Inhibition of Platelet-derived Growth Factor-induced Cell Growth Signaling by a Short Interfering RNA for EWS-Fli1 via Down-regulation of Phospholipase D2 in Ewing Sarcoma Cells*

Received for publication, October 12, 2004, and in revised form, May 25, 2005
Published, JBC Papers in Press, May 26, 2005, DOI 10.1074/jbc.M411626200

Satoshi Nozawa‡, Takatoshi Ohno‡, Yoshiko Banno‡, Taikoh Dobijima‡, Kazuhiko Wakahara‡, De-Gang Fan‡, and Katsuji Shimizu‡

From the Departments of Orthopaedic Surgery and Cell Signaling, Gifu University School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan

EWS-Fli1, a fusion gene resulting from a chromosomal translocation t(11;22, q24;q12) and found in Ewing sarcoma and primitive neuroectodermal tumors, encodes a transcriptional activator and promotes cellular transformation. However, the precise biological functions of its products remain unknown. To investigate the role of EWS-Fli1 in cell growth signaling, we transfected Ewing sarcoma TC-135 cells with short interfering RNAs for EWS-Fli1. EWS-Fli1 knockdown reduced cell growth and platelet-derived growth factor (PDGF)-BB-induced activation of the growth signaling enzymes. Interestingly, phospholipase D2 (but not the PDGF-BB receptor) showed marked down-regulation in the EWS-Fli1-knockdown TC-135 cells compared with the control cells. In Ewing sarcoma TC-135 cells, the PDGF-BB-induced phosphorylation of growth signaling involving extracellular signal-regulated kinase, Akt, p70S6K, and extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase; and poly(G) and poly(U) RNA. The binding activity of EWS is located in the RGG box, which is in the carboxyl-terminal region. The amino-terminal region of EWS regulates the RNA binding activity of EWS protein (5). Functional characterization of the EWS-Fli1 and EWS-erg chimeric proteins has suggested that they act as sequence-specific transcriptional activators and are capable of transforming cells (6–8). Antagonizing EWS fusion gene expression in tumors results in reduced tumorigenicity and clonogenicity, suggesting that their chimeric products need to be expressed at levels above a certain threshold to maintain oncogenicity (9–11).

Phospholipase (PL)3-D cleaves phosphatidylcholine in response to a variety of cell stimuli. This cleavage generates phosphatic acid (12–14), which acts as a second messenger and can be further converted into the messenger molecules 1,2-diacylglycerol and lyso-phosphatic acid (12–14). PLD2 is constitutively active and has been shown to require phosphatidylinositol 4,5-biphosphate in in vitro systems (13, 14); however, the precise mechanisms that regulate PLD2 activity are still undefined. PLD is thought to be involved in a variety of cellular responses, including cell proliferation and differentiation (15, 16). A recent report has also implicated PLD in the mammalian target of rapamycin (mTOR) pathway of cell growth signaling (17).

To improve understanding of the biological function of EWS-Fli1 in terms of cellular growth signal transduction, in the present study we examined the platelet-derived growth factor (PDGF)-BB signaling pathway in Ewing sarcoma cells transfected with short interfering RNAs (siRNAs) for EWS-Fli1 and PLD2. We found that EWS-Fli1 plays a role in PDGF-induced signaling by regulating PLD2 expression.

EXPERIMENTAL PROCEDURES

Cell Culture—The Ewing sarcoma cell line TC-135 was kindly supplied by Dr. T. J. Triche (University of Southern California, Los Angeles, CA). The cells were maintained in RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37 °C.

Cell Growth Assay—Cell growth was determined by a WST-8 assay kit (Kishida Kagaku, Osaka, Japan). Briefly, cells (5 × 10^4 cells/well) in 96-well plates were incubated overnight. Thereafter, the medium was replaced with new medium containing U0126, LY294002, rapamycin, or transfection reagent. After 48–96 h of incubation, the WST-8 reagents were added to the culture. After 1 h of incubation, the absorbance at 450

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Orthopaedic Surgery, Gifu University School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan. Tel.: 81-58-230-6333; Fax: 81-58-230-6334; E-mail: ohnota@cc.gifu-u.ac.jp.

