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# nRap GEP: A Novel Neural GDP/GTP Exchange Protein for Rap1 Small G Protein That Interacts with Synaptic Scaffolding Molecule (S-SCAM)[1]

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**Synaptic scaffolding molecule (S-SCAM) has six PDZ domains through which it interacts with *N*-methyl-D-aspartate receptors and neuroligin at synaptic junctions. We isolated here a novel S-SCAM-binding protein. This protein has one PDZ, one Ras association, one Ras GDP/GTP exchange protein (Ras GEP) domain, and one C-terminal consensus motif for binding to PDZ domains. We named it nRap GEP (neural Rap GEP). nRap GEP moreover has an incomplete cyclic AMP (cAMP)-binding (CAB) domain. The domain organization of nRap GEP is similar to that of Epac/cAMP–guanine nucleotide exchange factor (GEF) I, except that Epac/cAMP–GEFI has complete CAB and Ras GEP domains but lacks the other two domains and the C-terminal motif. nRap GEP showed GEP activity for Rap1 but did not bind cAMP. nRap GEP was specifically expressed in rat brain. Immunohistochemical analysis revealed that nRap GEP and S-SCAM were localized at synaptic areas of the cerebellum. These results suggest that nRap GEP is a novel neural Rap1-specific GEP which is associated with S-SCAM.** © 1999 Academic Press

Abbreviations used: PSD, postsynaptic density; SAP, synapse-associated protein; PDZ, PSD-95/Dlg-A/ZO-1; GK, guanylate kinase; NMDA, *N*-methyl-D-aspartate; SAPAP, SAP90/PSD-95-associated protein; GKAP, guanylate kinase-associated protein; DAP, hDLG-associated protein; S-SCAM, synaptic scaffolding molecule; GEP, GDP/GTP exchange protein; MAP, mitogen-activated protein; GAP, GTPase activating protein; TBST, Tris-buffed saline containing 0.05% Tween 20; pAb, polyclonal antibody; mAb, monoclonal antibody; RA, Ras association; CAB, cAMP-binding; GEF, guanine nucleotide exchange factor; GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; GFP, green fluorescent protein; SPM, synaptic plasma membrane.

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PSD-95/SAP90 contains three PDZ, one src-homology 3, and one GK domains and serves as a scaffolding molecule at synaptic junctions by interacting with many functional and structural proteins (reviewed in Refs. 1–3). Analysis of PSD-95/SAP90-deficient mice has indeed revealed its important role in synaptic plasticity (4). The PDZ domains of PSD-95/SAP90 directly interact with transmembrane proteins, such as NMDA receptors (5), $K^+$ channels (6), and neuroligin (7), with signaling molecules, such as synGAP (8), MAGUIN-1 (9), and citron (10), and with cytoskeletal proteins, such as CRIPT (11), whereas the GK domain directly interacts with SAPAP (also called GKAP and DAP) (12–14) and SPA-1-like protein (15).

We have recently isolated another synaptic scaffolding molecule which directly interacts with SAPAP and named it S-SCAM (16). S-SCAM has six PDZ, two WW, and one GK domains, and interacts with many molecules, such as NMDA receptors, neuroligin, and MAGUIN-1 (9, 16), which are also PSD-95/SAP90-binding molecules. Both PSD-95/SAP90 and S-SCAM are specifically expressed in neural tissues, but they have ubiquitous isoforms, named hDLG and MAGI-1/BAP1, respectively (17–20).

Accumulating evidence suggests that S-SCAM as well as PSD-95/SAP90 plays a role in organization of synaptic junctions. To explore the role of S-SCAM at synaptic junctions, we have first attempted here to

