled receptor	0.53
	NM_006121	Keratin 1 (epidermolytic hyperkeratosis)	0.53
	D50683	Transforming growth factor, beta receptor II (70-80 kDa)	0.62
	AB023420	Heat shock 70 kDa protein 4	0.63
	NM_001793	Cadherin 3, type 1, P-cadherin (placental)	0.65
	NM_004506	Heat shock transcription factor 2	0.66
	AF005392	Tubulin, alpha 2	1.57
	NM_000125	Estrogen receptor 1	1.87
	NM_000376	Vitamin D (1,25- dihydroxyvitamin D3) receptor	1.92
	X73502	Cytokeratin 20	1.93

in response to IL-13 (3.24-fold). However, transforming growth factor (TGF)-β-related genes were not significantly induced by IL-13 (TGF-β 1, 1.01-fold; TGF-β2, 0.34-fold; TGF-β3, 0.41-fold; TGF-β receptor type II, 0.62-fold; TGF-β receptor type III, 0.78-fold). This result suggested that TGF-β was not involved in the increase in the expression of the TN-C gene induced by IL-13.

TN-C expression in human dermal fibroblasts is upregulated by IL-13

Subsequently, we investigated the effects of IL-13 on the expression of TN-C in human dermal fibroblasts. First, we investigated the effects of various cytokines (IL-13, IL-4, TGF-β, and Oncostatin M; 10 ng/ml) on TN-C protein expression by dermal fibroblasts. Two polypeptides (320 and 220 kDa), corresponding to the two isoforms of TN-C, were detected in the conditioned medium. IL-13 induced TN-C expression modestly, whereas IL-4 had the strongest effect on TN-C protein expression (Figure 1a). The effect of TGF-β and Oncostatin M on TN-C expression was weaker than that of IL-13.

To determine whether the IL-13-mediated induction of TN-C protein expression was correlated with an increase of the mRNA level, mRNA expression was analyzed by Northern blotting. The TN-C mRNA level rose after stimulation with IL-13 for 3 hours, and peaked (3.90-fold) after 12 hours in comparison with the control level (Figure 1b). Thus, the

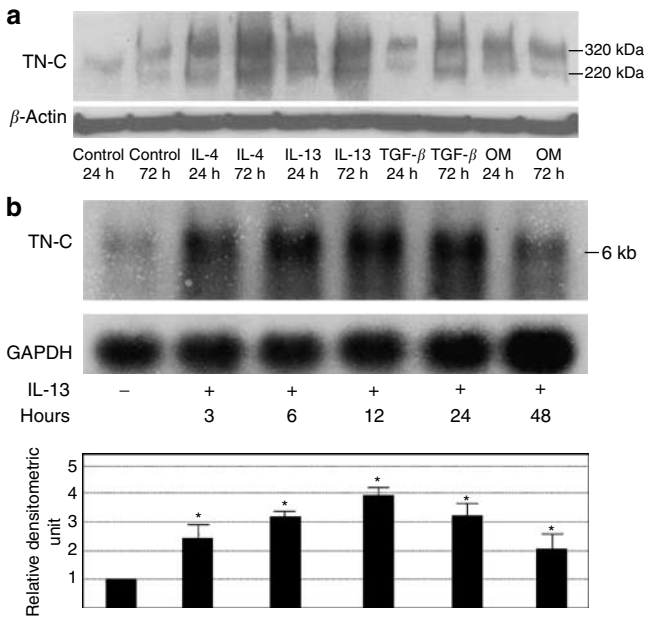

Figure 1. Induction of tenascin-C (TN-C) expression in human fibroblasts by IL-13. (**a**) Cultured fibroblasts were stimulated with the indicated cytokines for 24 or 72 hours. Conditioned media and cell lysates (normalized for protein concentrations as measured with the Bio-Rad reagent) were subjected to immunoblotting with TN-C antibody and anti-β-actin antibody, respectively. OM: Oncostatin M. (**b**) Cultured fibroblasts were incubated in the presence or absence of 10 ng/ml of IL-13 with the indicated time courses, and Northern blot analysis of TN-C mRNA expression was performed. Levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA are shown as a loading control. One experiment representative of three independent experiments is shown. TN-C mRNA levels quantitated by scanning densitometry and corrected for the levels of GAPDH in the same samples are shown relative to the level in untreated cells (1.0) (lower panel). Data are expressed as the mean ± standard deviation of three independent experiments. *$P<0.05$ as compared with the value in untreated cells.

effect of IL-13 on the TN-C protein level paralleled but was delayed relative to that on the mRNA level.

Mechanisms of IL-13-mediated TN-C upregulation

We determined whether the increase in TN-C mRNA levels after IL-13 treatment involves transcriptional activation. Actinomycin D completely blocked the IL-13-mediated increase in TN-C mRNA, suggesting that IL-13's stimulation of TN-C mRNA involves direct activation of the transcription of the TN-C gene and/or other genes involved in regulating the TN-C gene (Figure 2a). Cycloheximide, an inhibitor of protein synthesis, superinduced TN-C mRNA expression in IL-13-treated fibroblasts, suggesting that repressor protein(s) regulate transcription of the TN-C gene in human fibroblasts and IL-13-mediated upregulation of TN-C expression is independent of new protein synthesis.