§ The abbreviations used are: PL, phospholipase; mTOR, mammalian target of rapamycin; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; siRNA, short interfering RNA; rt, nucleotide(s); IR, irrelevant; PBut, [Hphosphatidylbutanoyl] P3K, phosphatidylinositol 3-kinase; MEK, mitogen-activated protein kinase; extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase.
A 19-nt sequence matching GL3 (nt 155–173) was chosen for GL3 RNA interference targeting as a negative control. The cells were then transiently transfected with 20 nm siRNA duplex. To determine transfection efficiencies, the cells were cotransfected with the siRNA expression vectors and pLEGFP-N1 (a green fluorescent protein expression construct; BD Biosciences) at a ratio of 9:1 using Lipofectamine 2000 (Invitrogen), following the manufacturer’s recommended procedure.

**Western Blot Analysis**—The control and test cells were harvested into ice-cold lysis buffer (1% Nonidet P-40, 0.5% sodium cholate, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 20 mM Heps, 1 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 20 mM β-glycerophosphate, 1 mM NaF, and 1 mM sodium orthovanadate, pH 7.4) and sonicated. Protein concentrations were assayed using the Bradford protein assay reagent (Bio-Rad). The total cell lysates (100 μg protein) were subjected to SDS/PAGE (9% gel) and transferred to polyvinylidene fluoride membranes (Millipore). The membranes were blocked with 5% skim milk. All proteins were determined by immunoblotting. The EWS-Fli1 fusion protein (68 kDa) was sensitively detected by Western blot analysis using an anti-Fli-1 antibody (Santa Cruz Biotechnology). Rabbit polyclonal antibodies against Ser473-phosphorylated Akt, Tyr204-phosphorylated PI3K p85, and Tyr204-phosphorylated ERK1/2, and mouse monoclonal antibody against cyclin D3 were obtained from Cell Signaling Technology (Boston, MA). Rabbit polyclonal antibodies against phosphorylated PDGFR were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibody against P3K p85 was from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal antibody against actin was obtained from Sigma. Rabbit polyclonal antibody against human PLD2 was prepared as described previously (19), and antibody against PLD1 was obtained from Cell Signaling Technology (Boston, MA). Rabbit polyclonal antibodies against the p70S6K platelet-derived growth factor receptor (PDGFR) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibody against P3K p85 was from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal antibody against actin was obtained from Sigma. Rabbit polyclonal antibody against human PLD2 was prepared as described previously (19), and antibody against PLD1 was obtained from Cell Signaling Technology (Boston, MA). Rabbit polyclonal antibodies against the p70S6K platelet-derived growth factor receptor (PDGFR) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibody against P3K p85 was from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal antibody against actin was obtained from Sigma. Rabbit polyclonal antibody against human PLD2 was prepared as described previously (19), and antibody against PLD1 was obtained from Cell Signaling Technology (Boston, MA). Rabbit polyclonal antibodies against the p70S6K platelet-derived growth factor receptor (PDGFR) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibody against P3K p85 was from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal antibody against actin was obtained from Sigma. Rabbit polyclonal antibody against human PLD2 was prepared as described previously (19), and antibody against PLD1 was obtained from Cell Signaling Technology (Boston, MA). Rabbit polyclonal antibodies against the p70S6K platelet-derived growth factor receptor (PDGFR) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibody against P3K p85 was from Upstate Biotechnology (Lake Placid, NY). Mouse monoclonal antibody against actin was obtained from Sigma. Rabbit polyclonal antibody against human PLD2 was prepared as described previously (19), and antibody against PLD1 was obtained from Cell Signaling Technology (Boston, MA). Rabbit polyclonal antibodies against the p70S6K platelet-derived growth factor receptor (PDGFR) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**RESULTS**

**Knockdown of EWS-Fli1 Protein by siRNA EWS-Fli1 and Growth Inhibition in TC-135 Cells**—To reduce EWS-Fli1 protein expression of EWS-Fli1, two siRNA EWS-Fli1 constructs were cotransfected with an enhanced green fluorescent protein expression vector into Ewing sarcoma TC-135 cells. When the expression level of EWS-Fli1 was examined by Western blot analysis using an antibody raised against Fli-1, there was significant loss of EWS-Fli1 protein expression in cells transfected with the siRNAs directed against both site I and site II compared with the control cells. The expression of EWS-Fli1 protein was decreased to 12.3% in site I siRNA-transfected cells and to 14.2% in site II siRNA-transfected cells. (Fig. 1A).