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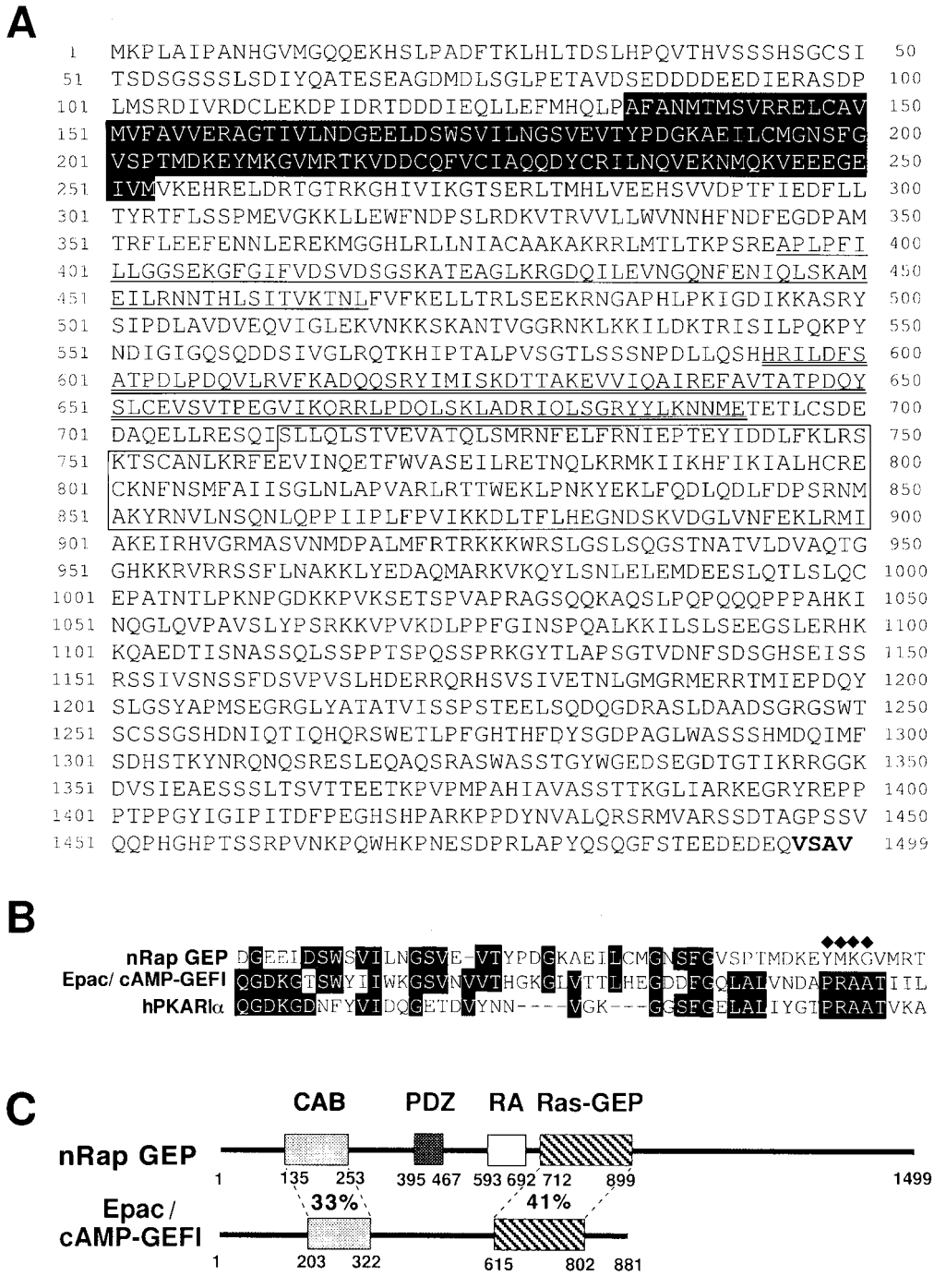


**FIG. 1.** Molecular structure of human nRap GEP. (A) Deduced aa sequence of human nRap GEP. *Black shading,* the CAB domain; *underlines,* the PDZ domain; *double underlines,* the RA domain; and *open box,* the Ras GEP domain. The C-terminal consensus sequence for binding to PDZ domains is shown in *bold letters.* These domains were identified by a module prediction program, SMART (32). (B) Alignment of the CAB domains of human nRap GEP, human Epac/cAMP-GEFI, and a human PKA regulatory subunit. *Black shading,* identical aa residues; and *black diamonds,* a PRAA motif that is present in the cAMP-binding pocket of PKA (reviewed in Ref. 31). *hPKARIα,* human PKA regulatory subunit type I-alpha (AF125193; GenBank/EMBL/DDBL). It should be noted that the CAB domain of nRap GEP is incomplete and does not bind cAMP. (C) Comparison of domain structures of nRap GEP and Epac/cAMP-GEFI. The aa sequence identities in the CAB and Ras GEP domains are 33 and 41%, respectively.

