Suprachiasmatic Nucleus Circadian Oscillatory Protein, a Novel Binding Partner of K-Ras in the Membrane Rafts, Negatively Regulates MAPK Pathway*

Kimiko Shimizu‡, Masato Okada§, Katsuya Nagaï, and Yoshitaka Fukada‡†

From the ‡Department of Biophysics and Biochemistry, Graduate School of Science, The University of Tokyo and Japan Science and Technology Corporation, Core Research for Evolutional Science and Technology, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan and the §Division of Protein Metabolism, Institute for Protein Research, Osaka University, 3-2 Yamada-Oka, Suita, Osaka 565-0871, Japan

Suprachiasmatic nucleus circadian oscillatory protein (SCOP) is a member of the leucine-rich repeat (LRR)-containing protein family. In addition to circadian expression in the rat hypothalamic suprachiasmatic nucleus, SCOP is constitutively expressed in neurons throughout the rat brain. Here we found that a substantial amount of SCOP was localized in the brain membrane rafts, in which only K-Ras was abundant among Ras isoforms. SCOP interacted directly through its LRR domain with a subset of K-Ras in the guanine nucleotide-free form that was present in the raft fraction. This interaction interfered with the binding of added guanine nucleotide to K-Ras in vitro. A negative regulatory role of SCOP for K-Ras function was examined in PC12 cell lines stably overexpressing SCOP or its deletion mutants. Overexpression of full-length SCOP markedly down-regulated ERK1/ERK2 activation induced by depolarization or phorbol ester stimulation, and this inhibitory effect of overexpressed SCOP was dependent on its LRR domain. These results strongly suggest that SCOP negatively regulates K-Ras signaling in the membrane rafts, identifying a novel mechanism for regulation of the Ras-MAPK pathway.

SCOP is (suprachiasmatic nucleus (SCN) circadian oscillatory protein) was originally identified in differential display screening of genes whose expression is regulated in a circadian manner within the rat hypothalamic SCN (1). The mRNA and protein levels of SCOP in the rat SCN increase during subjective night with a peak at midnight under constant dark conditions (1). In addition to circadian expression in the SCN, SCOP is expressed at a constant level in neurons throughout the rat brain (1), but its function in neurons including the SCN is yet to be elucidated. SCOP is a large polypeptide (1,966 amino acids) containing a pleckstrin homology (PH) domain, leucine-rich repeat (LRR), protein phosphatase 2C (PP2C)-like domain and glutamine (Q)-rich region (see Fig. 2), suggesting its unique role in intracellular signaling. The LRRs are characterized by multiple repeats of a sequence harboring leucine residues at invariable positions (2), and LRR-containing proteins are thought to contribute to diverse biological functions through the LRR-mediated protein-protein interactions (2). The LRR in SCOP is composed of 18-fold repeats of a short stretch unit with a relatively conserved but variable sequence, LXXLXNXXLXXLXXXXX, where L, N, P, A, and X denote leucine, asparagine, proline, aliphatic, and any amino acid, respectively (1). Yeast Saccharomyces cerevisiae adenylate cyclase contains 26-fold repeats of a similar unit (3), to which Ras binds for Ras-dependent enzymatic activation (4, 5). The homologous LRR is also found in human and Caenorhabditis elegans SUR-8 (6), which interacts directly with Ras to regulate its signaling (6, 7). It is thus predicted that SCOP may also interact with Ras to regulate Ras-mediated signaling pathway.