When the cell growth in each culture was measured four days after transfection, the cells that had been transfected with the site I siRNA showed inhibition of proliferation to 38.8% of the level seen in those transfected with the irrelevant siRNA. The corresponding percentage for cells transfected with the site II siRNA was 36.7% (Fig. 1B). These results showed that reduction of EWS-Fli1 resulted in growth inhibition of Ewing sarcoma TC-135 cells.

**Characterization of the PDGF-BB-induced p70S6K Activation Pathway in TC-135 Cells**—It has been reported that PDGF-BB signaling is important for cell proliferation in Ewing sarcoma (20) and that some pathways in growth signaling involving p70S6K depend on stimulants and cell type (21). The stimulation of the cells with PDGF-BB (10 ng/ml) induced phosphorylation of ERK1/2, Akt, and p70S6K in a time-dependent manner within 30 min. The ERK1/2 phosphorylation was increased at 2 min after stimulation with PDGF-BB and peaked at 10 min. The phosphorylations of Akt and p70S6K were significantly increased at 5 min, with a peak response at 10 min (Fig. 2A). To determine the p70S6K activation pathways(s) mediated by PDGF-BB, the effects of inhibitors of P3K and MEK were examined. Pretreatment of the cells with U0126 (5 μM), an inhibitor of MEK, or LY294002 (10 μM), an inhibitor of P3K, completely blocked the downstream enzymes ERK1/2 and Akt, respectively. U0126 showed no inhibitory effect on the PDGF-BB-induced Akt phosphorylation. PDGF-BB-induced p70S6K phosphorylation was partially inhibited by both the MEK (U0126) and P3K (LY294002) inhibitors. Moreover, a combination of the two inhibitors induced an additive inhibitory effect on p70S6K phosphorylation (Fig. 2B). These results suggest that the ERK1/2- and Akt-activating pathways are
both partially involved in PDGF-BB-induced p70S6K activation, but that these pathways act independently.

It is known that the growth response to p70S6K is dependent on mTOR (21–23). To investigate the role of mTOR in PDGF-BB-induced p70S6K activation, TC-135 cells were treated with rapamycin, an inhibitor of mTOR. The PDGF-BB-induced phosphorylation of p70S6K was indeed down-regulated by rapamycin in a dose-dependent manner, inhibiting ~90% (Fig. 2C). However, PDGF-BB-induced ERK1/2 phosphorylation was not affected by the inhibitor. This suggests that mTOR acts upstream of p70S6K during PDGF-BB-mediated signaling in TC-135 cells.

To examine the effects of signaling pathway inhibitors on TC-135 cell growth, a cell proliferation assay was performed with or without various inhibitors. Signaling pathway inhibitors, such as U0126, LY294002, and rapamycin, partially af-
fected cell growth inhibition, and a combination of the three inhibitors had an additive effect on cell growth inhibition (Fig. 2D). This result shows that these three signaling pathways are important in the PDGF-BB-induced growth of TC-135 cells.

PDGF-BB-induced Growth Signaling in TC-135 Cells with EWS-Fli1 Knockdown—As shown in Fig. 1, the growth of TC-135 cells was suppressed in EWS-Fli1 knockdown cells. We then examined the effects of EWS-Fli1 siRNA on PDGF-BB-induced ERK1/2, Akt, p70S6K signaling, and expression of cyclin D3 in TC-135 cells. PDGF-BB-stimulated ERK1/2 and Akt phosphorylation were diminished in EWS-Fli1 knockdown cells compared with those in control cells (Fig. 3). However, the levels of ERK1/2 and Akt protein were unaffected, confirming the specificity of the siRNAs for EWS-Fli1. PDGF-BB-induced P38 p85 phosphorylation was also partially suppressed by siRNAs for EWS-Fli1 (data not shown). PDGF-BB-stimulated p70S6K phosphorylation and expression of cyclin D3 were significantly reduced in the EWS-Fli1 knockdown TC-135 cells, although the total protein level remained unchanged. These data suggest that EWS-Fli1 is involved in the PDGF-BB-mediated growth signaling pathways involving ERK1/2, Akt, and p70S6K.