identify a novel S-SCAM-binding protein, and isolated a novel cAMP-independent GEP specific for Rap1 small G protein, named nRap GEP. Rap1 directly binds to c-Raf-1 and B-Raf, which are known to activate the MAP kinase pathway, but Rap1 antagonizes the Ras-induced activation of c-Raf-1 (reviewed in Ref. 21),

whereas both Rap1 and Ras activate B-Raf (22, 23). Rap1 and Ras are activated by GEP and inactivated by GAP (21). Many GEPs and GAPs for these small G proteins have been isolated and characterized. Of these, synGAP, a GAP for Ha-Ras (8), and SPA-1-like protein, a GAP for Rap1A (24), interact with PSD-95/SAP90 (8, 15). The property that nRap GEP directly interacts with S-SCAM has provided additional evidence that Rap1 also plays a role at the synapse.

## MATERIALS AND METHODS

*Expression cloning of nRap GEP.* Expression cloning was done as described (25). Briefly, the [$^{35}$S]methionine-labeled probe of S-SCAM was prepared using the TNT T7 quick coupled transcription/translation system (Promega) with pCIneo-Myc-S-SCAM-3 as a template. A fetal mouse brain expression cDNA library (Novagen) was plated at a density of $2 \times 10^5$ plaques/22-cm$^2$ dish. The plates were overlaid with nitrocellulose filters previously saturated with 10 mM isopropyl-$\beta$-D-thiogalactoside and allowed to grow at 37°C for 6 h. Filters were blocked at room temperature for 1 h in TBST containing 1% nonfat dry milk. Filters were then incubated with the probe in TBST containing 1% nonfat dry milk at 4°C overnight. Filters were washed in TBST four times and a strong signal was obtained after an overnight exposure. A positive phage was excised as a plasmid according to the manufacturer's protocol (Novagen).

*Assay for GEP activity.* GEP activity of nRap GEP was assayed by measuring the dissociation of [$^3$H]GDP from the lipid-modified form of each small G protein (2 pmol in a 100-$\mu$l reaction mixture). The dissociation of [$^3$H]GDP was assayed at 25°C by the filtration assay as described (26).

*Construction of expression vectors.* The cDNA of nRap GEP (KIAA0313; AB002311; GenBank/EMBL/DDBL) was kindly supplied from Dr. T. Nagase, Kazusa DNA Research Institute, Japan. Prokaryotic and eukaryotic expression vectors were constructed in pGEX (Amersham Pharmacia Biotech), pCIneo (CLONTECH, Palo Alto, CA), and pEGFP (CLONTECH) using standard molecular biological methods. pCIneo-Myc vector was constructed as described (16). The following constructs of nRap GEP contain the following aa residues: pCIneo-Myc-nRap GEP-1, full length, aa 1–1499; pCIneo-Myc-nRap GEP-4, aa 1338–1499; pEGFP-nRap GEP-1, full length, aa 1–1499; pGEX-nRap GEP-2, aa 1–464; and pGEX-nRap GEP-3, aa 691–1499. The GST-fusion protein was purified using glutathione-Sepharose beads according to the manufacturer's protocol (Amersham Pharmacia Biotech). The pCIneo-Myc constructs of S-SCAM were obtained as described (16).

*Overlay assay.* The [$^{35}$S]methionine-labeled probe of nRap GEP (pCIneo-Myc-nRap GEP-4) was prepared as described above. Immunoprecipitated samples were transferred on the nitrocellulose membrane, and the overlay assay was performed (27).

*Antibodies.* A rabbit anti-nRap GEP pAb was raised against GST-nRap GEP-2 (aa 1–464, the anti-nRap GEP pAb). The anti-SAPAP1 and -S-SCAM pAbs were obtained as described (13, 16). The anti-Myc mAb (9E10) was purchased from American Type Culture Collection (Rockville, MD). Second antibodies were obtained from Chemicon International, Inc. (Temecula, CA).