As a molecular switch, Ras cycles between a GTP-bound active state and a GDP-bound inactive state via GTP hydrolysis and GDP/GTP exchange steps. Two major types of Ras-interacting molecules have been described that modulate the Ras GTP-GDP cycle through regulation of either the activation or inactivation step. The former is mediated by a class of proteins, guanine nucleotide-exchange factors (GEFs), that interact with the GDP-bound form of Ras to reduce its affinity for GDP. This facilitates formation of the guanine nucleotide-free form of Ras, to which GTP binds for its activation. The latter is accelerated by GTPase activating proteins (GAPs), which increase the intrinsic GTPase activity of Ras and thereby promote the inactivation of Ras. Although the regulatory elements of Ras proteins may not be fully understood yet, these steps should provide a mechanism that manipulates the timing and strength of Ras signaling to ensure appropriate signaling output (8). In addition to these regulatory steps, the presence of Ras isoforms may contribute to a well ordered framework of Ras-mediated signaling. Despite a high degree of sequence similarity among Ras isoforms, accumulating evidence points to a preferential activation of specific effectors by each Ras isoform (9, 10). This issue seems to be associated with protein clustering in membrane microdomains such as rafts (11, 12). Membrane rafts are small platforms composed of sphingolipids, cholesterol, and a given set of proteins and are important for regulation of cellular signaling (11, 12). Selective localization of
SCOP Negatively Regulates K-Ras-MAPK Pathway

14921

the Ras isoforms at different membrane microdomains reinforces the idea of distinct functionality and regulation of these proteins (13). Knockout mouse for each of the Ras isoforms showed that K-Ras, but not H-Ras or N-Ras, is essential for development (14–16), and these results further support the idea that Ras isoforms play distinct roles.

The mitogen-activated protein kinase (MAPK) pathway is one of the best-characterized effector systems downstream of Ras (17, 18). Ras-MAPK-mediated signaling plays critical roles in cell proliferation, differentiation, and migration in response to extracellular signals. Recent studies have demonstrated an important contribution of Ras-MAPK cascade to the circadian clock system in the rodent SCN (19) and the chick pineal gland (20, 21). Here we show that SCOP functions as a negative regulator of the Ras-MAPK pathway by interacting with the nucleotide-free form of K-Ras in the membrane rafts to downregulate the nucleotide binding for its activation. The results presented in this study illustrate a novel type of regulatory mechanism for Ras signaling and suggest a contribution of SCOP to a variety of cellular functions.

EXPERIMENTAL PROCEDURES

Preparation of Detergent-insoluble Membrane Raft Fraction—The raft fraction was prepared as described (22) with modifications. Whole brains from adult male rats (7 weeks) were homogenized in buffer A (50 mM Tris-HCl, 1 mM EDTA, 50 mM NaCl, 2.5 mM β-mercaptoethanol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4) with a 10× volume of the brain mass. The resulting homogenate was centrifuged at 1,000 × g for 15 min to sediment tissue debris. The unpregnated material was centrifuged at 100,000 × g for 1 h. The resulting membrane pellet was suspended in buffer A with 1% (v/v) Triton X-100 at a volume equal to that used for the initial homogenate and then centrifuged for 1 h at 10,000 × g and combined with an equal volume of buffer B with 80% (v/v) sucrose. The mixture was divided into 3-mI samples. Each sample was overlaid sequentially with buffer B with 35% (v/v) sucrose (5 ml) and 5% (v/v) sucrose (2 ml), respectively. After centrifugation at 35,000 rpm for 20 h at 4 °C in a Beckman SW41 swing type rotor, the Triton X-100 insoluble cloudy material located at the interface between 5 and 35% sucrose solutions was collected (“raft fraction”).

Preparation of Fusion Proteins—Various SCOP fragments (see Fig. 2A) were produced in Escherichia coli as GST fusion proteins. These include (i) GST-PHF>LRR containing the PH domain and LRR (amino acids 420–1241 in SCOP), (ii) GST-LRR-PHOSQ containing the LRR, PHOS, and Q-rich domains (amino acids 588–1896), (iii) GST-LRR containing a part (the 7th through the 18th repeat) of LRR (amino acids 735–1027), and (iv) GST-PHOS containing the PHOS-like domain (amino acids 1082–1384). The GST fusion proteins were purified with glutathione-Sepharose 4B (Amersham Biosciences) according to the manufacturer’s protocol. The GST and MBP fusion proteins of full-length K-Ras were produced in E. coli. The latter was purified by amylase resin (New England Biolabs) according to the manufacturer’s protocol.

