Versatile Roles of R-Ras GAP in Neurite Formation of PC12 Cells and Embryonic Vascular Development

Received for publication, November 17, 2006, and in revised form, December 12, 2006

Published, JBC Papers in Press, December 19, 2006, DOI 10.1074/jbc.C600293200

Shintaro Iwashita,‡,¶1, Mariko Kobayashi,‡ Yuya Kubo,§,¶1, Yoshimi Hinohara,‡,¶1, Mariko Sezaki,‡ Kenji Nakamura,‡ Rika Suzuki-Migishima,‡ Minesuke Yokoyama,‡§, Showbu Sato,‡ Mitsunori Fukuda,‡ Masayuki Obha,‡ Chieko Kato,‡ Eiji Adachi,‡ and Si-Young Song‡

From the ‡Mitsubishi Kagaku Institute of Life Sciences (MITLS), Machida, Tokyo 194-8511, Japan, the ¶Yokohama National University, Yokohama, Kanagawa 240-8501, Japan, the §Brain Science Institute, RIKEN, Wako, Saitama 351-0198, Japan, and the ¶¶3Kitasato University School of Medicine, Sagamihara, Kanagawa 228-8555, Japan

Ras GTPase-activating proteins (GAP) are negative regulators of Ras that convert active Ras-GTP to inactive Ras-GDP. R-Ras GAP is a membrane-associated molecule with stronger GAP activity for R-Ras, an activator of integrin, than H-Ras. We found that R-Ras GAP is down-regulated during neurite formation in rat pheochromocytoma PC12 cells by nerve growth factor (NGF), which is blocked by the transient expression of R-Ras gap or dominant negative R-ras cDNA. By establishing a PC12 subclone that stably expresses exogenous R-Ras GAP, it was found that NGF reduced endogenous R-Ras GAP but not exogenous R-Ras GAP, suggesting that down-regulation of R-Ras GAP occurs at the transcription level. To clarify the physiological role of R-Ras GAP, we generated mice that express mutant Ras GAP with knocked down activity. While heterozygotes are normal, homozygous mice die at E12.5–13.5 of massive subcutaneous and intraperitoneal bleeding, probably due to underdeveloped adherens junctions between capillary endothelial cells. These results show essential roles of R-Ras GAP in development and differentiation: its expression is needed for embryonic development of blood vessel barriers, whereas its down-regulation facilitates NGF-induced neurite formation of PC12 cells via maintaining activated R-Ras.

Biochemical reactions mediated by receptors in the plasma membrane not only produce signaling for cell growth and differentiation but also down-regulate this signaling for the maintenance of cellular homeostasis (1). Ras GTPase-activating proteins (GAPs), representative molecules responsible for such down-regulation signaling, are negative regulators that convert active Ras-GTP to inactive Ras-GDP (2). Whereas H-Ras is a central regulator of cell proliferation and differentiation, R-Ras is an activator of integrins, cell-surface receptors for the extracellular matrix (3). R-Ras and H-Ras exert opposite effects on integrin activation (4), thus producing different effects, for example, on the interaction between endothelial cells and monocytes (5). Activated H-Ras, but not R-Ras, induces extensive neuronal differentiation of rat pheochromocytoma PC12 cells (6), while dominant active R-Ras promotes neurite outgrowth of late embryonic retinal neurons (7). For the proper formation of the neuronal network, R-Ras inactivation is also required to induce growth cone collapse via the R-Ras GAP activity of plexin-B1, a Semaphorin 4D receptor (8). These results implicate the need for a balance between R-Ras and H-Ras activation/inactivation in long term biological processes such as neuronal differentiation and homeostasis.

