Centaurin-α₁ Is a Phosphatidylinositol 3-Kinase-dependent Activator of ERK1/2 Mitogen-activated Protein Kinases*

Centaurin-α₁ is known to be a phosphatidylinositol 3,4,5-triphosphate (PIP₃) binding protein that has two pleckstrin homology domains and a putative ADP ribosylation factor GTPase-activating protein domain. However, the physiological function of centaurin-α₁ is still not understood. Here we have shown that transient expression of centaurin-α₁ in COS-7 cells results in specific activation of ERK, and the activation is inhibited by co-expression of a dominant negative form of Ras. We have also found that a mutant form of centaurin-α₁ that is unable to bind PIP₃ fails to induce ERK activation and that a phosphatidylinositol 3-kinase inhibitor LY294002 inhibits centaurin-α₁-dependent ERK activation. Furthermore, transient knockdown of centaurin-α₁ by small interfering RNAs results in reduced ERK activation after epidermal growth factor stimulation in T-REX 293 cells. These results suggest that centaurin-α₁ contributes to ERK activation in growth factor signaling, linking the P13K pathway to the ERK mitogen-activated protein kinase pathway through its ability to interact with PIP₃.

The ERK² MAP kinase cascade plays a pivotal role downstream of growth factor signaling (1–7). Extracellular stimuli, such as growth factors, induce sequential activation of Ras, Raf-1 MAP kinase kinase kinase, MEK MAP kinase kinase, and ERK MAP kinase. It is known that there are two isoforms of ERK, ERK1 and ERK2, and they phosphorylate and activate various transcription factors, such as Elk-1 and Elk-2. These transcription factors regulate expression of diverse genes that relates to the proliferation and growth of the cells.

A previous large scale functional cDNA screening that was performed to identify molecules involved in the MAP kinase signaling pathways identified many potential activators of the MAP kinase cascades, which include known activators, such as the MAP kinase kinase family members, molecules with functions not previously linked to the MAP kinase pathways, and molecules with no previously revealed functions (8, 9). Among these potential activators, centaurin-α₁ was shown to be able to activate the ERK MAP kinase cascade when overexpressed (9).

Centaurin-α₁ was originally identified as a PIP₃-binding protein (10, 11). Human centaurin-α₁ is composed of 373 amino acids and has a molecular mass of 43 kDa. Its N-terminal region contains a zinc finger motif that is highly homologous to the ARF (ADP ribosylation factor)-GAP (GTPase-activating protein) domain. Recently, it was reported that this potential ARF-GAP domain exhibited GAP activity for ARF6 in mammalian cells (12). The C-terminal region of centaurin-α₁ contains two PH domains, which have been reported to bind PIP₃ (13). The expression of centaurin-α₁ is ubiquitous and especially high in the brain. The distribution of centaurin-α₁ in the cell is concentrated in the nucleus (13, 14). Despite such information, the physiological function of centaurin-α₁ is still unknown.

In this study, we showed that centaurin-α₁ can activate ERK, and this activation needs the P13K-dependent recruitment of centaurin-α₁ to the plasma membrane. We also showed that centaurin-α₁ contributes to ERK activation induced by epidermal growth factor (EGF) in 293 cells. These results indicate that centaurin-α₁ plays a role in the growth factor signaling upstream of the ERK MAP kinase.

**EXPERIMENTAL PROCEDURES**

Reagents and Plasmids—Anti-c-Myc antibody was from Santa Cruz Biotechnology. Anti-HA antibody and anti-GFP antibody were from Clontech. Anti-phospho-p44/42 mitogen-activated protein kinase protein was from Cell Signaling. Anti-MAP kinase2/ERK2 was from Upstate Biotechnology. LY294002 was from Cell Signaling. U0126 was from Promega. EGF was from BD Biosciences. The open reading frame of human centaurin-α₁ was subcloned into pSRα-HA vector, pSRα-Myc vector, pcDNA3.1 (+) vector, and pEGFP-C1 vector. The RC1RC2 mutant was generated by site-directed mutagenesis. The sequences of the primers used for mutating Arg-149 to Cys are 5'-CGGG CCA GTT TTT GAG CTG CAA GTT TGT GCT GAC AG-3' and 5'-CTG TCA GCA CAA AGC TGC TCA AAA ACT GCC CG-3'. The sequences of the primers used for mutating Arg-273 to Cys are 5'-GAA GGA GGC TTC CGG AGT CTG TCC TTC ATC ATG G-3' and 5'-CCA TGG TGA ACC AGC TCC GGA AGT CGG CAG AGG AAG TGG GTG TTC ACC ATG G-3'. Small interfering RNA (siRNA) expression plasmid was constructed using pCEP4 (Invitrogen). The selection marker was replaced from the original hygromycin-resistant gene to a puromycin-resistant gene by a PCR-based cloning approach. Human H1 promoter was inserted into the plasmid, and one tetracycline operator (tetO₂) sequence was introduced between the TATA box.