In addition, we wished to determine whether IL-13 increased the stability of TN-C mRNA. Following the inhibition of transcription by actinomycin D, the loss of IL-13-induced TN-C mRNA expression was not significantly different from that observed in the untreated cells (Figure 2b). The failure of IL-13 to increase the half-life of TN-C mRNA suggests that the IL-13-mediated induction of TN-C expression is regulated at the level of transcription. In fact, direct measurement of the specific gene transcription using the nuclear run-on transcription analysis revealed an elevation in the rate of TN-C gene transcription in fibroblasts treated with IL-13 (3.85-fold) (Figure 2c). Thus, we examined the effect of IL-13 on the promoter activity of the human TN-C gene by transient transfection. IL-13 stimulated the promoter activity of TN-C (2.28-fold) (Figure 2d).

Wortmannin, LY294002, or Calphostin C inhibits IL-13-mediated upregulation of TN-C protein and mRNA expression

We investigated which signaling pathways are involved in the IL-13-mediated TN-C protein or mRNA induction. Pretreatment of cells with phosphoinositide 3-kinase (PI3K) inhibitors, wortmannin and LY294002, or an inhibitor of classical and novel protein kinase C (PKC) isoforms and phospholipase D, Calphostin C, blocked the IL-13-mediated upregulation of TN-C protein expression (Figure 3a). These inhibitors also blocked the IL-13-mediated upregulation of TN-C mRNA expression, whereas rapamycin did not (Figure 3b). However, they did not affect the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, demonstrating that at the indicated concentration, these inhibitors did not have generalized toxic effects. These results suggest that the PI3K, PKC, or phospholipase D signaling pathway is essential for the IL-13-mediated expression of TN-C.

PI3K/Akt signaling pathway is activated following IL-13 treatment, and wortmannin, LY294002, and Akt inhibitor reduce this induction by IL-13

To confirm the involvement of the PI3K/Akt signaling pathway in the IL-13-mediated expression of TN-C, we determined whether the forced overexpression of a dominant-negative mutant of Akt could overcome or inhibit the effect of IL-13. Immunoblotting revealed that IL-13-induced TN-C expression was downregulated by the transfection of the dominant-negative mutant of Akt (Figure 4a).

Next, we investigated whether IL-13 actually activated the PI3K/Akt signaling pathway. PI3K is composed of the catalytic subunit p110 and the regulatory subunit p85. The activity of PI3K is regulated through tyrosine phosphorylation of p85. Immunoprecipitation using anti-PI3K p85 antibodies revealed significant phosphorylation of p85 after 5–30 minutes of treatment with IL-13, followed by a slow decrease in the cellular level of phosphorylated p85 (Figure 4b). Also, immunoblotting using anti-phospho-Akt (Ser 473) antibody revealed significant phosphorylation (3.27-fold) of Akt after 15 minutes of treatment with IL-13 (Figure 4c). Immunoblotting for total Akt protein demonstrated that the amount of Akt did not change in the presence of IL-13.

Furthermore, the Akt kinase assay showed that the level of phosphorylated glycogen synthase kinase-3β was maximal after 15 minutes (3.03-fold) of incubation with IL-13 (Figure 4d). Moreover, cells were pretreated with wortmannin, LY294002, or a selective inhibitor of Akt, Akt inhibitor, for 1 h before stimulation with IL-13, and then the Akt kinase