*Immunoprecipitation.* Immunoprecipitation experiments for the overlay and coimmunoprecipitation assays for COS7 cells were done as follows: the eukaryotic expression constructs were transfected to COS7 cells using Superfect reagent (QIAGEN) in various combinations. COS7 cells expressing the indicated proteins were lysed in 500 $\mu$l of a lysis buffer (20 mM Tris–Cl at pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% (w/v) Triton X-100, 10 $\mu$g/ml of leupeptin, and 10 $\mu$M $\alpha$-amidino-phenylmethanesulfonyl fluoride hydrochloride) for each 10-cm dish with rotation at 4°C for 40 min. The lysates were centrifuged at 100,000*g* at 4°C for 30 min. The supernatant was incubated with the mouse anti-Myc mAb-coupled protein A–Sepharose beads at 4°C for 2 h. After the beads were extensively washed with the lysis buffer, the bound proteins were eluted by boiling the beads in an SDS sample buffer for 10 min. The samples were then subjected to SDS–PAGE, followed by the overlay assay or Western blot analysis.

The crude synaptosomal fraction was prepared from two rat brains as described (13). The fraction was homogenized in 4 ml of the lysis buffer described above and centrifuged at 100,000*g* for 30 min to collect the supernatant. 0.6-ml of aliquots of the supernatant were incubated with the anti-nRap GEP pAb or control IgG at 4°C for 3 h. Protein G–Sepharose beads (Amersham Pharmacia Biotech) were added to the sample, and incubation was further performed at 4°C for 1 h. After the beads were extensively washed with the lysis buffer, the bound proteins were eluted by boiling the beads in an SDS sample buffer for 10 min. The samples were then subjected to SDS–PAGE, followed by Western blot analysis.

*Immunohistochemistry.* Immunofluorescence microscopy of frozen sections of rat brain was done as described (28). Briefly, samples were frozen using liquid nitrogen, and the frozen sections were cut in a cryostat. The samples were mounted on glass slides, air dried, and fixed with 95% (v/v) ethanol at 4°C for 30 min and with acetone at room temperature for 1 min. The samples were washed with PBS containing 10 mg/ml of BSA, incubated with either the anti-S-SCAM or -nRap GEP pAb, washed again with PBS containing 10 mg/ml of BSA and 0.01% (w/v) saponin, and incubated with Texas red-conjugated second antibodies (Amersham Pharmacia Biotech). The samples were washed with PBS, embedded, and viewed with a confocal imaging system MRC-1024 (Bio-Rad Laboratories).

*Other procedures.* The lipid-modified forms of Rap1B, Ha-Ras, RhoA, and Rab3A were obtained from *Spodoptere frugiperda* cells expressing each small G protein (29, 30). The plasmid for GST-C3G containing the Ras GEP domain was kindly supplied from Dr. A. Wittinghofer, Max Planck Institute, Germany, and the protein was

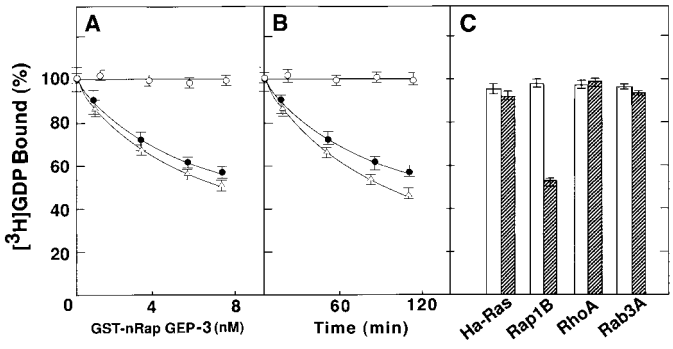


**FIG. 2.** GEP activity of nRap GEP. (A) Dose-dependent activity for Rap1B. The dissociation of [$^3$H]GDP from the lipid-modified form of Rap1B was assayed by incubation for 1 h with various doses of GST, GST-nRap GEP-3 containing the Ras GEP domain, or GST-C3G. (●), GST-nRap GEP-3; (○), GST alone; and (△) GST-C3G. The values are means ± S.E. of three independent experiments. (B) Time-dependent activity for Rap1B. The dissociation of [$^3$H]GDP from the lipid-modified form of Rap1B was assayed by incubation for various times with GST, GST-nRap GEP-3, or GST-C3G (5 nM each). (●), GST-nRap GEP-3; (○), GST alone; and (△) GST-C3G. The values are means ± S.E. of three independent experiments. (C) Small G protein specificity of nRap GEP. The dissociation of [$^3$H]GDP from the lipid-modified form of each small G protein was assayed by incubation for 1 h with 7 nM of GST or GST-nRap GEP-3. *Hatched columns,* GST-nRap GEP-3; and *open columns,* GST alone. The values are means ± S.E. of three independent experiments.