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1. These abbreviations are: ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; PIP₃, phosphatidylinositol 3,4,5-triphosphate; ARF, ADP ribosylation factor; GAP, GTPase-activating protein; PI(3,4,5)P₃, phosphatidylinositol (3,4,5)-trisphosphate; PI(3,4)P₂, phosphatidylinositol (3,4)-bisphosphate.

2. The abbreviations used are: ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; PIP₃, phosphatidylinositol 3,4,5-triphosphate; ARF, ADP ribosylation factor; GAP, GTPase-activating protein; PI(3,4,5)P₃, phosphatidylinositol (3,4,5)-trisphosphate; PI(3,4)P₂, phosphatidylinositol (3,4)-bisphosphate.
sequence and the transcription initiation site in the H1 promoter (15, 16).

Cell Cultures and Transfection—COS-7 cells, 293-EBNA cells (Invitrogen), and T-REx 293 cells (Invitrogen) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 5 or 10% fetal calf serum. The cells were maintained in 5% CO₂ at 37 °C. The cells were seeded on a 6 wells/cell culture dish at a density of 1.5 × 10⁵ cells/well. Transfection of these cells was performed using Lipofectamine Plus reagent (Invitrogen) or FuGENE 6 reagent (Roche Applied Science) according to the manufacturer’s protocols. The total amount of DNA was adjusted using pSR-HA, pSRα-Myc, or pcDNA3.1(+) vector.

EGF Stimulation—3 h after transfection using Lipofectamine Plus reagent, the medium was exchanged to DMEM containing 10% fetal calf serum, and the cells were incubated for 18 h. Thereafter, the cells were cultured in low serum medium (DMEM supplemented with 0.35% glucose and 1% heat-inactivated horse serum) for 20 h. The cells were stimulated with 30 nM EGF for 2 min at 37 °C.

Kinase Assays and Immunoblotting—21 h after transfection, COS-7 cells were lysed and immune complex kinase assay was performed as previously described (17). The following proteins were used as substrates: myelin basic protein for ERK2 and ERK5, glutathione S-transferase-c-Jun-(1–79) for c-Jun N-terminal kinase (JNK), and p38. After SDS-PAGE, radioactivity was analyzed using an image analyzer (Bio-Rad). The samples were subjected to immunoblotting with anti-HA, anti-GFP, or anti-Myc antibodies. Immunoblotting was also performed as previously described (17).

Cell Staining—Cells were fixed in 3.7% formaldehyde for 10 min. The coverslips were washed three times with phosphate-buffered saline for 5 min. Fluorescence images were observed using a Confocal Microscope (Bio-Rad).

Lipid Delivery—Synthetic phospholipids PI(3,4,5)P₃ (diC₁₆) and PI(4,5)P₂ (diC₁₆) (Echelon) were resolved in water at 300 μM and mixed with 100 μM histone (Echelon) for 5 min. COS-7 cells transiently transfected with GFP-fused centaurin-α₁ were treated with histone-phospholipid complexes diluted 1:10 with normal culture medium (DMEM supplemented with 10% fetal calf serum). After 2 min, the cells were fixed, and fluorescence images were observed.

Luciferase Assays—293-EBNA cells were seeded into 5% fetal calf serum-containing DMEM in a 96-well cell culture plate to a final density of 1 × 10⁶ cells/well and cultured for 24 h. Thereafter, the cells were transfected with 50 ng of pcDNA3.1-human centaurin-α₁, 60 ng of pFR-Luc (Stratagene), 0.25 ng of pFA2-Elk-1 (Stratagene), and 5 ng of siRNA expression plasmid or an empty vector in each well using FuGENE6. Twenty-four h after transfection, the reporter activity was measured using PicaGene LT2.0 (Toyo Ink) according to the manufacturer’s instructions.