Pull-down Assay of Ras Proteins with SCOP Fragments—The raft fraction (30 μl) was mixed with 1 ml of buffer A containing 60 mM n-octyl-β-D-glucoside (nOG) and incubated for 1 h at 4 °C. In the presence or absence of guanine nucleotide, the nOG-solubilized raft fraction was incubated for 4 h at 4 °C with each GST fusion protein of the SCOP fragments (0.5 μg) premixed with glutathione-Sepharose beads (10 μl). The beads were then washed three times with buffer A containing 60 mM nOG. Bound proteins were separated by SDS-PAGE, followed by immunoblot analysis with anti-K-Ras monoclonal antibody (1:400, Santa Cruz Biotechnology). In experiments with recombinant K-Ras, each GST fusion protein of the SCOP fragments (0.5 μg) pre-bound to gluthione-Sepharose beads was incubated with purified MBP-K-Ras (1 μg) for 4 h at 4 °C in a binding buffer (20 mM Tris-HCl, 200 mM NaCl, 5 mM EDTA, 1% (v/v) Nonidet P-40, 5% (v/v) glycerol, 2 mg/ml bovine serum albumin, 5 mM β-mercaptoethanol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, pH 7.4). The beads were washed three times with the binding buffer, once with the binding buffer without the presence of bovine serum albumin, and treated with SDS sample buffer for the immunoblot analysis.

Detection of Active Form of K-Ras—The raft fraction (30 μl) was mixed with 1 ml of buffer A containing 60 mM nOG and 10 mM MgCl2 and incubated for 1 h at 4 °C. The raft fraction thus solubilized was incubated with 10 μl of GST-RBD (Ras-binding domain of Raf-1) coupled to glutathione-agarose beads (Upstate Biotechnology). The beads were washed three times with buffer A, and the bound proteins were subjected to SDS-PAGE and immunoblot analysis with the anti-K-Ras antibody.

GT/P-S Binding Assay—The guanine nucleotide binding activity of GST-K-Ras was evaluated at 4 °C by incubating with 20 μM [35S]GTP-γ-S (3 Ci/mmol, PerkinElmer Life Sciences) and the raft fraction (1 μl) in the presence or absence of GST-LRR or GST in a total of 30 μl of buffer B (25 mM Tris-HCl, 100 mM NaCl, 1 mM dithiothreitol, 10 mM MgCl2, 60 mM nOG, pH 7.4). The GTP-γ-S binding reaction was quenched by the addition of 170 μl of a washing buffer (100 mM Tris-HCl, 1 mM MgCl2, pH 7.4) containing 3 mM GTP. The reaction mixture was transferred to a multiscreen filter cup (Millipore, 0.45-μm cellulose membranes, type WE) and washed. The filter was immediately washed five times with 0.2 ml of the washing buffer, dried, and solubilized by adding 100 μl of 2-methoxyethanol for scintillation counting with 800 μl of scintillation cocktail ACSII (Amersham Biosciences).

Plasmid Construction and Functional Analysis of SCOP in PC12 Cells—The cDNA encoding mutant E. coli’s medium containing 10% horse serum and 5% fetal bovine serum. The cultured cells were transfected with the expression plasmid using LipofectAMINE Plus (Invitrogen). The transfected cells were selected in the presence of G418 (0.5 mg/ml) as a selective antibiotic to obtain several lines of stable transformants. The expression level of SCOP protein in each cell line was evaluated by immunoblot analysis with αEC and αCB antibodies that recognize the LRR- and PP2C-like domains of SCOP, respectively. Wild-type PC12 cells or the stable transformants thus established were plated on 35-mm culture dishes at a density of 8 × 104 cells/cm2 and cultured for 20 h at 37 °C. Cells were exposed to 50 mM KC1 or 200 mM α-tetradecanoylphorbol-13-acetate (TPA, from Sigma) and collected at the indicated time point in 100 μl of ice-cold RIPA buffer (20 mM Tris-HCl, 150 mM NaCl and 1% (v/v) Nonidet P-40, 1% (w/v) sodium deoxycholate, 0.1% (v/v) β-mercaptoethanol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 1 mM Na3VO4, pH 7.4). After a 30-min incubation on ice for solubilization, the lysate was centrifuged at 15,000 × g for 20 min at 4 °C. Supernatant was subjected to immunoblot analysis using anti-P-MAPK antibody (New England Biolabs) or Pan-ERK antibody (Transduction Laboratories) with the aid of a chemiluminescence detection system (PerkinElmer Life Sciences).