Down-regulation of PLD2 Expression and PDGF Signaling in TC-135 Cells with EWS-Fli1 Knockdown—It has been reported that activation of phosphatidylinositol-specific PLC-γ1 and PLD plays an important role in PDGF-stimulated intracellular signaling leading to proliferation in various cells (24–26). A previous study has demonstrated that tyrosine phosphorylation of PLC-γ1 is increased in PDGF-BB-stimulated Ewing sarcoma cells (20). Our previous study showed that transfection of TC-135 cells with antisense EWS-Fli1 had no apparent effect on PLC-γ1 (27). Several reports have suggested that PLDs are involved in the extracellular ligand-induced activation of ERK1/2, Akt, and mTOR-mediated growth and survival signaling pathways (28, 29). Therefore, we examined the role of PLD in PDGF-BB signaling in TC-135 cells. To examine the subtype of PLD expressed in TC-135 cells, the cell lysates were subjected to Western blot analysis with specific antibodies against PLD1 and PLD2.
As shown in Fig. 4A, the mixed antibodies of PLD1 and PLD2 clearly detected recombinant PLD1a, -1b, and -2, and PLD2 was distinct in TC135 cell lysates, whereas PLD1, which corresponds to PLD1a, was hardly detectable, indicating that PLD2 is the main form expressed in TC-135 cells.

Interestingly, PLD2 showed marked down-regulation in the EWS-Fli1 knockdown TC-135 cells compared with the control cells (Fig. 4B). The expression of PLD2 was reduced to 19.7% by the EWS-Fli1 site I siRNA and to 23.4% by the site II construct. On the other hand, expression of the PDGF receptor (PDGFR) in the EWS-Fli1 knockdown cells did not differ from that in the control cells.

To confirm the implication of PLD2 on proliferation signaling, we used PLD2 siRNA. Transfection of TC-135 cells with PLD2 siRNA caused a significant decrease of PLD2 expression (to 30% that of the control) (Fig. 5A). The phosphorylation of ERK1/2 and Akt was reduced by 30–40% in PLD2 down-regulated TC-135 cells, whereas the expression levels of ERK1/2 and Akt were unaffected (Fig. 5A).

A previous study has demonstrated that the level of cyclin D3 protein, known to be an activator of G1–S phase transition in the cell cycle, is aberrantly high in cells overexpressing PLD1 and PLD2 in comparison with control cells (30). The phosphorylation of P70S6K was further decreased to 40% of the control, and cyclin D3 expression was further reduced (to 50% of the control) in PLD2 down-regulated TC-135 cells compared with the control cells. These data suggested that PLD2 could play a role in growth signaling involving p70S6K and cyclin D3 expression in Ewing sarcoma cells. In fact, PLD2 siRNA exerted a strong influence on suppression of cell growth in Ewing sarcoma TC-135 cells (Fig. 5B).

Effect of PLD Inhibitor on PDGF-BB-induced ERK1/2, Akt, and p70S6K Activation in TC-135 Cells—To examine the activation of PLD in response to PDGF-BB in TC-135 cells, we determined cellular PLD activity by measuring the formation of PBut in the presence of 1-butanol via the trans-phosphatidyl reaction in cells prelabeled with [3H]palmitic acid. As shown in Fig. 6A, when the TC-135 cells transfected with siRNA for IR were stimulated with PDGF-BB (10 ng/ml) for 10 min, PBut formation was significantly increased (2.5-fold). In TC-135 cells transfected with siRNA for EWS-Fli1 site I, PDGF-induced PBut formation was largely reduced, suggesting that EWS-Fli1 was involved in regulation of PDGF-induced PLD activation.
To examine the involvement of PLD in PDGF-BB-induced growth signaling activation in TC-135 cells, we examined the effects of treatment with 1-butanol (which is an inhibitor of phosphatidic acid formation by PLD activation and t-butyl alcohol), negative control on PDGF-BB-induced ERK1/2, Akt, and p70S6K activation, and expression of cyclin D3. Pretreatment of the cells with 1-butanol (0.3%) completely abolished PDGF-BB-induced phosphorylation of p70S6K and the decrease of cyclin D3 expression, whereas t-butyl alcohol (0.3%) had no effect (Fig. 6B). The PDGF-induced phosphorylation of ERK1/2 and Akt was partially suppressed by 1-butanol. These results further suggested that PLD is implicated in PDGF-BB-induced growth signaling in TC-135 cells.