Establishment of Cell Lines Expressing siRNA for Centaurin-α₁—The siRNA expression plasmids were introduced into T-REx 293 cells using FuGENE 6. Twenty-four h after transfection, the medium was exchanged to DMEM containing 1 μg/ml puromycin and 5% fetal calf serum. Seven days later, the puromycin-resistant cells were collected and used as cell lines in which siRNAs of human centaurin-α₁ were induced by doxycycline.

Quantitative Analysis of Centaurin-α₁ mRNA—The quantitative real-time PCR reaction was performed with an ABI PRISM 7000 sequence detection system according to the manufacturer’s instructions. The sequence of dual fluorophore-labeled probe for human cen-
Centaurin-α1 is a PI3K-dependent Activator of ERK

Centaurin-α1 is a PI3K-dependent Activator of ERK—In the previous report, it has been shown that overexpression of human centaurin-α1 can activate ERK in mammalian cells (9). To examine whether this activation is specific for the ERK MAP kinase cascade among the four major MAP kinase cascades, HA epitope-tagged or GFP-fused centaurin-α1 was co-expressed with four kinds of Myc-tagged MAP kinases, ERK2, ERK5, c-Jun N-terminal kinase, or p38 in COS-7 cells, and the activity of these MAP kinases was measured by in vitro kinase assays. As a result, only ERK2 showed the enhanced kinase activity when co-expressed with centaurin-α1 (Fig. 1A). This result indicates that centaurin-α1 is a specific activator of ERK among four MAP kinases.

Ras Is Involved in the Activation of ERK by Centaurin-α1—It is known that Ras, a low molecular weight GTP-binding protein, activates the Raf-MEK-ERK kinase pathway. To examine whether Ras is involved in the activation of ERK by centaurin-α1, we co-transfected COS-7 cells with centaurin-α1 and each of four kinds of dominant negative forms of low molecular weight G-proteins Ras, Cdc42, Rho, and Rac and measured the activity of ERK MAP kinase. As a result, centaurin-α1 failed to activate ERK when co-expressed with a dominant negative form of Ras (Fig. 1B). On the other hand, dominant negative forms of other low molecular weight G-proteins did not affect the activation of ERK by centaurin-α1 (Fig. 1B). A MEK inhibitor U0126 also inhibited the activation of ERK by centaurin-α1. These results indicate that the activation of ERK by the overexpression of centaurin-α1 in COS-7 cells is mediated by Ras.

PH Domains in Centaurin-α1 Are Important for ERK Activation—Centaurin-α1 has two PH domains in its N-terminal region. It has been reported that the PH domains are necessary for the ability of centaurin-α1 to bind PIP3 (13). Moreover, the RC1RC2 mutant form of centaurin-α1, in which Arg-149 and Arg-273 in the PH domains are mutated to Cys, is no longer recruited to the plasma membrane after EGF stimulation (18). To evaluate the role of the PH domains in the activation of ERK by centaurin-α1, we constructed the RC1RC2 mutant and examined its ability to activate ERK. Overexpression of the RC1RC2 mutant resulted in much weaker activation of ERK than that of wild-type centaurin-α1 in COS-7 cells (Fig. 2A). To investigate the localization of centaurin-α1, in the cell and to study the role of the PH domain for the translocation of centaurin-α1 by EGF stimulation, COS-7 cells were transfected with a GFP-fused form of wild-type or RC1RC2 mutant centaurin-α1. Before and after EGF stimulation, we determined their subcellular localization using the laser scanning confocal microscope. Before the stimulation, both wild-type and the RC1RC2 mutant centaurin-α1 largely localized to the nucleus and a small part of wild-type centaurin-α1 localized to the plasma membrane (Fig. 2B). After the stimulation, although wild-type centaurin-α1 was strongly recruited to the plasma membrane, the RC1RC2 mutant did not change their localization (Fig. 2B). These results suggest that the recruitment of centaurin-α1 to the plasma membrane, which is regulated by its PH domains, is important for the activation of ERK in COS-7 cells.