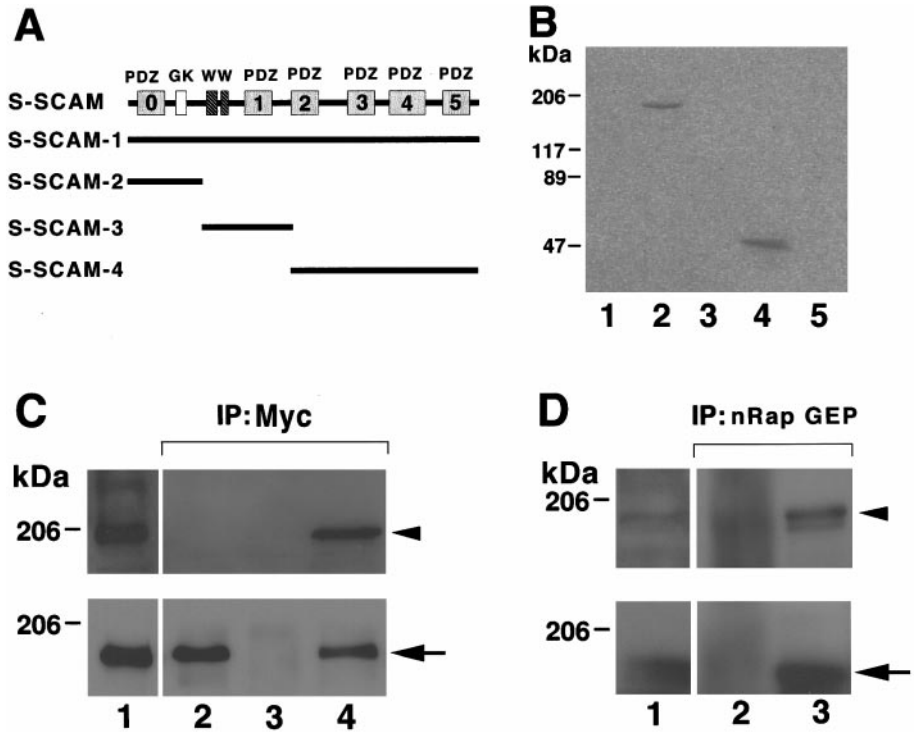


**FIG. 3.** Direct binding of nRap GEP to S-SCAM. (A) Various pCIneo-Myc constructs of S-SCAM. (B) Binding of nRap GEP to S-SCAM by the blot overlay assay. Immunoprecipitated samples of COS7 cells expressing various mutants of S-SCAM were overlaid with [$^{35}$S]methionine-labeled probe of nRap GEP. *Lane 1,* Mock; *lane 2,* pCIneo-Myc-S-SCAM-1, full length; *lane 3,* pCIneo-Myc-S-SCAM-2; *lane 4,* pCIneo-Myc-S-SCAM-3 containing the second PDZ and WW domains; and *lane 5,* pCIneo-Myc-S-SCAM-4. (C) Immunoprecipitation from COS7 cells expressing GFP-nRap GEP and/or Myc-S-SCAM. Immunoprecipitated samples of COS7 cells expressing various combinations of GFP-nRap GEP and Myc-S-SCAM were subjected to SDS-PAGE (8% polyacrylamide gel), followed by Western blot analysis with the anti-Myc mAb for S-SCAM and with the anti-nRap GEP pAb for nRap GEP. *IP,* immunoprecipitation. *Lane 1,* input; *lane 2,* pCIneo-Myc-S-SCAM-1 alone; *lane 3,* pEGFP-nRap GEP-1 alone; and *lane 4,* pEGFP-nRap GEP-1 and pCIneo-Myc-S-SCAM-1. *Arrowhead,* nRap GEP; and *arrow,* S-SCAM. Input contains 25% of the extract used for the assay. (D) Immunoprecipitation from rat brain. The Triton X-100 extract of the rat crude synaptosomal fraction was subjected to immunoprecipitation with the anti-nRap GEP pAb or control IgG. The immunoprecipitate was then subjected to SDS-PAGE (8% polyacrylamide gel), followed by Western blot analysis with the anti-S-SCAM or -nRap GEP pAb. *IP,* immunoprecipitation. *Lane 1,* input; *lane 2,* with control IgG; and *lane 3,* with anti-nRap GEP pAb. *Arrowhead,* nRap GEP; and *arrow,* S-SCAM. Input contains 25% of the extract used for the assay.