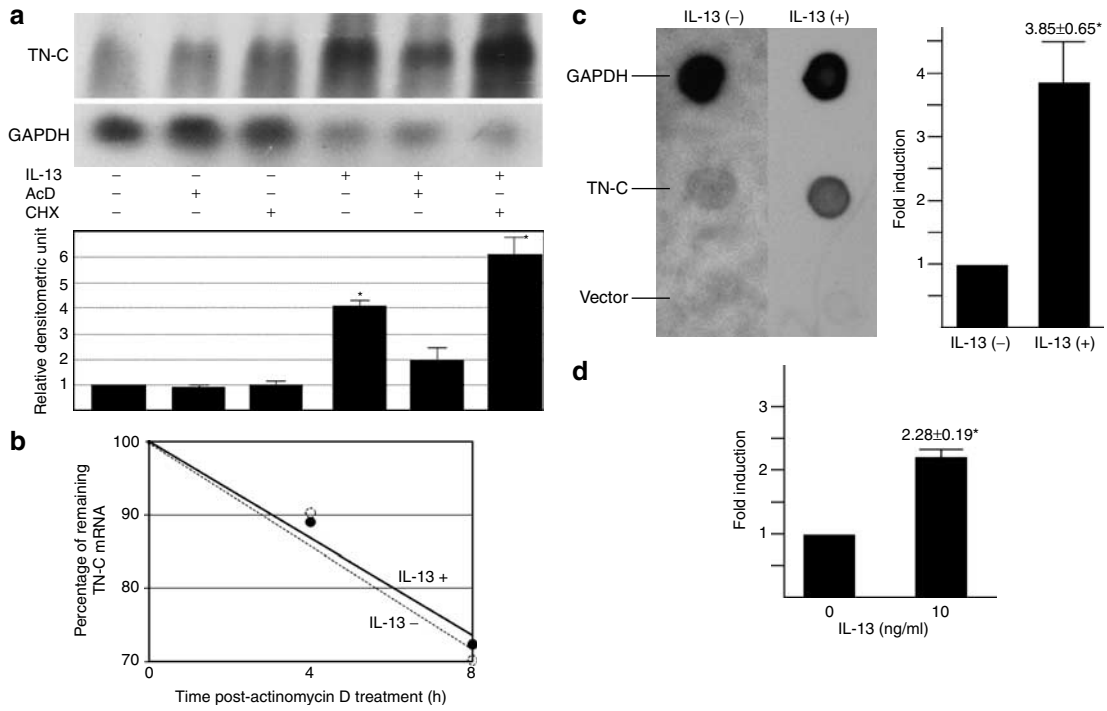

Figure 2. Effects of actinomycin D or cycloheximide on IL-13-mediated increase in tenascin-C (TN-C). (**a**) Human dermal fibroblasts were serum-starved for 24 hours and incubated in the absence or presence of 2.5 μg/ml of actinomycin D or 10 μg/ml of cycloheximide, with or without 10 ng/ml of IL-13, for 12 hours. Northern blot analysis of TN-C mRNA expression was performed. TN-C mRNA levels quantitated by scanning densitometry and corrected for the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same samples are shown relative to the level in untreated cells (1.0) (lower panel). *$P<0.05$ as compared with the value in untreated cells. AcD; actinomycin D, CHX; cycloheximide. (**b**) Effect of IL-13 on the half-life of TN-C mRNA was examined. Human dermal fibroblasts were serum-starved for 24 hours and incubated in the absence or presence of 10 ng/ml of IL-13 for 12 hours before the addition of 2.5 μg/ml of actinomycin D. mRNA was extracted from the cells at the indicated time points after actinomycin D was administrated. Northern blot analysis of TN-C mRNA expression was performed. The corrected density by GAPDH was expressed as a percentage of the value at time 0 and plotted on a scale. The solid line indicates levels in IL-13-treated cells, and the dotted line indicates control (untreated) levels. Statistical analysis was performed comparing the value of each time point with the Mann–Whitney *U*-test. (**c**) Dermal fibroblasts were serum-starved for 24 hours and treated with 10 ng/ml of IL-13 for 12 hours. Nuclei were prepared as described in ''Materials and Methods''. GAPDH is a positive control, and the corresponding vector is a negative control. One experiment representative of three independent experiments is shown. The TN-C gene transcription levels quantitated by scanning densitometry and corrected for the levels of GAPDH transcription in the same samples are shown relative to the level in untreated cells (1.0). (**d**) The plasmid carrying a −2.1 kb fragment of the human TN-C promoter was used in the transient transfection in the presence or absence of IL-13. The number shows the promoter activity stimulated by IL-13 relative to the promoter activity without IL-13, which was set at 1. The means ± standard deviation of four independent experiments are shown. *$P<0.05$ as compared with the value in untreated cells.

assay was performed. Wortmannin (100 nM), LY294002 (30 μM), or Akt inhibitor (20 μM) significantly inhibited the Akt-induced phosphorylation of glycogen synthase kinase-3β, whereas rapamycin (10 μM) did not (Figure 4e). These results suggest that the treatment with IL-13 results in the activation of the PI3K/Akt signaling pathway in human dermal fibroblasts and that wortmannin, LY294002, or Akt inhibitor inhibits the expression of TN-C in human dermal fibroblasts stimulated by IL-13.

Activation of PKC in IL-13-stimulated fibroblasts

We investigated whether the activation of PKC is involved in the IL-13-mediated production of TN-C protein and which isozyme of PKC is activated by IL-13.

Pretreatment of cells with the selective PKC-δ inhibitor Rottlerin (Gschwendt *et al.,* 1994) blocked the IL-13-mediated upregulation of TN-C protein expression (Figure 5a), whereas the selective PKC-α inhibitor Gö6976 (Martiny-Baron *et al.,* 1993) did not. Furthermore, the overexpression of dominant-negative PKCs had no significant effect on TN-C protein expression in the absence of IL-13, whereas IL-13's effect on TN-C protein was abrogated by the dominant-negative PKC-δ as well as -ε (Figure 5b). These results suggest that IL-13 regulates the expression of TN-C protein through PKC signaling, especially PKC-δ and -ε, in human dermal fibroblasts.

It is generally accepted that inactive PKC isoforms are located in the nucleus or cytoplasm, and translocated to the plasma membrane and/or cytoskeleton after activation (Mochly-Rosen *et al.,* 1990; Tourkina *et al.,* 2001). Next, to examine the activation of PKC-α, -δ, and -ε by IL-13, immunofluorescence microscopy using anti-PKC-α, -δ, and -ε antibodies was performed. The result revealed that only PKC-δ and -ε were translocated to the plasma membrane in IL-13-treated fibroblasts (Figure 5c).