Four types of Ras GAPs have been identified (9), and targeted mice have been generated for three of them. Null mice of p120gap (10) or Nf1 (neurofibromin) (11, 12) are embryonic lethal apparently due to defects in the higher organization of endothelial and neuronal cells. Mutant mice of Syngap, a brain-specific Ras gap, are postnatal lethal apparently due to improper neuronal development (13). The fourth type, GAP1 family members (9), share conserved domains: tandem C2 domains, a GAP-related domain (GRD), and a pleckstrin homology domain. R-Ras GAP (also called GAP1 HRPB), a member of the fourth family, is membrane-associated (14) and has a stronger GAP activity against R-Ras than H-Ras (15). An arginine situated in the GRD loop, the “arginine finger,” has been identified as essential for all Ras GAP activity (16). Based on this result, we generated mice mutant for R-Ras gap, which lacks the region including the arginine finger, to clarify its physiological significance in vivo. In this paper, we show a positive correlation between the down-regulation of R-Ras GAP and neurite outgrowth of PC12 cells and the embryonic lethality of R-Ras gap mutant mice accompanied with massive hemorrhage.

EXPERIMENTAL PROCEDURES

All experimental procedures are described in the supplemental data.

RESULTS

Positive Correlation between the Down-regulation of R-Ras GAP and Neurite Outgrowth of PC12 Cells—We examined transcript levels of three Ras gaps (R-Ras gap, p120gap, and Nf1)
Physiological Role of R-Ras GAP

FIGURE 1. Positive correlation between down-regulation of R-Ras GAP and neurite outgrowth of PC12 cells. A, measurement of Ras gap transcripts. The transcript levels of R-Ras gap were measured by quantitative RT-PCR in PC12 cells treated with NGF (20 ng/ml), EGF (10 ng/ml), or CPTcAMP (0.25 mM). The mean values ± S.E. are shown with the number of independent experiments in parentheses. B, Northern blot analysis. One mg of total RNA from rat brain or PC12D cells treated with or without NGF for 24 h was analyzed using a R-Ras gap cRNA probe. C, immunoblot analysis. Immunoprecipitates obtained with anti-R-Ras GAP peptide #2 Ab were separated by a two-dimensional gel electrophoresis followed by immunoblotting. Panels a–c, rat brain (a) and PC12 cells untreated (b) or treated with NGF (c) are shown. D and E, analysis of neurite formation. The indicated amounts of R-ras V38, R-ras N43, R-Ras gap, or H-ras N17 cDNA were co-transfected into PC12D cells with green fluorescence protein vector. After culturing for 2 days, cells were incubated in the presence of NGF for 2 days (D) or 1 day (E). Green fluorescence protein-positive cells were scored as bearing neurites, if they had processes two times longer than the diameter of the cell body. Each value represents the mean ± S.E. of multiple independent experiments as indicated by the number in parentheses (D) or in triplicate (E).

by quantitative RT-PCR in nerve growth factor (NGF)-treated PC12D cells. R-Ras gap transcript levels decreased by about 80% after treatment with NGF for 20 h, when neurites are substantially extended. Epidermal growth factor (EGF), a growth-promoting factor in PC12 cells, had no effect (Fig. 1A). A reduction in the R-Ras gap transcript was also induced by another differentiating reagent, 8-(4-chlorophenylthio)cAMP (CPTcAMP) (Fig. 1A). On the other hand, the amounts of p120GAP or Nf1 transcripts showed little change by treatment with NGF (Table 1). After incubation with NGF for 4 days, the R-Ras gap transcript returned to initial levels. Northern blot analysis confirmed the NGF-mediated suppression of R-Ras gap mRNA (Fig. 1B). The amount of R-Ras GAP protein was also decreased by NGF, but not EGF, whereas that of p120GAP was unchanged (Fig. 1C and supplemental Fig. 1O).

These results show a good correlation between the down-regulation of R-Ras GAP and neurite outgrowth of PC12D cells.