purified as described above. Other procedures, including subcellular fractionation of rat brain, determination of protein concentrations, and SDS–PAGE, were performed as described (7, 13). Prestained markers used on SDS–PAGE were myosin (206 kDa), $\beta$-galactosidase (117 kDa), BSA (89 kDa), and ovalbumin (47 kDa).

## RESULTS

We first attempted to identify a novel S-SCAM-binding protein using the expression cloning strategy. We screened $2 \times 10^6$ clones from a mouse fetal brain cDNA library using a fragment of S-SCAM containing the second PDZ and WW domains as a probe. We identified one positive clone and determined its nucleotide sequence. Isolated clone was 2.5 kbp and had a coding region (450 bp) which had a homology with the C-terminal portion of a human cDNA (KIAA0313; AB002311; GenBank/EMBL/DDBL) (78.3% nucleotide sequence identity). Thus, subsequent experiments were performed using this human cDNA. Its open reading frame encoded a protein of 1,499 aa and possessed several domains of high homology to other signaling molecules (Figs. 1A–1C). This protein has one PDZ, one RA, and one Ras GEP domains, and one C-terminal consensus motif for binding to PDZ domains. We named it nRap GEP (neural Rap GEP). nRap GEP has moreover an incomplete CAB domain (Fig. 1B). The domain organization of nRap GEP is similar to that of Epac/cAMP-GEFI (33, 34), except that Epac/cAMP-GEFI has complete CAB and Ras GEP domains but lacks the other two domains and the C-terminal motif (Fig. 1C).

We then examined GEP activity of nRap GEP for Rap1B in comparison with that of C3G, which is a well-known Rap1 GEP (36). Both nRap GEP and C3G stimulated the dissociation of [$^3$H]GDP from Rap1B (Figs. 2A and 2B) and the binding of [$^{35}$S]GTP$\gamma$S to Rap1B (data not shown) in dose- and time-dependent manners. The activities of these two GEPs were nearly