DISCUSSION

TN-C is a polymorphic high-molecular mass extracellular matrix glycoprotein composed of six similar subunits joined

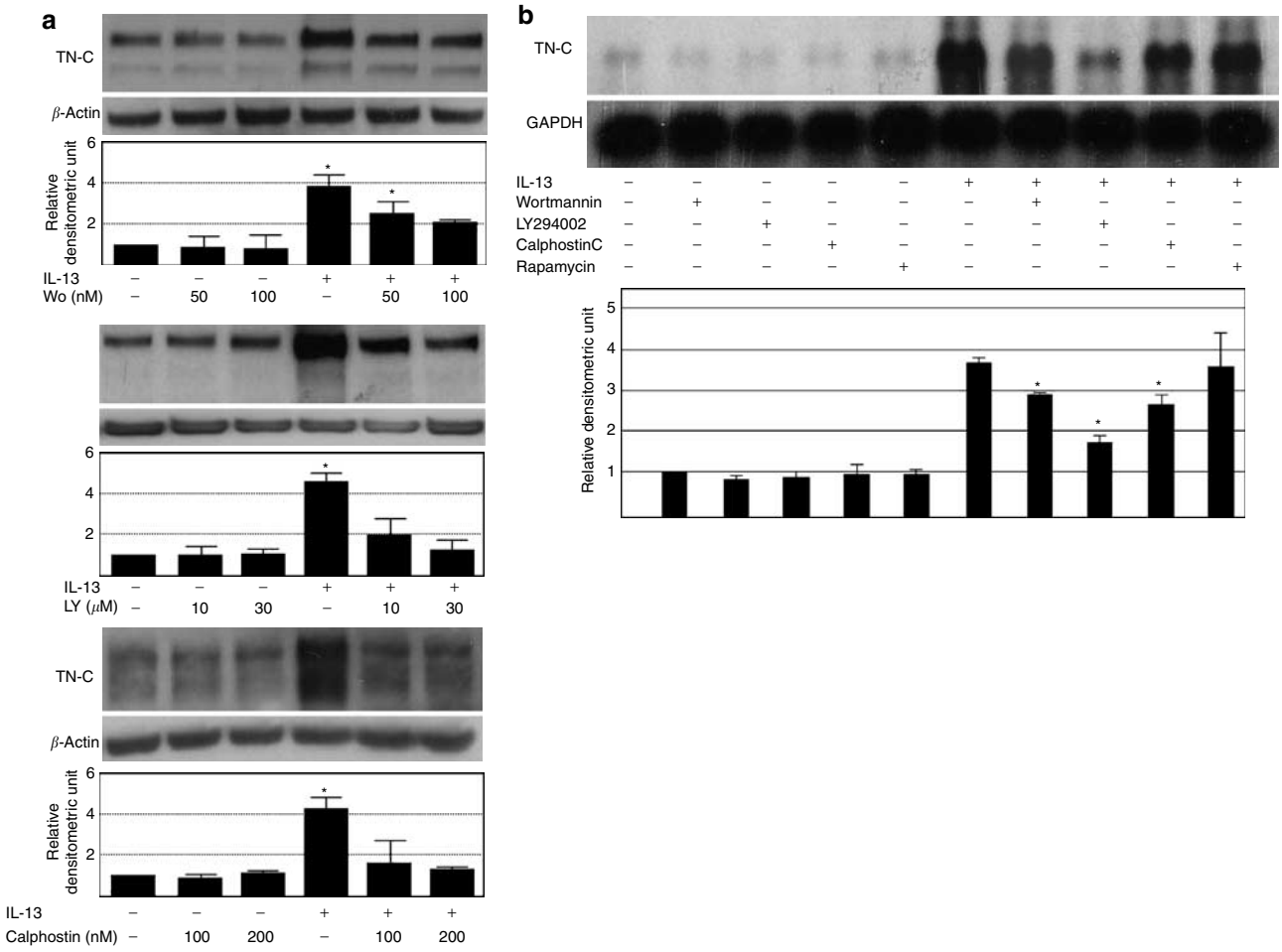

Figure 3. Effects of wortmannin, LY294002, and Calphostin C on IL-13-induced tenascin-C (TN-C) expression. (**a**) Human dermal fibroblasts were serum-starved for 24 hours and pre-treated with 50 or 100 nM wortmannin, 10 or 30 μM LY294002, or 100 or 200 nM Calphostin C, for 1 h before the addition of 10 ng/ml of IL-13 for 72 hours. Conditioned media and cell lysates (normalized for protein concentrations as measured with the Bio-Rad reagent) were subjected to immunoblotting with TN-C antibody (conditioned media) and anti-β-actin antibody (cell lysates), respectively. One experiment representative of three independent experiments is shown. The levels of TN-C quantitated by scanning densitometry and corrected for the levels of β-actin are shown relative to the level in untreated (1.0). *$P<0.05$ as compared with the value in untreated cells. Wo; wortmannin, LY: LY294002. (**b**) A Northern blot analysis of TN-C mRNA expression was performed. Wortmannin (100 nM), LY294002 (30 μM), Calphostin C (200 nM), and rapamycin (10 μM) were added 1 h before the addition of 10 ng/ml of IL-13. After 24 h, cells were collected. One experiment representative of three independent experiments is shown. TN-C mRNA levels quantitated by scanning densitometry and corrected for the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same samples are shown relative to the level in untreated cells (1.0) (lower panel). *$P<0.05$ as compared with the value in IL-13-treated cells without inhibitors.

together at their NH_2 terminus by disulfide bonds (Erickson, 1993). Although its exact biological functions are still unknown, TN-C has been shown to inhibit cell adhesion to fibronectin and included among ''antiadhesive molecules'', which seem to play roles in the processes of morphogenesis, wound healing, and cancer progression (Borsi *et al.,* 1994).