To address the biological significance of R-Ras GAP down-regulation in neurite outgrowth, we employed transient transfection experiments to examine the effects of Ras or Ras GAP on NGF-mediated neurite outgrowth of PC12 cells. Whereas dominant positive R-ras (V38) rather enhanced outgrowth, dominant negative R-ras (N43) significantly inhibited neurite outgrowth in a dose dependent manner (Fig. 1D). Furthermore, transfection of R-Ras gap cDNA substantially suppressed neurite outgrowth similarly to H-ras (N17), a dominant negative H-Ras, a blocker of neuronal differentiation of PC12 cells (17) (Fig. 1E). These results imply that the down-regulation of R-Ras GAP is relevant to the long-term activation of R-Ras, which seems to be critical for neurite outgrowth of PC12D cells. To examine which step the down-regulation of R-Ras GAP occurs, we established a PC12D subclone that stably expressed FLAG-tagged R-Ras GAP. Because exogenous R-Ras GAP with a FLAG tag (DYKDDDDK) is bigger than endogenous R-Ras GAP by 1 kDa of molecular size and has an extra three negative charges, the two R-Ras GAPs can be distinguished by two-dimensional analysis of Western blotting. The exogenous R-Ras GAP was found to be much more resistant to NGF treatment than the endogenous R-Ras GAP (supplemental Fig. 2), suggesting that down-regulation of R-Ras GAP was regulated at the transcriptional level. High Expression of R-Ras gap mRNA in the Brain and Spleen—The tissue distribution of the R-Ras gap transcript was examined using a specific cRNA probe. The mRNA was detected at high levels in the brain and spleen of 4-day-old rats, and high expression was maintained in the brain of 4-week-old rats (supplemental Fig. 3A). On the other hand, gap1m (18), a structurally related Ras gap, expressed rather ubiquitously in these tissues (data not shown). We next examined the cellular distribution of the R-Ras gap transcript in 4-week-old rat brain by in situ hybridization (supplemental Fig. 3B). The signals were distributed ubiquitously in all layers of the cerebral cortex but not in the white matter. In the cerebellum, the signal was strong in the Purkinje cell layer and moderate in the...
granule cell layer but not present in the white matter. In the olfactory bulb, the signal was strong in the mitral cell and glomerular layers and moderate in the granule cell layer. In the hippocampal region, the CA regions and dentate gyrus showed strong signals. These results show that R-Ras gap is highly expressed in the brain, mainly in neuronal cells.

Generation and Characterization of R-Ras gap Mutant Mice—To explore further the physiological role of R-Ras GAP in vivo, we tried to generate R-Ras gap mutant mice. The gene comprises 24 exons with its GRD in exons 10–16 (Fig. 2A–1). An arginine located in exon 12 (arginine finger) is critical for Ras GAP activity (16). To generate mutant mice with knocked down R-Ras GAP activity, a targeting vector was constructed by replacing exons 11–12 with Neo cassette. Since these exons consist of 149 and 115 nucleotides, respectively, the transcript of the targeted R-Ras gap should lack 264 nucleotides corresponding to 88 amino acids without frameshift (see Fig. 2A). So far more than 150 heterozygotes have been bred, and all have grown normally with average life span, indicating that the truncated R-Ras GAP molecule does not interfere with proper Ras signaling. However, no viable R-Ras gap<sup>−/−</sup> offspring were born from R-Ras gap<sup>+/−</sup> intercrosses, raising the possibility that R-Ras gap is essential for embryonic development. We found that homozygotes die at E12.5–13.5. All homozygotes, more than 50, dissected at E13.5 showed massive subcutaneous and intraparenchymal bleeding, while no heterozygotes showed such findings.

### TABLE 1

Down-regulation of the R-Ras gap transcript by neurite forming reagents

| Extracellular ligand | Ras gap transcripts |
|----------------------|---------------------|
| NGF                  | 101 ± 29 (3)        |
| CPTcAMP              | ND                  |
| EGF                  | ND                  |