RESULTS

Colocalization and Interaction of SCOP with K-Ras in Membrane Rafts—Functional studies on the LRRs in yeast adenylylate cyclase (3–5) and SUR-8 (6, 7) suggested the possibility that SCOP interacts with Ras through the LRR domain. On the other hand, the PH domain has been implicated in membrane localization of proteins (23–26), and it seems to confer raft association in some cases (27, 28). We therefore paid special attention to the LRR and PH domains and explored possible localization of SCOP with Ras protein in the membrane rafts. Rat brain membranes were treated with 1% (v/v) Triton X-100 and subjected to a discontinuous sucrose gradient centrifugation. The raft fraction was recovered as Triton X-100-insoluble materials at the interface between 5 and 35% sucrose solutions (29). This fraction contained a substantial amount of SCOP and K-Ras, whereas the majority of H-Ras and N-Ras proteins were detected in the other fractions (Fig. 1). We examined the interaction of SCOP fragments with K-Ras recovered in the raft fraction. The raft fraction was treated with 60 mM nOG for solubilization and mixed with a GST-SCOP fragment fusion protein (Fig. 2A and B) that was pre-bound to glutathione-Sepharose beads. Immunoblot analysis of the precipitated proteins demonstrated that GST-PHF>LRR and GST-LRR associated with K-Ras but neither GST-PHOS nor GST alone did (Fig. 2C). Neither H-Ras nor N-Ras was detected in the same blot by the specific antibodies (data not shown). Similar experiments were performed with 1% Triton X-100-
edly increased after incubation with 10 mM GTP (Fig. 3A). We then determined whether the nucleotide binding state of K-Ras affected its interaction with SCOP LRR. First we measured the amount of active form of K-Ras in the nOG-solubilized raft fraction bound with MBP (Fig. 3A, top panel), indicating that a certain amount of K-Ras in the fraction can be converted to the active GTPγS-bound form only by incubation with GTPγS. This is indicative of either a difference in the state of K-Ras protein between the raft and non-raft fractions or of alternate involvement of raft-specific component(s) in this interaction. The latter possibility was excluded by in vitro pull-down experiments in which both GST-LRR/PHOS/Q and GST-LRR were shown to precipitate MBP-K-Ras (but not MBP) expressed in E. coli (Fig. 2D).

Using RBD, a specific probe for the GTP-bound active form of Ras, we then determined whether the nucleotide binding state of K-Ras affected its interaction with SCOP LRR. First we measured the amount of active form of K-Ras in the nOG-solubilized raft fraction and found an intermediate level of K-Ras capable of binding with GST-RBD (Fig. 3A, top panel, none). The amount of K-Ras precipitated with GST-RBD markedly increased after incubation with 10 μM GTPγS (Fig. 3A, top panel), indicating that a certain amount of K-Ras in the fraction can be converted to the active GTPγS-bound form only by incubation with GTPγS. On the other hand, K-Ras in the GTP-bound form in the original fraction was converted to a form incapable of binding with RBD (probably the GDP-bound form) only by incubation with 10–100 μM GDP (Fig. 3A, top panel). These preparations of K-Ras were then tested for binding selectivity of SCOP LRR by incubating with GST-LRR/PHOS/Q or GST-LRR. We found that a certain amount of K-Ras in the original nOG-solubilized raft fraction bound with both GST-LRR/PHOS/Q and GST-LRR (Fig. 3A, panels 2 and 3, none). This interaction was abrogated when the raft fraction was preincubated not only with GDP but also with GTPγS (Fig. 3A, top panel). These results strongly suggest that SCOP LRR has no or very low affinity for both GDP- and GTPγS-bound forms of K-Ras. It is most probable that the LRR domain binds to the nucleotide-free form of K-Ras that is probably present in the membrane rafts.