It is known that the expression of TN-C is increased under fibrotic conditions such as scarring (Harty *et al.,* 2003). Upregulation of TN-C expression is also observed in the dermis or lungs of patients with systemic sclerosis, an autoimmune disorder characterized by the excessive deposition of extracellular matrix components in the skin and internal organs (Rodnan *et al.,* 1979; LeRoy, 1992; Tourkina *et al.,* 2001). On the other hand, IL-13 has also been implicated in the pathogenesis of fibrotic disease in several organs (Lee *et al.,* 2001). We previously reported that IL-13 induced the expression of type I collagen to the same extent as IL-4 (Jinnin *et al.,* 2004b). Previous studies showed that IL-13 induced hepatic or pulmonary fibrosis in mice *in vivo* (Chiaramonte *et al.,* 1999; Zhu *et al.,* 1999). In addition, Kolodsick *et al.* (2004) reported that IL-13-deficient mice were protected from fluorescein isothiocyanate-induced fibrosis of the lung because of impaired collagen synthesis by fibroblasts, whereas IL-4-deficient mice were not protected. IL-13 may play more important roles in the regulation of fibrosis *in vivo* than IL-4. It was reported that serum levels of IL-13 increased reflecting systemic inflammation in patients with systemic sclerosis (Hasegawa *et al.,* 1997). Furthermore, the amount of IL-13 protein was shown to be increased in the culture supernatants of alveolar macrophages from systemic sclerosis patients compared to control subjects (Hancock *et al.,* 1998). These findings suggest that