DISCUSSION

PDGF-BB-induced Growth Signaling in Ewing Sarcoma Cells—Recent studies have shown that aberrant fusion products from the chromosomal rearrangements seen in Ewing sarcoma and PNET may be responsible for these malignancies (1–3, 6–8). In the present study, we have demonstrated that knockdown of the fusion protein EWS-Fli1 by siRNAs directed against specific sites in its gene caused a significant reduction in cell growth. A recent study has demonstrated that expression of β-PDGFR, which is a functional and potentially crucial component of the signaling pathway, is important for the growth of Ewing sarcoma cells (20). However, we observed that there was no significant difference in PDGFR expression between EWS-Fli1 knockdown cells and the control TC-135 cells. Constitutive ERK1/2 activation is also evident in several human Ewing sarcoma-derived cell lines (31), and ribosomal p70S6K is known to be important for growth signaling (21). To elucidate the mechanism underlying the inhibition of cell growth caused by EWS-Fli1 knockdown, we examined the signaling pathway stimulated by PDGF-BB in TC-135 cells. In TC-135 cells, the PDGF-BB-induced growth signaling pathway leading to the phosphorylation of p70S6K was inhibited by the inhibitors LY294002 (a PI3K inhibitor), U0126 (a MEK inhibitor), and rapamycin (a mTOR inhibitor), suggesting that at least three independent signaling pathways (the MEK-ERK1/2-p70S6K, PI3K-Akt-p70S6K, and mTOR-p70S6K pathways) are mediated by PDGF-BB in TC-135 cells (Fig. 7). In fact, the combination of LY294002 with U0126 caused additive inhibition of PDGF-BB-induced p70S6K phosphorylation. These PDGF-BB-mediated signaling pathways were suppressed by transfection of TC-135 cells with EWS-Fli1 siRNA. The decrease of p70S6K activation was most evident in EWS-Fli1 knockdown cells. Tyrosine phosphorylation of PLC-γ1 has been reported in PDGF-BB-stimulated Ewing sarcoma cells (20). Our previous study has demonstrated down-regulation of
PLC-β in Ewing sarcoma cells transfected with an antisense EWS-Fli1 sequence, whereas there was no apparent difference in PLC-γ1 (27), suggesting that the PLC-γ signaling pathway may not be involved in growth signaling of EWS-Fli1.