SCOP LRR Is a Negative Regulator for Guanine Nucleotide Binding to K-Ras—We then asked whether the interaction of K-Ras with the LRR domain may affect its guanine nucleotide binding by examining the effect of postincubation of the LRR-K-Ras complex with GDP or GTPγS. Once bound to GST-LRR in the absence of guanine nucleotides, most of the K-Ras continued to be associated with LRR even after postincubation with 10 μM GDP (Fig. 3B, lanes 3 and 4). This contrasted sharply with the effect of the preincubation (lane 2). Similar results were obtained in pre- and postincubation experiments with 10 μM GTPγS (Fig. 3B, lanes 5–7), although a minimal amount of K-Ras was dissociated from GST-LRR after the postincubation (lanes 6 and 7). These observations suggest the LRR-dependent inhibition of guanine nucleotide binding to the nucleotide-free form of K-Ras in the raft fraction.

soluble fraction (100,000 × g supernatant prepared from the rat brain homogenate; see “Experimental Procedures”), and we found that none of the Ras isoforms coprecipitated with any of the SCOP fragments examined (data not shown). These results demonstrate the LRR-mediated interaction of SCOP with K-Ras only in the membrane rafts. This is indicative of either a difference in the state of K-Ras protein between the raft and non-raft fractions or of alternate involvement of raft-specific component(s) in this interaction. The latter possibility was excluded by in vitro pull-down experiments in which both GST-LRR/PHOS/Q and GST-LRR were shown to precipitate MBP-K-Ras (but not MBP) expressed in E. coli (Fig. 2D).

Using RBD, a specific probe for the GTP-bound active form of Ras, we then determined whether the nucleotide binding state of K-Ras affected its interaction with SCOP LRR. First we measured the amount of active form of K-Ras in the nOG-solubilized raft fraction and found an intermediate level of K-Ras capable of binding with GST-RBD (Fig. 3A, top panel, none). The amount of K-Ras precipitated with GST-RBD markedly increased after incubation with 10 μM GTPγS (Fig. 3A, top panel), indicating that a certain amount of K-Ras in the fraction can be converted to the active GTPγS-bound form only by incubation with GTPγS. On the other hand, K-Ras in the GTP-bound form in the original fraction was converted to a form incapable of binding with RBD (probably the GDP-bound form) only by incubation with 10–100 μM GDP (Fig. 3A, top panel). These preparations of K-Ras were then tested for binding selectivity of SCOP LRR by incubating with GST-LRR/PHOS/Q or GST-LRR. We found that a certain amount of K-Ras in the original nOG-solubilized raft fraction bound with both GST-LRR/PHOS/Q and GST-LRR (Fig. 3A, panels 2 and 3, none). This interaction was abrogated when the raft fraction was preincubated not only with GDP but also with GTPγS (Fig. 3A, top panel). These results strongly suggest that SCOP LRR has no or very low affinity for both GDP- and GTPγS-bound forms of K-Ras. It is most probable that the LRR domain binds to the nucleotide-free form of K-Ras that is probably present in the membrane rafts.