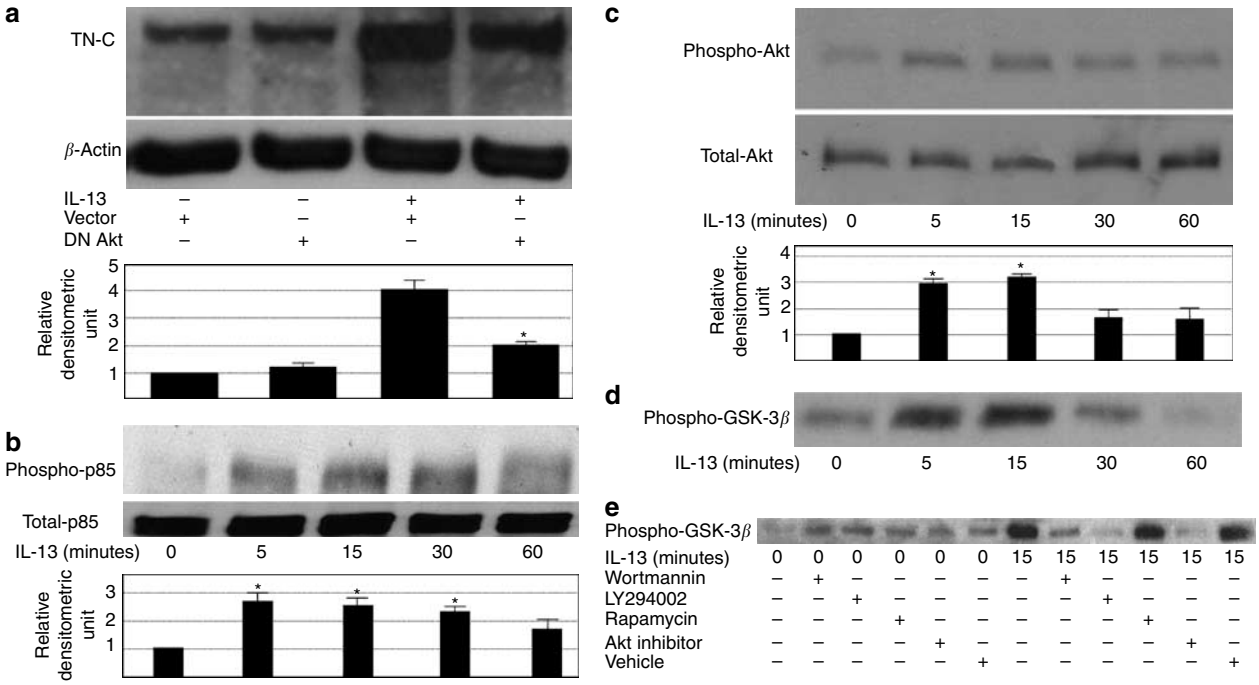

Figure 4. Activation of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway by IL-13 in human dermal fibroblasts. (**a**) For immunoblot analysis, the overexpression of the dominant-negative mutant of Akt in human dermal fibroblasts was performed by electroporating as described in ''Materials and Methods''. Twenty-four hours after the transfection, cells were treated with IL-13 (10 ng/ml) and then 24 hours later, lysed. The lysates were electrophoresed through a 10% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with anti-tenascin-C (TN-C) antibody. The same membrane was then stripped and reprobed with anti-β-actin antibody as a loading control. One experiment representative of three independent experiments is shown. The levels of TN-C quantitated by scanning densitometry and corrected for the levels of β-actin in the same samples are shown relative to the level in untreated cells transfected with vector (1.0) (lower panel). *$P<0.05$ as compared with the value in IL-13-treated cells transfected with empty vector. DN; dominant-negative. (**b**) Human dermal fibroblasts were serum-starved for 24 hours and treated with 10 ng/ml of IL-13 for the periods indicated. Cell lysates were immunoprecipitated with anti-p85 antibody, then immunoblotted with anti-phosphotyrosine (4G10) antibody. The same membrane was stripped and reprobed with anti-p85 antibody to determine the abundance of total p85 protein. One experiment representative of three independent experiments is shown. The levels of phosphorylated p85 quantitated by scanning densitometry and corrected for the levels of total p85 in the same samples are shown relative to the level in untreated cells (1.0) (lower panel). Data are expressed as the mean $\pm$ standard deviation of three independent experiments. *$P<0.05$ as compared with the value in untreated cells. (**c**) Cell lysates (30 μg of protein/sample) were subjected to immunoblotting with anti-phospho-Akt (Ser 473) antibodies. That the amounts of Akt proteins were unchanged was confirmed by immunoblotting using anti-Akt antibodies. One experiment representative of three independent experiments is shown. The levels of phosphorylated Akt quantitated by scanning densitometry and corrected for the levels of total Akt in the same samples are shown relative to the level in untreated cells (1.0) (lower panel). (**d**) To determine the time course of Akt kinase activity, human dermal fibroblasts were serum-starved for 24 hours and treated with 10 ng/ml of IL-13 for the period indicated. After the treatment, Akt was collected by immunoprecipitation, and subjected to an *in vitro* kinase assay in the presence of glycogen synthase kinase-3β. The samples were subjected to immunoblotting with anti-phospho-glycogen synthase kinase-3β antibodies. (**e**) Cells were also preincubated for 1 h with 100 nM wortmannin, 30 μM LY294002, 10 μM rapamycin, or 20 μM Akt inhibitor and then stimulated for 0.25 hours with 10 ng/ml of IL-13, before a kinase assay was performed.

TN-C expression is upregulated in systemic sclerosis due to IL-13 signaling, and that blockade of the PI3K or PKC signaling pathway may also have therapeutic value by reducing the amount of TN-C produced during fibrosis.

Makhluf *et al.* (1996) indicated that an IL-4-induced increase in TN-C mRNA was controlled at the level of transcription. IL-13 shares many biological properties with IL-4 as described above, and in the present study, we obtained similar results using IL-13. In addition, we showed that IL-13 induced the transcription of the TN-C gene.

PI3K/Akt is activated in response to a variety of stimuli, including growth factors and cytokines (Hemmings, 1997). Our results showed that treatment with PI3K inhibitors, wortmannin, and LY294002 blocked the IL-13-mediated upregulation of TN-C protein and mRNA expression, whereas treatment with rapamycin did not. This suggested that the mammalian target of rapamycin is not involved in the effect of IL-13 on TN-C expression, and that other downstream signaling cascades including glycogen synthase kinase-3 may mediate the upregulation of TN-C expression by IL-13.

The PKC family of proteins is comprised of at least 10 isozymes with diverse functions that are involved in numerous important cellular processes (Jimenez *et al.*, 2001). Studies have established that the activation of PKC is necessary for the stimulation of gene expression in response to various cytokines, and that several celluar responses to IL-13 are regulated by a PKC-dependent signal transduction pathway. For example, PKC-δ and -ζ mediate IL-4/IL-13-induced germline ε transcription in human B cells (Ikizawa *et al.*, 2001). In addition, Tourkina *et al.* (2001) reported that thrombin stimulated TN-C expression via opposing PKC-ε signaling mechanisms in systemic sclerosis and normal lung fibroblasts. Thus, the PKC