The Relationship between EWS-Fli1 and PLD Expression in Cell Growth Signaling—Numerous studies have suggested that PLD might be involved in tumorigenesis (15, 32). For example, enhanced expression and increased activity of two PLD isoforms have been found in cancer cells and tissues (33–35). We have observed that ADP-ribosylation factor-activated PLD activity was transiently increased in regenerating liver nuclei and that PLD2 levels were markedly elevated in the nuclei of hepatoma and kidney tumor cells (35, 36). There is also evidence that PLD2 levels are markedly elevated in the caveolae of stimulated oncogenic cells and multidrug-resistant cancer cells (37, 38). Furthermore, overexpression of PLD1 or PLD2 in mouse fibroblasts induces colony formation in soft agar, and both transformants induce undifferentiated sarcoma when transplanted into nude mice (30). Moreover, elevated expression of either PLD1 or PLD2 prevents cell cycle arrest induced by high intensity Raf signals (39) and promotes actinomycin D-induced apoptosis (38). These studies have suggested that PLDs are implicated in cell proliferation and survival signaling. A number of studies have demonstrated that PLD directly regulates mTOR/p70S6K signaling (22, 40) or participates in the activation of ERK1/2 via Raf-1 translocation to the membrane (28, 29, 41), as well as in PI3K and Akt stimulation (42). Our finding that the expression of PLD2, the main form of PLD expressed in TC-135 cells, was markedly reduced in EWS-Fli1 knockdown cells raised the possibility that the PDGFinduced growth signaling pathways might be down-regulated by a decrease of PLD2 expression. In PLD2-reduced TC-135 cells induced by transfection with the siRNA, phosphorylation of p70S6K and the level of cyclin D3 expression were further suppressed, suggesting a role of PLD2 in growth signaling in Ewing sarcoma TC-135 cells. In fact, the PLD2 siRNA transfection as well as EWS-Fli1 siRNA caused suppression of TC-135 cell growth. The PDGFB-mediated growth signaling pathways in TC-135 cells were inhibited by 1-butanol (which inhibits phosphatidic acid formation after PLD activation) but not by t-butyl alcohol. These results obtained with the two types of butanol further support the notion that PLD has a role in the PDGFB-induced p70S6K activation pathways involving ERK1/2 and Akt activation in TC-135 cells. Although PLD2 is implicated in EWS-Fli1-induced growth signaling, it cannot be ruled out that PLD1 is involved in PDGFB-mediated growth signaling in TC-135 cells, because PLD1 was present in small amounts in TC-135 cells, and the inhibition of growth signaling by 1-butanol was much stronger than that obtained by transfection with PLD2 siRNA. In this study, we demonstrated down-regulation of PLD2 expression in EWS-Fli1 knockdown TC-135 cells, suggesting an association of PLD2 expression with EWS-Fli-1. To gain more insight into the role of EWS-Fli1 in the expression of PLD, we examined the effects of EWS-Fli1 overexpression on the expression of PLD in NIH3T3 cells. Stable cell lines transfected with cDNA encoding EWS-Fli1 or vector alone were established. Significant up-regulation of PLD2 expression was found in EWS-Fli-1-overexpressing cells compared with the vector control. On the other hand, Western blot analysis with the anti-PLD1 antibody showed no significant change of PLD1 expression in both cell lines (data not shown). This result suggests that the promoter region of PLD2 may be regulated by the target sequence of EWS-Fli1 protein.

The Effect of cyclin D Expression on Ewing Sarcoma Cells—Some previous studies have shown that EWS-Fli1 is correlated with cyclin D expression (43–46). Also, a recent study has demonstrated distinct regulation of D-type cyclins in Ewing tumor cells; in TC-71 Ewing sarcoma cells, inhibition of EWS-Fli1 decreases the level of cyclin D1 but increases that of cyclin D3 (43). However, in TC-135 cells, we showed that cyclin D3 expression was down-regulated by transfection with EWS-Fli1 siRNA. The reason for the difference in cyclin D3 expression between these two cell lines remains to be elucidated. Furthermore, cell- and tissue-specific patterns of D-type cyclin expression have been reported (43). In Ewing tumors and rhabdomyosarcomas, both the Ras-ERK and PI3K-Akt pathways regulate cyclin D expression (43). In this study, we found that EWS-Fli1 knockdown caused a decrease of cyclin D3 expression, which may have been due to down-regulation of p70S6K pathways through a decrease of PLD2 expression. Furthermore, PDGFB-BB-induced phosphorylation of PI3K was reduced in cells transfected with EWS-Fli1 siRNA (data not shown). From these results, it may be suggested that EWS-Fli1 regulates cyclin D3 expression by p70S6K through the Ras-ERK, PI3K/Akt, and mTOR growth signaling pathways.

In conclusion, the present study using EWS-Fli1 siRNAs has demonstrated that EWS-Fli1 may play a role in regulating the expression of PLD2, which is a critical regulator of proliferation and survival signaling in TC-135 Ewing sarcoma cells. The down-regulation of PLD2 induced by transfection with EWS-Fli1 siRNA may suppress the growth of TC-135 cells.