SCOP LRR Is a Negative Regulator for Guanine Nucleotide Binding to K-Ras—We then asked whether the interaction of K-Ras with the LRR domain may affect its guanine nucleotide binding by examining the effect of postincubation of the LRR-K-Ras complex with GDP or GTPγS. Once bound to GST-LRR in the absence of guanine nucleotides, most of the K-Ras continued to be associated with LRR even after postincubation with 10 μM GDP (Fig. 3B, lanes 3 and 4). This contrasted sharply with the effect of the preincubation (lane 2). Similar results were obtained in pre- and postincubation experiments with 10 μM GTPγS (Fig. 3B, lanes 5–7), although a minimal amount of K-Ras was dissociated from GST-LRR after the postincubation (lanes 6 and 7). These observations suggest the LRR-dependent inhibition of guanine nucleotide binding to the nucleotide-free form of K-Ras in the raft fraction.
The LRR-mediated negative regulation of K-Ras activation (GTP binding) was investigated directly by measuring the rate of GTP\(\gamma\)S binding to recombinant GST-K-Ras protein. The GTP\(\gamma\)S binding to GST-K-Ras absolutely required the presence of the rafts (Fig. 4A), which likely provide a guanine nucleotide exchange factor activity for recombinant K-Ras. In the presence of a constant amount of the raft fraction, GTP\(\gamma\)S binding to GST-K-Ras showed a nearly linear relationship with the amount of GST-K-Ras protein at any time point of incubation (Fig. 4A, solid symbols). We investigated the effect of GST-LRR and found that the addition of increasing amounts of GST-LRR reduced the amount of GTP\(\gamma\)S binding (Fig. 4B). The presence of a 10-fold molar excess of GST-LRR (60 pmol) over GST-K-Ras (6 pmol) inhibited the binding to a level of ~40% of that observed in the presence of an equivalent amount of GST (Fig. 4B).

**SCOP Suppresses the Activation of MAPK Pathway**—To explore the function of SCOP in *vivo*, we established several lines of PC12 cells stably overexpressing full-length SCOP (Fig. 5A, SCOP) and examined the effect of Ras-stimulating treatment on extracellular signal-regulated kinase 1 and 2 (ERK1/2) that are well established targets downstream of Ras. In PC12 cells, membrane depolarization elicits voltage-sensitive calcium channel-dependent calcium ion influx that triggers ERK phosphorylation/activation via activation of Ras (30). Treatment of wild-type PC12 cells with 50 mM KCl stimulated phosphorylation of ERK1/2, which reached a maximal level 5 min after KCl application (Fig. 5B, WT). Phosphorylation of ERK decreased to an intermediate level that was sustained for at least 60 min after the onset of the stimulation. In SCOP-overexpressing cells, KCl treatment also elevated ERK1/2 phosphorylation (Fig. 5B, SCOP). However, with the exception of the 2-min time point, the level of phosphorylation observed was significantly lower than that seen in KCl-stimulated wild-type cells. Moreover, the kinetics for phosphorylation of ERK1/2 in SCOP-overexpressing cells lacked the sharp peak seen in WT cells upon KCl treatment (Fig. 5B, SCOP). To determine whether these differences were dependent on the LRR domain of SCOP, we established two other PC12 cell lines (Fig. 5A), each overexpressing a SCOP deletion mutant devoid of either the LRR (SCOP-\(\Delta$$LRR$$) or PP2C-like plus Q-rich domain (SCOP-$$\Delta$$PHOS\(\gamma\)). The KCl-stimulated PC12 cells overexpressing SCOP-\(\Delta$$LRR$$ showed kinetics for ERK1/2 phosphorylation very similar to wild-type cells (Fig. 5B, compare SCOP-\(\Delta$$LRR$$ with WT). By contrast, the PC12 cells overexpressing SCOP-\(\Delta$$PHOS\(\gamma\) exhibited a weak sustained response of ERK1/2 phosphorylation (Fig. 5B, SCOP-\(\Delta$$PHOS\(\gamma\)), as was observed in cells overexpressing full-length SCOP. This LRR-dependent inhibition of ERK1/2 phosphorylation by SCOP was reproduced for each construct in two other cell lines overexpressing each protein at different levels (data not shown). No significant change in ERK2 protein level was observed, not only among samples from different time points (< 3 h) but also among cells lines (Fig. 5B, lower panels in each pair).