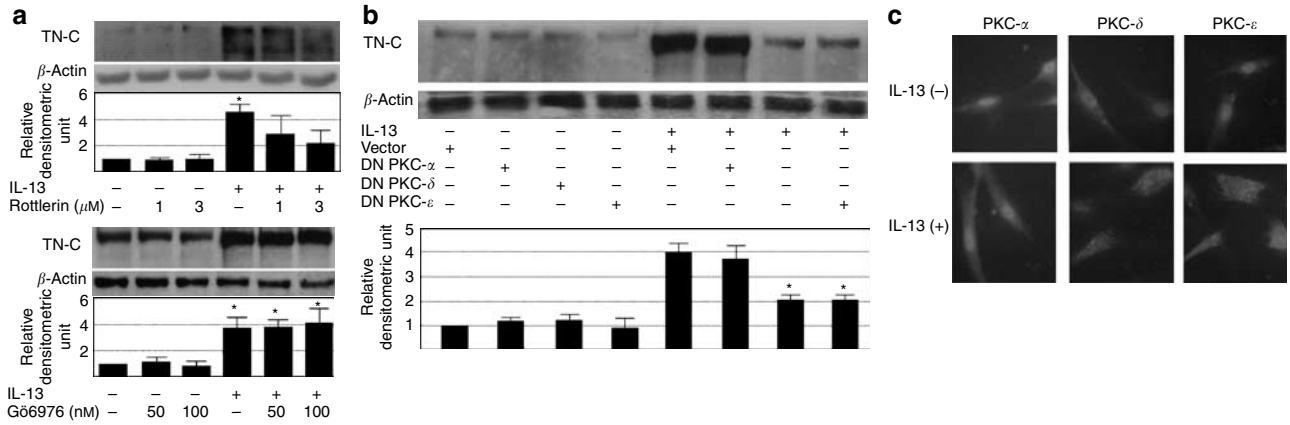

Figure 5. Activation of protein kinase C (PKC) signaling pathway by IL-13. (**a**) Human dermal fibroblasts were serum-starved for 24 hours and pre-treated with 1 or 3 μM Rottlerin, or 50 or 100 nM Gö6976, for 1 h before the addition of IL-13 (10 ng/ml) for 72 hours. Conditioned media and cell lysates (normalized for protein concentrations as measured with the Bio-Rad reagent) were subjected to immunoblotting with tenascin-C (TN-C) antibody and anti-β-actin antibody, respectively. One experiment representative of three independent experiments is shown. The levels of TN-C quantitated by scanning densitometry and corrected for the levels of β-actin are shown relative to the level in untreated (1.0), as described in Figure 3a. (**b**) For immunoblot analysis, the overexpression of the dominant-negative mutant of PKC-α, -δ, or -ε in human dermal fibroblasts was performed by electroporation as described in Figure 4a. DN; dominant negative. WT; wild type. (**c**) The subcellular localization of endogenous PKC-α, -δ, and -ε was visualized by immunofluorescence microscopy. Dermal fibroblasts were serum-starved for 24 hours and incubated in the presence or absence of IL-13 (10 ng/ml) for 1 hour.

family may be one of the main signaling pathways regulating TN-C levels. However, there is a limitation with the interpretation of our results. There are some reports that used higher concentrations of Rottlerin or Gö6976. Although the dominant-negative data support our conclusion, the possible involvement of another conventional PKC isoform, such as PKC-β, will need to be examined. In addition, the roles of the downstream signaling cascade of the PI3K/Akt or PKC signaling pathway and of crosstalk between these pathways and other signaling pathways in the IL-13-mediated induction of TN-C expression also must be examined.

MATERIALS AND METHODS

Reagents

Wortmannin, LY294002, Akt inhibitor II, Calphostin C, Rottlerin, and Gö6976 were purchased from Calbiochem (La Jolla, CA). Recombinant human IL-13 was obtained from R&D systems (Minneapolis, MN). Actinomycin D and cycloheximide were purchased from Sigma (St Louis, MO). Antibodies selective for Akt and phospho-Akt (Ser473), and the Akt kinase assay kits were from New England Biolabs (Beverly, MA). Anti-PI3K p85 antibodies and anti-phosphotyrosine (4G10) antibodies were from Upstate Biotechnology (Lake Placid, NY). Anti-PKC-α, -δ, or -ε antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell cultures

Fibroblasts were obtained by skin biopsy from healthy donors. All biopsies were obtained with institutional review board approval and written informed consent according to the Declaration of Helsinki Principles. Primary explant cultures were established in 25-cm^2 culture flasks in modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 μg/ml gentamycin, as described previously (Ihn *et al.*, 1996, 1997). Monolayer cultures were maintained at 37°C in 5% CO_2 in air. Fibroblasts between the third and sixth subpassages were used for experiments.

Microarray

The cDNA microarray hybridization experiments were performed using Intelligene Human Cancer CHIP Version 3.0 (codes X102) (Takara, Tokyo, Japan) according to the manufacturer's directions. The protocol used and a complete listing of the nearly 630 genes related to cancer on the Human Cancer CHIP are available on the Web. An approximately 300-bp cDNA region of each gene, which has minimal homology with other genes registered in the cDNA database, is spotted on this DNA chip. Poly $(A)^+$ mRNA was isolated and used to prepare cDNAs that were fluorescently labeled with either Cy3 (IL-13-untreated) or Cy5 (IL-13-treated) fluorescent dye. Samples were mixed and applied to DNA microarrays. The arrays were scanned using ScanArray (GS Lumonics), and the data were analyzed using Quant Array (BM BIO, Tokyo, Japan) (Hishikawa *et al.*, 2001). A gene expression ratio (Cy5/Cy3) >1.5 or <0.67 was considered significant.

Immunoblotting

Human dermal fibroblasts were cultured until they were confluent, then the media were collected. Aliquots of conditioned medium (normalized for cell numbers) were subjected to immunoblotting with an anti-human TN-C monoclonal antibody (NEC1b) (kindly provided by Dr Wolfgang Rettig) (Rettig and Garin-Chesa, 1989) (1:500 dilution) (Jinnin *et al.*, 2004a).