### FIGURE 2

Generation of R-Ras gap mutant mice. A-1, targeting vector. The top panel represents the relationship between exons and domains of R-Ras gap. A targeting vector was constructed by replacing the region of exons 11–12 with Neo cassette and by the addition of the diphtheria toxin A (DT-A) cassette. A-2, Southern blot analysis. Genomic DNA digested with KpnI was analyzed using an external 3′ probe (indicated in A-1). A 9.1- or 14.9-kb band is detected from wild-type or targeted allele, respectively. Abbreviations: +/+; wild type; +/−, R-Ras gap, heterozygotes; −/−, R-Ras gap homozygotes. A-3, genotyping of mice. PCR of genomic DNA from littermates was carried out using two sets of primers. Pooled PCR products were stained with ethidium bromide after separation in agarose gels. The upper and the lower bands denote products from the targeted allele and the wild-type allele, respectively. B, expression of spliced transcripts. The top panel represents a schematic presentation of transcripts and three expected PCR products with their sizes (bp). RT-PCR was performed using three sets of primers (supplemental Table 1) and total RNA from E12.5 of wild-type, heterozygote, or homozygote mice as indicated. M, size marker. C, truncated R-Ras GAP protein. Whole-brain lysates of wild-type (left) or heterozygote (right) mice were immunoprecipitated and followed by immunoblotting. The arrows indicate expected bands of wild-type (97 kDa) and truncated R-Ras GAP (88 kDa), respectively. The bar in the upper panel denotes the location of peptide#2 for Ab production.
ACCELERATED PUBLICATION: Physiological Role of R-Ras GAP

Some homozygote embryos dissected at E12.5 showed hemorrhaging in the brain and/or rump (Fig. 3A). No apparent macroscopic or microscopic abnormalities were noted in systemic organs at E12.5. Furthermore, no increased apoptosis and neural degeneration were observed in homozygotes of R-Ras gap mutant embryos (data not shown), which were described in p120gap KO mice (10). Then, because R-Ras gap is highly expressed in the brain, and severe hemorrhaging occurs frequently in the brains of homozygotes, we examined brain blood vessels by electron microscopy. We observed that the adherens junctions between capillary endothelial cells were less developed in homozygotes as compared with heterozygous or wild-type mice (Fig. 3B). Thus, the possible explanation for the massive hemorrhaging in homozygotes is increased permeability of blood vessels due to underdeveloped adherens junctions between endothelial cells as detected in the brain. We could not examine the effects of loss of function of R-Ras GAP on the neurite formation due to the embryonic lethality of mutant mice.

DISCUSSION

In this study we demonstrate that homozygous R-Ras gap mutant mice are embryonic lethal dying at E12.5–13.5. Mouse embryo fibroblasts established from the mutant mice dissected at E8.5 grow normally compared with those from heterozygotes and wild-type (data not shown), indicating that R-Ras gap is not essential for cell growth. The homozygote mice showed massive subcutaneous and intraparenchymal bleeding, probably due to underdeveloped adherens junctions between capillary endothelial cells. This phenotype is related to reports that p120gap (10) and NFI (11, 12) null mice are also embryonic lethal dying at E9 or E13.5–14.5 and show severe defects in the higher organization of endothelial cells in the vascular system and neuronal cells in the brain. Therefore, these Ras gap mutant mice may share a common defect involving abnormal migration or adhesiveness or organization of endothelial cells. Our R-Ras gap mutant mice lacked 88 amino acids, including the arginine finger loop responsible for both catalytic activity (16) and substrate specificity (19). Replacement of the arginine with lysine in p120GAP GRD results in a 1000th reduction in Ras GAP activity (16), suggesting that our mutant mice have the low Ras GAP activity of truncated R-Ras GAP. Because R-Ras GAP is a constitutively membrane-associated molecule, and all Ras signaling occurs only in plasma membrane, the knockdown of R-Ras GAP activity may lead to long-term activated R-Ras.

It has been established that R-Ras is mainly responsible for cell adhesiveness and H-Ras for cell migration (4), but many ras null mice are non-lethal with the exception of K-ras null mice (20). In non-lethal mice, such as H-ras null mice (21) and N-ras null mice (22), total levels of Ras protein (Pan Ras) are maintained as in wild-type mice. R-ras null mice are phenotypically normal with excessive vascular formation (23). These results indicate the mutual signaling networks and compensation among Ras family members. In contrast, the number of Ras GAP molecules is limited compared with Ras members, and all null or mutant mice of Ras GAP members generated so far are unviable. The lethality might be related to the broad substrate specificity of Ras GAP molecules. R-Ras GAP (15) and p120GAP (24) have stronger GAP activities for R-Ras than H-Ras, whereas NF1 has stronger GAP activity for H-Ras than R-Ras. Furthermore, Cullen and co-workers (9) have reported that R-Ras GAP and Ca²⁺-triggered Ras GAPs have GAP activity for Rap in addition to a proper target Ras. Therefore knockdown of a Ras GAP molecule may lead to the long-term activation of related-Ras molecules as well as one target Ras, resulting in wider aberrant Ras signaling that cannot be compensated for by the mutual Ras network mentioned above.