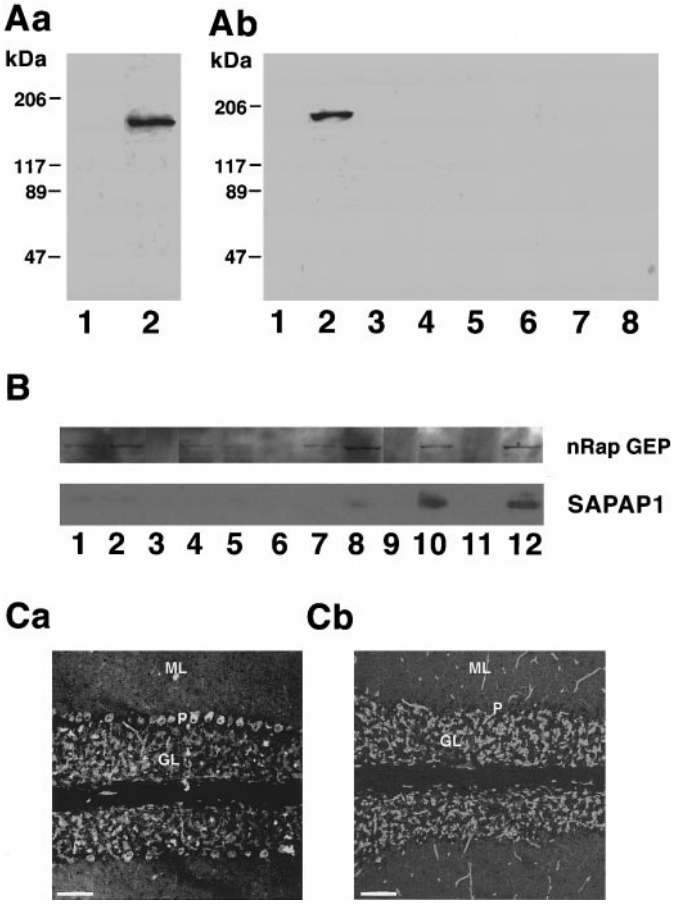


**FIG. 4.** Tissue distribution of nRap GEP in rat and its subcellular localization in rat brain. (A) Tissue distribution of nRap GEP. Equal aliquots of the Triton-X 100-soluble fractions of COS7 cells (20 $\mu$g of protein each) and the homogenates of the indicated rat tissues (60 $\mu$g of protein each) were subjected to SDS-PAGE (8% polyacrylamide gel), followed by Western blot analysis with the anti-nRap GEP pAb. (Aa) Expression of Myc-nRap GEP in COS7 cells. *Lane 1,* Mock; and *lane 2,* pCIneo-Myc-nRap GEP-1. (Ab) Tissue distribution. *Lane 1,* heart; *lane 2,* brain; *lane 3,* spleen; *lane 4,* lung; *lane 5,* liver; *lane 6,* skeletal muscle; *lane 7,* kidney; and *lane 8,* testis. (B) Subcellular localization of nRap GEP in rat brain. Equal aliquots of the subcellular fractions of rat brain (30 $\mu$g of protein each) were subjected to SDS-PAGE (8% polyacrylamide gel), followed by Western blot analysis with the anti-nRap GEP or -SAPAP1 pAb. *Lane 1,* the homogenate fraction; *lane 2,* the crude synaptosomal fraction; *lane 3,* the nuclear pellet fraction; *lane 4,* the synaptosomal cytosol fraction; *lane 5,* the crude synaptosomal pellet fraction; *lane 6,* the lysed synaptosomal membrane fraction; *lane 7,* the crude synaptic vesicle fraction; *lane 8,* the SPM fraction; *lane 9,* the 0.5% (w/v) Triton X-100-soluble fraction of the SPM; *lane 10,* the 0.5% (w/v) Triton X-100-insoluble fraction of the SPM; *lane 11,* the 1% (w/v) Triton X-100-soluble fraction of the SPM; and *lane 12,* the 1% (w/v) Triton X-100-insoluble fraction of the SPM. (C) Localization of nRap GEP in rat cerebellum. The photographs were obtained from serial sections of rat cerebellum. (Ca) nRap GEP; and (Cb) S-SCAM. *ML,* molecular layer; *P,* Purkinje cells; and *GL,* granular layer. *Bars,* 100 $\mu$m.

the same. nRap GEP did not show GEP activity for other small G proteins, including Ha-Ras, RhoA, and Rab3A (Fig. 2C). Epac/cAMP-GEFI shows cAMP-dependent GEP activity for Rap1A but not for Ha-Ras or R-Ras (33, 34). We next examined whether cAMP bound to the incomplete CAB domain of nRap GEP. cAMP did not bind to a GST-fusion protein containing the incomplete CAB domain of nRap GEP (aa 1–464) under the conditions where cAMP bound to GST-PKA regulatory subunit (data not shown). The Myc-tagged full length of nRap GEP immunoprecipitated from COS7 cells showed GEP activity for Rap1B, but the GEP activity was not affected by cAMP (data not shown). These results indicate that nRap GEP is a cAMP-independent, Rap1-specific GEP.

We confirmed the binding of nRap GEP to S-SCAM by the blot overlay and coimmunoprecipitation assays. In the blot overlay assay, the [$^{35}$S]methionine-labeled fragment containing the C-terminal consensus motif bound to the full length and a fragment of S-SCAM containing the second PDZ and WW domains (Figs. 3A and 3B). In the coimmunoprecipitation assay using COS7 cells, GFP-nRap GEP and Myc-S-SCAM were coimmunoprecipitated (Fig. 3C). Moreover, nRap GEP and S-SCAM were coimmunoprecipitated from rat brain (Fig. 3D). These results are consistent with the earlier observation that the PDZ domains bind to unique C-terminal motifs of four aa residues of membrane proteins (1, 3), and indicate that the second PDZ domain of S-SCAM directly binds to the C-terminal consensus motif of nRap GEP.