LY294002 Inhibits Centaurin-α1-dependent Activation of ERK—As PIP3, which binds to the PH domain, is a product of PI3K, PI3K activity may be required for ERK activation by centaurin-α1. We therefore examined the effect of a PI3K inhibitor, LY294002, on this centaurin-α1-dependent activation of ERK. COS-7 cells were co-transfected with centaurin-α1 and ERK and treated with LY294002. Immunoblot analysis showed that LY294002 is able to inhibit the phosphorylation of ERK in a dose-dependent manner (Fig. 3A). This indicates that PI3K activity is required for the activation of ERK induced by centaurin-α1. To investigate the role of PI3K activity on the subcellular localization of centaurin-α1, COS-7 cells expressing GFP-fused centaurin-α1 were treated with 20 μM LY294002. Although a small part of GFP-fused centaurin-α1 localized to the plasma membrane in the absence of LY294002, centaurin-α1 failed to localize to the plasma membrane in the presence of LY294002 (Fig. 3B). Furthermore, the translocation of wild-type centaurin-α1 to the plasma membrane by EGF stimulation was not detected in the presence of LY294002 (Fig. 3C). A MEK inhibitor U0126 did not inhibit the recruitment of centaurin-α1 to the plasma membrane (Fig. 3C). These results indicate that the EGF-dependent recruitment of centaurin-α1 requires PI3K activation but not ERK activation. We also demonstrated that exogenous PIP3 is able to induce the translocation of centaurin-α1 to the plasma membrane, whereas P(4,5)P2 does not induce such translocation (Fig. 3D). Without the histone carrier, phosphoinositides do not permeate the cell membrane (Fig. 3D). These results suggest that the PI3K- and PIP3-dependent recruitment of centaurin-α1 to the plasma membrane is required for the activation of ERK induced by centaurin-α1.

FIGURE 2. The RC1RC2 mutant fails to phosphorylate ERK and is not recruited to the plasma membrane by EGF stimulation. A, COS-7 cells were co-transfected with Myc-tagged ERK2 and the indicated amounts of either Myc-tagged centaurin-α1, or the Myc-tagged RC1RC2 mutant. 21 h after transfection, the cells were treated with either EGF or the RC1RC2 mutant. 21 h after transfection, the cells were serum-starved for 20 h and stimulated with EGF (30 nm) for 2 min. GFP-tagged proteins were imaged by confocal microscope before and after EGF stimulation. WT, wild-type.
Down-regulation of Centaurin-α₁ by siRNA Suppresses the Activation of ERK by EGF—To study the function of endogenous centaurin-α₁, we employed the siRNA approach. For the purpose of screening the sequences of siRNA that have an ability to knock down the expression of centaurin-α₁ mRNA, 293-EBNA cells were co-transfected with each of the siRNA expression plasmids, a centaurin-α₁ expression plasmid, pFR-Luc, and pFA2-Elk-1. As a result, we found that two sequences (si3 and si5) of 25 sequences we tested were able to suppress the reporter gene activity to less than half of that in the control experiment in which the empty vector was used instead of an siRNA expression vector (Fig. 4). The other 23 sequences including si4 had little or no effect on the reporter activity (Fig. 4 and data not shown).

We tried to obtain 293-EBNA stable cell lines that constitutively express the double-stranded RNA having the sequence si3, si4, or si5. Although we were able to establish si4-expressing cell lines, we were unable to obtain si3- or si5-expressing cell lines. This may suggest that centaurin-α₁ plays an important role in cell survival or proliferation.
Thereafter, we constructed the T-REx 293 cell lines in which the expression of short hairpin RNA (shRNA) of si3, si4, or si5 is induced by doxycycline. In T-REx 293 cells, the tetracycline repressor is constitutively expressed, and therefore shRNA is not expressed in the absence of doxycycline. To know the suppressive effect of siRNA on the expression of endogenous centaurin-α1 mRNA, we examined the expression level of centaurin-α1 mRNA in these cell lines after doxycycline treatment. As shown in Fig. 5A, si3 and si5 significantly attenuated the expression level of endogenous centaurin-α1 mRNA. On the other hand, si4 had only a small effect on the mRNA level (Fig. 5A). Next, we examined the contribution of centaurin-α1 in EGF signaling. The inducible siRNA-expressing cells were transfected with pFR-Luc, pFA2-Elk-1, and phRL-tk 24 h after doxycycline treatment. One day after the transfection, the cells were stimulated with EGF for 3 h, and luciferase activity was measured (Fig. 5B). The cells expressing shRNA si3 or si5 (but not those expressing si4) showed markedly decreased luciferase activity compared with the cells without induction of siRNA. This result indicates that centaurin-α1 is required for the EGF-dependent activation of Elk-1 in T-REx 293 cells. To examine whether centaurin-α1 is necessary for ERK activation by EGF, the phosphorylation state of ERK was analyzed after EGF stimulation with or without the pretreatment of cells with doxycycline. As shown in Fig. 5C, phosphorylation of ERK by EGF stimulation was decreased when the expression of centaurin-α1 was reduced by siRNA si3 or si5. In contrast, we could not detect the decrease of phosphorylated ERK in cells in which si4 was expressed (Fig. 5C). This result indicates that centaurin-α1 contributes to the phosphorylation of ERK in the EGF signaling pathway.