It is noteworthy that R-Ras stimulates, whereas H-Ras inhibits, the interaction between monocytes and endothelial cells (5), which facilitates transmigration of monocytes across endothelial cells (25). These results suggest the importance of a balance between R-Ras and H-Ras activation in blood barrier homeostasis. Although it is not evident how R-Ras GAP is directly involved in the organization of adherens junctions during development, the blood vessel leakiness of mutant mice at E12.5 (Fig. 3A) might be induced by an imbalance between R-Ras and H-Ras activation. In our homozygote mice, this imbalance is caused by maintaining activated R-Ras and other Ras-related molecules due to a lack of proper R-Ras GAP. Massive hemorrhaging first detected at E12.5 seems to be closely related to the fact that cardiac ventricular pressure in mouse embryos increases significantly around embryonic day 12 (26). The blood barrier of the mutant mice may not endure the increased blood pressure. We did not examine the embryos...
We show a positive correlation between differentiation factor-mediated down-regulation of R-Ras GAP, but not p120GAP, and neurite outgrowth of PC12 cells (Fig. 1, A–C). The down-regulation of R-Ras GAP may maintain long term activation of not only R-Ras but also Ras-related proteins, which may facilitate the NGF-mediated differentiation of PC12 cells similar to the outgrowth of retinal neurons (7). It is noteworthy that the down-regulation of R-Ras GAP, a negative regulator, may mediate slow and long positive signaling such as NGF-mediated neuronal differentiation of PC12 cells. This type of signaling may fulfill the requirements for the differentiation process where a constitutive stimulation occurs, while cellular homeostasis is maintained. So far we cannot explain the two critical functions of R-Ras GAP from a collective view: its expression is essential for blood barrier homeostasis, whereas its down-regulation may facilitate neurite formation of PC12 cells.

We expect to reveal a new aspect of signaling networks by clarifying the mechanism by which the balance in the activation process where a constitutive stimulation occurs, while cellular homeostasis is maintained. So far we cannot explain the two critical functions of R-Ras GAP from a collective view: its expression is essential for blood barrier homeostasis, whereas its down-regulation may facilitate neurite formation of PC12 cells. We expect to reveal a new aspect of signaling networks by clarifying the mechanism by which the balance in the activation of R-Ras, H-Ras, and other Ras-related molecules is maintained. The mechanism is involved in long term processes such as neuronal differentiation and homeostasis.

Acknowledgments—We thank M. Sano for providing PC12D cells; T. Yamakuni, A. Oomori, S. Kamijo, S. Ichinose, and S. Omichi for technical advice; T. Saitoh for help in electron microscopic analysis; and S. Yamakuni, A. Oomori, S. Kamijo, S. Ichinose, and S. Omichi for technical advice. We are indebted to M. Dooley-Ohto, K. K. Chada, and K. K. Iwata for editing the manuscript.