In another experiment, the PC12 cells were treated with TPA, which induces PKC activation (31). This treatment also stimulates phosphorylation and activation of MAPK in a Ras-dependent manner (32,33). Wild-type PC12 cells responded to TPA treatment with a sharp increase in ERK1/2 phosphorylation, peaking at 5 min, which then declined gradually over 180 min (Fig. 5C, WT). This sharp response of ERK1/2 phosphorylation to TPA treatment was blunted not only in the cells overexpressing full-length SCOP (Fig. 5C, SCOP) but also in SCOP-\(\Delta$$PHOS\(\gamma$$-overexpressing cells (Fig. 5C, SCOP-\(\Delta$$PHOS\(\gamma$$). On the other hand, the sharp response of ERK1/2 phosphorylation to TPA treatment was observed in PC12 cells overexpressing SCOP-\(\Delta$$LRR$$, again indicating the importance of the SCOP LRR domain for negative regulation of the MAPK. No significant change in ERK2 protein level was observed among the various samples (Fig. 5C, lower panels in each pair).

**DISCUSSION**

LRRs have been found in a variety of proteins with diverse functions and cellular locations (2). We first predicted the in-

![Fig. 4. The effect of SCOP LRR on GTP\(\gamma\)S binding to K-Ras.](image-url)
cultured PC12 cells were exposed to 50 mM KCl and subjected to immunoblot analysis with a mixture of anti-SCOP antibodies (Ref. 1, aEC and αCB). B and C, the cultured PC12 cells were exposed to 50 mM KCl (B) or 200 mM TPA (C) at time 0 and collected at the indicated time point for preparing soluble lysate in RIPA buffer. These samples were subjected to immunoblot analysis with anti-P-MAPK (upper panel in each pair) or Pan-ERK antibody (lower panels). The latter antibody displayed a low sensitivity to ERK1, and therefore an ERK1 protein band is not evident in the blot as compared with its immunoreactivity to anti-P-MAPK.

The effects of overexpressed SCOP and its deletion mutants on ERK phosphorylation in PC12 cells. A, the RIPA-solubilized lysate of wild-type PC12 cells (WT) or its stable cell lines transformed with full-length SCOP (SCOP), LRR-deleted SCOP (SCOP-ΔLRR), and PP2C-like and Q-rich domain-deleted SCOP (SCOP-ΔPHOS/Q) were subjected to immunoblot analysis with a mixture of anti-SCOP antibodies (Ref. 1, aEC and αCB). B and C, the cultured PC12 cells were exposed to 50 mM KCl (B) or 200 mM TPA (C) at time 0 and collected at the indicated time point for preparing soluble lysate in RIPA buffer. These samples were subjected to immunoblot analysis with anti-P-MAPK (upper panel in each pair) or Pan-ERK antibody (lower panels). The latter antibody displayed a low sensitivity to ERK1, and therefore an ERK1 protein band is not evident in the blot as compared with its immunoreactivity to anti-P-MAPK.

interaction of SCOP LRR (18 repeats) with Ras because of its similarity in sequence to the LRR of yeast adenylate cyclase (26 repeats) (3) and of SUR-8 (18 repeats) (6), both of which are known to bind with Ras for functional regulation. The present study provides evidence for the direct interaction of Ras with SCOP LRR, but the LRR domain of SCOP was functionally different from those of yeast adenylate cyclase and SUR-8 in terms of Ras regulation. Yeast adenylate cyclase interacts with the GTP-bound form of Ras and is thereby activated (4, 34), whereas the interaction of SUR-8 with the GTP-bound form of Ras results in activation of MAPK pathway (7). In contrast, SCOP associates with the nucleotide-free form of K-Ras in the membrane rafts to down-regulate the Ras-MAPK pathway. This is a novel mechanism for negative regulation of Ras protein function through interaction with LRRs.

Despite the high sequence similarity among Ras isoforms, SCOP selectively associates with K-Ras, a unique isoform that is abundant in the raft fraction of the rat brain homogenate (Fig. 1). It has been recognized that activation of different Ras isoforms results in distinct signaling outputs (9, 14, 15, 35–37), and the membrane microdomains such as rafts and caveolae most likely contribute to the segregated signaling (11, 12). It should be noted that in some cell lines established from non-neural tissues H-Ras operates in membrane rafts, whereas K-Ras seems to be located outside rafts (13, 38), in contrast with the selective enrichment of K-Ras in the rafts of the rat brain membranes (Fig. 1). This may be ascribed to cell type-specific compositions of rafts (for example, the existence of neuron-specific proteins such as SCOP), and hence neural membrane rafts might be unique because they lack caveolin (11).