Acknowledgments—We thank Dr. T. J. Triche (University of Southern California, Los Angeles, CA) for providing the Ewing sarcoma cells, E. Wada for assistance, and our colleagues at the Department of Orthopaedic Surgery, Gifu University School of Medicine, for encouragement.

REFERENCES
1. Arvand, A., and Denny, C. T. (2001) Oncogene 20, 5747–5754
2. de Alava, E., and Gerald, W. L. (2000) J. Clin. Oncol. 18, 204–213
3. Delattre, O., Zucman, J., Plougastel, B., Desmaze, C., Morot, T., Peter, M., Kover, H., Joubert, I., de Jong, P., Rouleau, G., Auriol, A., and Thomas G. (1992) Nature 359, 162–165
4. Sorensen, P. H., Lessnick, S. L., Lopez-Terrada, D., Liu, X. F., Triche, T. J., and Denny, C. T. (1994) Nat. Genet. 6, 146–151
5. Ohno, T., Ouchi, M., Lee, L., Gatalica, Z., Rao, V. N., and Reddy, E. S. (1994) Oncogene 9, 3087–3097
6. Bailly, R. A., Bosselut, R., Zucman, J., Cormier, F., Delattre, O., Roussel, M., Thomas, G., and Ghysdael, J. (1994) Mol. Cell. Biol. 14, 3230–3241
7. May, W. A., Lessnick, S. L., Braun, B. S., Klemaz, M., Lewis, B. C., Lunsofar, L. B., Thomas, R., and Denny, C. T. (1995) Mol. Cell Biol. 15, 7395–7399
8. Ohno, T., Rao, V. N., and Reddy, E. S. (1993) Cancer Res. 53, 5859–5863
9. Ouchi, M., Ohno, T., Fujimura, Y., Rao, V. N., and Reddy, E. S. (1995) Oncogene 11, 1049–1054
10. Tanaka, K., Iwakuma, T., Harimaya, K., Sato, H., and Iwamoto, Y. (1997) J. Clin. Investig. 99, 239–247
11. Toretzky, J. A., Connell, Y., Neckers, L., and Bhat, N. K. (1997) J. Neurooncol. 31, 9–16
12. Exton, J. H. (1998) Biochim. Biophys. Acta 1436, 105–115
13. Liscovitch, M., Czarney, M., Fiucci, G., and Tung, X. (2000) Biochem. J. 345, 401–415
14. Cockcroft, S. (2001) Cell. Mol. Life Sci. 58, 1674–1687
15. Banno, Y. (2002) J. Biochem. (Tokyo) 131, 901–906
16. Nozawa, Y. (2002) Biochim. Biophys. Acta 1585, 77–86
17. Kam, Y., and Exton, J. H. (2004) FASEB J. 18, 311–319
18. Dojima, T., Lee, N. S., Li, H., Ohno, T., and Rossi, J. J. (2003) Mol. Ther. 7, 811–816
19. Banno, Y., Fujita, H., Ono, Y., Nakashima, S., Ito, Y., Kurumaki, N., and Nozawa, Y. (1999) J. Biol. Chem. 274, 27385–27391
20. Uren, A., Merchant, M. S., Sun, C. J., Vitolo, M. I., Sun, Y., Tsokos, M., Illei, P. B., Ladanyi, M., Passaniti, A., Mackall, C., and Toretsky, J. A. (2003) Oncogene 22, 2334–2342
21. Pullen, N., and Thomas, G. (1997) FEBS Lett. 410, 78–82
22. Fang, Y., Park, I. H., Wu, A. L., Du, G., Huang, P., Frohman, M. A., Walker, S. J., Brown, H. A., and Chen, J. (2003) Oncogene 22, 2334–2342
23. Seufferlein, T., and Rozengurt, E. (1996) Cancer Res. 56, 3895–3897
24. Alimandi, M., Heidaran, M. A., Gutkind, J. S., Zhang, J., Ellmore, N., Valius, M., Kazlauskas, A., Pierre, J. H., and Li, W. (1997) Oncogene 15, 585–593