**DISCUSSION**

It is well known that, after growth factor stimulation, both the PI3K pathway and the ERK MAP kinase pathway are activated (19, 20).
Although these two pathways are activated independently, cross-talk between the PI3K and ERK pathways has been reported (21). However, the signaling molecules linking the PI3K pathway to the ERK MAP kinase cascade have been unclear. In this report, we have shown that centaurin-α1 may act as an adaptor molecule that links the PI3K activation to the ERK MAP kinase cascade. Our results reported here suggest that, upon growth factor stimulation, PIP$_3$, which is produced by activated PI3K, promotes the recruitment of centaurin-α1 to the plasma membrane via the PH domains of centaurin-α1, and thereafter centaurin-α1 activates Ras, resulting in the activation of the ERK MAP kinase cascade (see Fig. 6). Molecular mechanisms by which centaurin-α1 activates Ras remain elucidated.

When centaurin-α1 is overexpressed in COS-7 cells, a small part of wild-type centaurin-α1 localized to the plasma membrane without EGF stimulation. This may be due to the naturally excess amount of centaurin-α1 protein in the cells. Given that the RC1RC2 mutant form of centaurin-α1 hardly localized to the plasma membrane and that LY294002 treatment decreased the localization of centaurin-α1 to the plasma membrane, the basal activity of PI3K and the steady-state level of PIP$_3$ abundance may contribute to this localization.

In RNAi experiments, we used the HEK293 cell line to evaluate the role of centaurin-α1 in EGF signaling, as we found that both centaurin-α1 and the EGF receptor are endogenously expressed in this cell line (data not shown). The large scale functional genomics approaches identified centaurin-α1 as a putative AP-1 regulatory oncogene and suggested that centaurin-α1 could function to augment cell proliferation in a primary cell type and mediate cellular transformation (22). Our results demonstrating that centaurin-α1 has an ability to activate the ERK MAP kinase cascade can well explain this oncogenic activity of centaurin-α1. In a more recent report (12), it was shown that centaurin-α1 negatively regulates ARF6 activity through its function as an in vivo PIP$_3$-dependent ARF6 GAP. In this case, centaurin-α1 negatively regulates membrane trafficking and actin cytoskeleton architecture by inactivating ARF6. It has also been reported that centaurin-α1 is a neuronal actin-binding protein and regulates the actin cytoskeleton (23). Given that one of the functions of centaurin-α1 is to activate ERK MAP kinase, centaurin-α1 may regulate the cell fate by promoting cell proliferation and inhibiting various cellular activities, including membrane trafficking and actin cytoskeleton organization.

In humans, centaurin-α1 is strongly expressed in the brain, especially in neurons (24). In Alzheimer disease, the intraneuronal expression of centaurin-α1 protein was shown to be elevated. It has also been reported that the immunostaining of centaurin-α1 is concentrated in neuritic plaques, the neuropathological hallmark of Alzheimer disease (25, 26). Centaurin-α1 is for the most part in the nucleus, but during neuritogenesis centaurin-α1 becomes localized more in the neurites and the cytoplasm. This may suggest the possibility that centaurin-α1 recruited to the plasma membrane plays some role in neurite outgrowth (24). The activation of ERK by centaurin-α1 may contribute to these phenomena. Interestingly, when we overexpressed centaurin-α1 in COS-7 or PC12 cells, we often observed a number of neurite-like processes growing around the cell membrane. Centaurin-α1 is expressed ubiquitously and could regulate cell fate by acting as an adaptor molecule linking the PI3K pathway to the ERK MAP kinase cascade. The present functional characterization of centaurin-α1 could provide new insights into the understanding of growth factor signaling in mammalian cells.

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