When Myc-tagged full length of nRap GEP was overexpressed in COS7 cells, a protein band with a molecular mass of about 180 kDa was detected with the anti-nRap GEP pAb (Fig. 4Aa). Western blot analysis in various rat tissues showed that a protein band with a similar molecular mass was detected only in rat brain (Fig. 4Ab). These results suggest that the cDNA of nRap GEP (KIAA0313) encodes the full length and that nRap GEP is a neural tissue-specific protein. In various subcellular fractions of rat brain, nRap GEP was enriched in the SPM fraction (Fig. 4B). This subcellular distribution of nRap GEP was similar to that of S-SCAM (16), although nRap GEP was not as enriched as SAPAP in the PSD fraction. Immunohistochemical analysis showed that the immunoreactivities of nRap GEP and S-SCAM were detected at the glomeruli in the granular layer, suggesting that both the proteins are localized at the synapse (Fig. 4C, a and b). It may be noted that the immunoreactivity of nRap GEP was also detected in the soma of Purkinje cells, whereas that of S-SCAM was detected at the dendrites.

## DISCUSSION

We have isolated here a novel Rap1-specific GEP, named nRap GEP. This protein has at least four domains, including one incomplete CAB, one PDZ, one RA, and one Ras GEP domains, and one C-terminal consensus motif for binding to PDZ domains. The incomplete CAB domain of nRap GEP lacks the aa se-

quences (PRAA) that is present in the cAMP-binding pocket of PKA (31) and Epac/cAMP-GEFI (Fig. 1B) (33, 34). Consistent with this structure, nRap GEP does not bind cAMP and its GEP activity is independent on cAMP. This property is different from that of Epac/cAMP-GEFI of which GEP activity for Rap1A is dependent on cAMP. The RA domain has originally been identified in Ral GDS and AF6 as a Ras-binding consensus sequence (reviewed in Ref. 34). It has recently been shown that the $K_d$ values of the RA domain of AF6 for GTP-Rap1A and GTP-Ha-Ras are $10^{-7}$ and $10^{-6}$ M orders, respectively (35). These values are markedly higher than the Kd value for Ha-Ras of c-Raf-1, which is a well-established downstream target of Ras (21), ($10^{-8}$ M order) (38), and thus the physiological significance of the binding of Ras and Rap1 to the RA domain of AF6 remains unknown. Therefore, we have not examined whether GTP-Ras and/or GTP-Rap1 also bind to nRap GEP. The PDZ domain is a well-known protein module that binds to unique C-terminal consensus sequences of membrane proteins (1, 3). The PDZ domain is also known to interact with other PDZ domains (1, 3). We have not yet identified a molecule that interacts with the PDZ domain of nRap GEP.

Many GEPs and GAPs specific for Ras and/or Rap1 have thus far been isolated and characterized (21). Among them, synGAP, a GAP for Ha-Ras (8), and SPA-1-like protein, a GAP for Rap1A (22), interact with PSD-95/SAP90. Both Ras and Rap1 regulate gene expression through the MAP kinase pathway (22–23), which may play important roles in synaptic plasticity (reviewed in Ref. 39). We have shown here that nRap GEP interacts with S-SCAM and is specifically expressed in neural tissue where it may be localized at the synapse. Moreover, MAGUIN-1, which interacts with PSD-95/SAP90 and S-SCAM (9), has a similar domain structure of *Drosophila* CNK (40). CNK binds to D-Raf and functions in the MAP kinase pathway, suggesting that MAGUIN-1 also plays a similar role at synaptic junctions. Taken together, nRap GEP, SPA-1-like protein, synGAP, and MAGUIN-1, all of which regulate the MAP kinase pathway, may form a multiple complex with receptors, channels, and adhesion molecules, through scaffolding molecules, such as PSD-95/SAP90 and S-SCAM, and play roles at the synapse.

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