25. Plevin, R., Cook, S. J., Palmer, S., and Wakelam, M. J. (1991) Biochem. J. 279, 559–565
26. Sekiya, F., Poulin, B., Kim, Y. J., and Bhee, S. G. (2004) J. Biol. Chem. 279, 32181–32190
27. Dojima, T., Ohno, T., Banno, Y., Nozawa, Y., Wen-yi, Y., and Shimizu, K. (2000) Br. J. Cancer 82, 16–19
28. Rizzo, M. A., Shone, K., Vasudev, C., Stolz, D. B., Sung, T. C., Frohman, M. A., Watkins, S. C., and Romero, G. (1999) J. Biol. Chem. 274, 1131–1139
29. Hong, J. H., Oh, S. O., Lee, M., Kim, Y. R., Kim, D. U., Hur, G. M., Lee, J. H., Lim, K., Hwang, B. D., and Park, S. R. (2001) Biochim. Biophys. Res. Commun. 281, 1337–1342
30. Min, D. S., Kwon, T. K., Park, W. S., Chang, J. S., Park, S. K., Ahn, B. H., Ryoo, Z. Y., Lee, Y. H., Lee, Y. S., Rhee, D. J., Yoon, S. H., Hahn, S. J., Kim, M. S., and Jo, Y. H. (2001) Carcinogenesis 22, 1641–1647
31. Silvany, R. E., Eliazer, S., Wolff, N. C., and Ilaria, R. L., Jr. (2000) Oncogene 19, 4523–4530
32. Foster, D. A., and Xu, L. (2003) Mol. Cancer Res. 1, 789–800
33. Noh, D. Y., Ahn, S. J., Lee, R. A., Park, I. A., Kim, J. H., Suh, P. G., Ryu, S. H., Lee, K. H., and Han, J. S. (2000) Cancer Lett. 161, 207–214
34. Yoshida, M., Okamura, S., Kodaki, T., Mori, M., and Yamashita, S. (1998) Oncol. Res. 10, 399–406
35. Zhao, Y., Ebara, H., Akao, Y., Shamoto, M., Nakagawa, Y., Banno, Y., Deguchi, T., Ohishi, N., Yagi, K., and Nozawa, Y. (2000) Biochem. Biophys. Res. Commun. 278, 140–143
36. Banno, Y., Tamiya-Koizumi, K., Oshima, H., Morikawa, A., Yoshida, S., and Nozawa, Y. (1997) J. Biol. Chem. 272, 5208–5213
37. Fiucci, G., Czarny, M., Lavié, Y., Zhao, D., Berse, B., Blusztajn, J. K., and Liscovitch, M. (2000) Int. J. Cancer 85, 882–888
38. Yamada, M., Banno, Y., Takawa, Y., Koda, M., Hara, A., and Nozawa, Y. (2004) Biochem. J. 378, 649–656
39. Joseph, T., Bryant, A., Frankel, P., Wood, R., Kerkhoff, E., Rapp, U. R., and Foster, D. A. (2002) Oncogene 21, 3651–3658
40. Fang, Y., Vililla-Bach, M., Bachmann, R., Flanigan, A., and Chen, J. (2001) Science 294, 1942–1945
41. Slaaby, R., Du, G., Alshuller, Y. M., Frohman, M. A., and Seedorf, K. (2000) Biochem. J. 351, 613–619
42. Banno, Y., Takawa, Y., Akao, Y., Okamoto, H., Osawa, Y., Nagatani, T., Nakashima, S., Suh, P. G., and Nozawa, Y. (2001) J. Biol. Chem. 276, 35622–35628
43. Zhang, J., Hu, S., Schofield, D. E., Sorensen, P. H., and Triche, T. J. (2003) Cancer Res. 64, 6026–6034
44. Dauphinot, L., De Oliveira, C., Melot, T., Sevenet, N., Thomas, V., Weissman, B. E., and Delattre, O. (2001) Oncogene 20, 3258–3265
45. Matsumoto, Y., Tanaka, K., Nakastani, F., Matsunobu, T., Matsuda, S., and Iwamoto, Y. (2001) Br. J. Cancer 84, 768–775
46. Eliazer, S., Spencer, J., Ye, D., Olsen, E., and Ilaria, R. L., Jr. (2003) Mol. Cell. Biol. 23, 482–492