REFERENCES
1. Iwashita, S., and Kobayashi, M. (1992) Cell. Sig- nal. 4, 123–132
2. Donovan, S., Shannon, K. M., and Bollag, G. (2002) Biochim. Biophys. Acta. 1602, 23–45
3. Zhang, Z., Vuori, K., Wang, H. G., Reed, J. C., and Ruoslahti, E. (1996) Cell 85, 61–69
4. Kimbara, K., Goldfinger, L. E., Hansen, M., Chou, F. L., and Ginsberg, M. H. (2003) Nat. Rev. Mol. Cell Biol. 4, 767–778
5. Cole, A. L., Subbanagounder, G., Mukhopadhyay, S., Berliner, J. A., and Vora, D. K. (2003) Arterioscler. Thromb. Vasc. Biol. 23, 1384–1390
6. Self, A. J., Caron, E., Paterson, H. F., and Hall, A. (2001) J. Cell Sci. 114, 1357–1366
7. Ivins, J. K., Yurchenco, P. D., and Lander, A. D. (2000) J. Neurosci. 20, 6551–6560
8. Ito, Y., Onuma, I., Katoh, H., Kaibuchi, K., and Negishi, M. (2006) EMBO Rep. 7, 704–709
9. Kupzig, S., Deaconescu, D., Bouyoucuf, D., Walker, S. A., Liu, Q., Polte, C. L., Daumke, O., Ishizaki, T., Lockyer, P. J., Wittinghofer, A., and Cullen, P. (2006) J. Biol. Chem. 281, 9891–9900
10. Henkemeyer, M., Rossi, D. J., Holmyard, D. P., Puri, M. C., Mambalam, G., Harpal, K., Shih, T. S., Jacks, T., and Pawson, T. (1995) Nature 377, 695–701
11. Brannan, C. I., Perkins, A. S., Vogel, K. S., Ratner, N., Nordlund, M. L., Reid, S. W., Buchberg, A. M., Jenkins, N. A., Parada, L. F., and Copeland, N. G. (1994) Genes Dev. 8, 1019–1029
12. Jacks, T., Shih, T. S., Schmitt, E. M., Bronson, R. T., Bernards, A., and Weinberg, R. A. (1994) Nat. Genet. 7, 353–361
13. Kim, J. H., Lee, H. K., Takamiya, K., and Huganir, R. L. (2003) J. Neurosci. 23, 1119–1124
14. Cozier, G. E., Lockyer, P. J., Reynolds, J. S., Kupzig, S., Bottomley, I. R., Millard, T. H., Banting, G., and Cullen, P. J. (2000) J. Biol. Chem. 275, 28261–28268
15. Yamamoto, T., Matsui, T., Nakafuku, M., Iwamatsu, A., and Kaibuchi, K. (1995) J. Biol. Chem. 270, 30557–30561
16. Ahmadian, M. R., Kiel, C., Stege, P., and Scheffzek, K. (2003) J. Mol. Biol. 329, 699–710
17. Szebeneyi, J., Cai, H., and Cooper, G. M. (1990) Mol. Cell. Biol. 10, 5324–5332
18. Maekawa, M., Li, S., Iwamatsu, A., Morishita, T., Yokota, K., Inai, Y., Kohsaka, S., Nakamura, S., and Hattori, S. (1994) Mol. Cell. Biol. 14, 6879–6885
19. te Biesebeke, R., Krab, I. M., and Parmeggiani, A. (2001) Biochemistry 40, 7474–7479
20. Koera, K., Nakamura, K., Nakao, K., Miyoshi, J., Toyoshima, K., Hatta, T., Otani, H., Aiba, A., and Katsuki, M. (1997) Oncogene 15, 1151–1159
21. Ise, K., Nakamura, K., Nakao, K., Shimizu, S., Harada, H., Ichise, T., Miyoshi, J., Gondo, Y., Ishikawa, T., Aiba, A., and Katsuki, M. (2000) Oncogene 19, 2951–2956
22. de Castro, I. P., Diaz, R., Malumbres, M., Hernandez, M. I., Jagirdar, I., Jimenez, M., Ahn, D., and Peller, A. (2003) Cancer Res. 63, 1615–1622
23. Komatsu, M., and Ruoslahti, E. (2005) Nat. Med. 11, 1346–1350
24. Li, S., Nakamura, S., and Hattori, S. (1997) J. Biol. Chem. 272, 19328–19332
25. Ridley, A. J. (2001) FEBS Lett. 498, 168–171
26. Ishiwata, T., Nakazawa, M., Pu, W. T., Tevosian, S. G., and Izumo, S. (2003) Circ. Res. 93, 857–865