Although SCOP and K-Ras were also present in the Triton X-100-soluble (non-raft) fraction, we observed no significant binding of SCOP LRR to K-Ras present in non-raft fractions. This observation suggests the idea that SCOP and K-Ras may become associated only in the membrane rafts or alternatively that their interaction may induce the rafts formation. However, direct involvement of a third (raft-specific) component is inconsistent with the results of our pull-down experiments in which recombinant SCOP and K-Ras proteins were both expressed in E. coli (Fig. 3D). Instead, SCOP LRR seems to associate selectively with the nucleotide-free form of K-Ras present in the membrane rafts but not with the GTPγS- nor GDP-bound forms that were produced by adding the guanine nucleotides after solubilization of the rafts. These data indicate that in the membrane rafts, just as in the E. coli lysate, at least a part of K-Ras is kept in the nucleotide-free form or in a form with an extremely low affinity for the guanine nucleotides. The postincubation experiment with GTPγS or GDP (Fig. 3B) strongly suggests that SCOP LRR “traps” the nucleotide-free state (or a low affinity state) of K-Ras that is generated by GDP release after receiving signals. This binding probably down-regulates the K-Ras function (Fig. 5) by inhibiting the GTP binding reaction (Fig. 4B). In this regard, the action of SCOP provides a striking contrast with that of CDC25p, which is also supposed to bind and stabilize the nucleotide-free state of Ras but promotes its activation (39).

In PC12 cells, SCOP suppressed activation of ERK1 and -2 (Fig. 5), which are well characterized effectors downstream of Ras. In both cortical neurons and PC12 cells, calcium triggers ERK activation via activation of Ras (30, 40). It is interesting to note that although KCl and TPA treatment activate Ras through different pathways (30–33) the effects of SCOP overexpression on the stimulus-dependent change in ERK1/2 phosphorylation were similar to each other (Fig. 5). This suggests that SCOP acts on Ras itself or on a step downstream of Ras in the signaling pathway. The finding that SCOP binds directly to K-Ras (Fig. 2B) supports K-Ras as the target site of SCOP.

In rodent SCN, SCOP-mediated regulation of the K-Ras-MAPK pathway may be important for the maintenance of circadian rhythm (19–21, 41). Both the protein level of SCOP (1) and the phosphorylation (and activation) of ERK1/2 (19) display a circadian variation in rodent SCN. MAPK activity has its peak late in the subjective day when the protein level of SCOP is low, whereas MAPK activity is minimum at mid-late subjective night when SCOP expression is highest. Therefore, we hypothesize that SCOP inhibition of ERK1/2 contributes to the circadian oscillation of ERK1/2. In addition to the circadian
expression in the SCN, a wide distribution of SCOP in the rat brain (1) suggests that SCOP may play a general role in regulation of the K-Ras-MAPK pathway and contribute to a variety of physiological functions such as neural plasticity and neuronal survival. SCOP expression is not circadian-regulated in non-SCN areas of the rat brain (1), and there must be an as yet unidentified regulatory mechanism for SCOP function. A possible regulatory step is the translocation of SCOP between the membrane rafts and cytosol, which could be modulated by the PH domain and/or affected by post-translational modifications such as phosphorylation. In preliminary experiments, we detected phosphorylated SCOP in the brain homogenate and 3T3 cell lysate. Future studies on each domain function of SCOP would help to understand a new aspect of regulatory processes of the K-Ras-MAPK pathway not only in the SCN but also in other brain areas.

Acknowledgments—We thank Maiko Kataeda for excellent technical assistance, Isaac Sterling, Prof. Daniel R. Storm, and Dr. Guy C.-K. Chan for careful reading and many helpful comments on the manuscript, and colleagues in the laboratories for technical support and encouragement.

REFERENCES