For immunoblotting using antibodies against phospho-Akt, membranes were incubated with anti-phospho-Akt (Ser473) monoclonal antibody (1:1,000) overnight at 4°C. As a loading control, immunoblotting was also performed using antibodies against total Akt (1:1,000).

Preparation of RNA and Northern blot analysis

Total RNA was extracted using an acid guanidinium thiocyanate–phenol–chloroform method and analyzed by Northern blotting as described previously (Ihn *et al.*, 1997). The following cDNA probes were used: a human TN-C 1431-bp *Eco*RI fragment (kindly provided by Dr Mario Bourdon, La Jolla Institute for Experimental Medicine,

La Jolla, CA) and a GAPDH 437-bp *Hind*III–*Not*I fragment. The membranes were then washed and exposed to X-ray film.

Nuclear run-on transcription

The nuclear run-on transcription analysis was performed according to a modified protocol that uses digoxigenin-labeled uridine triphosphate (Bentley and Groudine, 1986; Celano *et al.,* 1989; Merscher *et al.,* 1994; Jinnin *et al.,* 2004b).

Transient transfection

Fibroblasts were grown to 50% confluence in 100-mm dishes in modified Eagle's medium with 10% fetal calf serum. The media were replaced with serum-free medium, and fibroblasts were transfected with the TN-C promoter constructs driving chloramphenicol acetyltransferase expression, employing FuGENE6 as described previously (Ihn *et al.,* 2002). In order to normalize for minor variations in transfection efficiency, 1 μg of pSV-β-galactosidase vector (Promega, Madison, WI) was included in all transfections. After 24 hours of incubation, cells were incubated for another 24 hours in the absence or presence of 10 ng/ml IL-13. Subsequently, cells were harvested in 0.25 M Tris-HCl (pH 8) and fractured by freeze–thawing. Extracts, normalized for protein concentrations as measured with the Bio-Rad reagent, were incubated with butyl-CoA and [^{14}C]-chloramphenicol for 90 minutes at 37°C. Butylated chloramphenicol was extracted using an organic solvent (2:1 mixture of tetramethylpentadecane and xylene) and quantitated by scintillation counting. The data were standardized based on β-galactosidase activity. Each experiment was performed in duplicate.

For the immunoblot analysis using a dominant-negative mutant of Akt, PKC-α, -δ, or -ε, a transient transfection was performed by electroporating into human dermal fibroblasts, using a Bio-Rad Gene Pulser apparatus. Cells (3×10^6) were resuspended in 500 μl of phosphate-buffered saline containing 10 μg of DNA and electroporated at 950 μF and 300 V.

Immunoprecipitation

Phosphorylated levels of p85 were examined by immunoprecipitation using antibody for p85 and phosphotyrosine (Yamane *et al.,* 2003). The same membrane was then stripped and reprobed with anti-p85 antibody to show the total amount of p85.

Assay of Akt activation

The activation of Akt was examined using an Akt kinase assay kit according to the manufacturer's instructions (Yamane *et al.,* 2003).

Plasmids

The dominant-negative mutant form of Akt was kindly provided by Dr Brian A. Hemmings (Alessi *et al.,* 1996; Andjelkovic *et al.,* 1997). The dominant-negative mutant forms of PKC-α, -δ, and -ε were obtained from Dr Jae-Won Soh (Soh *et al.,* 2001), Dr Weiqun Li (Li *et al.,* 1996), and Dr Alex Toker (Deaconess Medical Center, Boston, MA) (Berrier *et al.,* 2000), respectively. A full-length TN-C promoter construct (bp $+75$ to -2100 relative to the transcription start site) linked to the chloramphenicol acetyltransferase reporter gene (PTN-I-CAT) was kindly provided by Dr Roberto Gherzi (Gherzi *et al.,* 1995).

Plasmids used in the transient transfection assays were purified twice on CsCl gradients, as described previously (Ihn *et al.,* 1997). At least two different plasmid preparations were used for each experiment.

Immunofluorescence microscopy

Dermal fibroblasts were grown in four-well LAK TEK chambers (Nunc, Naperville, IL) to subconfluence as described above. After 24 hours of serum starvation, cells were fixed with 3.7% formaldehyde, permeabilized with 0.5% Triton X-100 in phosphate buffered saline, and blocked with 10% fetal calf serum in Triton X-100 in phosphate buffered saline. Cells were stained with anti-PKC-α, -δ, or -ε antibodies as the primary antibody, washed, and incubated with FITC-conjugated secondary antibodies. To visualize the antibody staining, Zeiss fluorescent microscope was used (Asano *et al.,* 2004). For the positive control of PKC-α, -δ, and -ε, we also examined the cells stimulated with phorbol myristic acetate, an activator of PKC (data not shown) (Li *et al.,* 2002; Runyan *et al.,* 2003).

Statistical analysis

Data presented as bar graphs are the means $\pm$ standard deviation of at least three independent experiments. Statistical analysis was performed using the Mann–Whitney *U*-test ($P < 0.05$ was considered significant).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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The protocol used and a complete listing of the nearly 630 genes on Human Cancer CHIP Version 3.0 (Takara, Tokyo, Japan) are available at http://bio.takara.co.jp/BIO_EN/catalog_d.asp?C_ID=C1145.

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