Characterization of STEF, a Guanine Nucleotide Exchange Factor for Rac1, Required for Neurite Growth*

Naoki Matsuo§§, Mikio Hoshino¶¶, Masato Yoshizawa‡, and Yo-ichi Nabeshima‡

From the ‡Department of Pathology and Tumor Biology, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan, and the ¶¶Percusory Research for Embryonic Science and Technology PRESTO, Japan Science and Technology Corporation, Kawaguchi 332-0012, Japan

Received for publication, July 3, 2001, and in revised form, October 12, 2001
Published, JBC Papers in Press, November 13, 2001, DOI 10.1074/jbc.M106186200

Rho family GTPases (i.e. Rho, Rac, Cdc42) have been shown to be key regulators of actin dynamics that underlie orchestrated changes in cell morphology (1) including those of neurons during the formation of the nervous system (2). Neurite growth is an essential developmental process, and the importance of these GTPases has been demonstrated by the disruption of this process upon ectopic expression of their constitutively active or dominant-negative mutants in individual domains: PHnTSS for membrane association, DH for enzymatic activity, and PHc for promoting catalytic activity. Ectopic expression of STEF in N1E-115 neuroblastoma cells induced neurite-like processes containing F-actin, βIII tubulin, MAP2, and GAP43 in a Rac1-dependent manner even under the serum-containing neurite-inhibiting conditions. We further found that a PHnTSS STEF fragment specifically inhibited the function of both STEF and Tiam1, a closely related Rac1 guanine nucleotide exchange factor. Suppression of endogenous STEF and Tiam1 activities in N1E-115 cells by ectopically expressed PHnTSS STEF resulted in inhibition of neurite outgrowth in serum-starved conditions, which usually induce neurite formation. Furthermore, these inhibitory effects were rescued by exogenously expressed STEF or Tiam1, suggesting that STEF and Tiam1 are involved in neurite formation through the activation of Rac1 and successive cytoskeletal reorganization of neuronal cells during development.

Accumulating evidence suggests that Rho family GTPases play critical roles in the organization of the nervous system. We previously identified a guanine nucleotide exchange factor of Rac1, STEF (SIF and Tiam 1-like exchange factor), which can induce ruffling membrane in KB cells and is predominantly expressed in the brain during development. Here, we characterize the molecular nature of STEF and its involvement in neurite growth. Deletion analyses revealed distinct roles for individual domains: PHnTSS for membrane association, DH for enzymatic activity, and PHc for promoting catalytic activity. Ectopic expression of STEF in N1E-115 neuroblastoma cells induced neurite-like processes containing F-actin, βIII tubulin, MAP2, and GAP43 in a Rac1-dependent manner even under the serum-containing neurite-inhibiting conditions. We further found that a PHnTSS STEF fragment specifically inhibited the function of both STEF and Tiam1, a closely related Rac1 guanine nucleotide exchange factor. Suppression of endogenous STEF and Tiam1 activities in N1E-115 cells by ectopically expressed PHnTSS STEF resulted in inhibition of neurite outgrowth in serum-starved conditions, which usually induce neurite formation. Furthermore, these inhibitory effects were rescued by exogenously expressed STEF or Tiam1, suggesting that STEF and Tiam1 are involved in neurite formation through the activation of Rac1 and successive cytoskeletal reorganization of neuronal cells during development.

* This work was supported in part by grants-in-aid for scientific research on priority areas (C) Advanced Brain Science Project, (C) Genome Science, and (A) Research for Comprehensive Promotion of Study of Brain (to M. H.) from the Ministry of Education, Culture, Sports, and Science and Technology, Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by a research fellowship from the Japan Society for the Promotion of Science (JSPS) for Young Scientists.

¶ To whom correspondence should be addressed: Dept. of Pathology and Tumor Biology, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan. Tel.: 81-75-753-4426; Fax: 81-75-753-4676; E-mail: mikio@mls.med.kyoto-u.ac.jp.

© 2002 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

This paper is available on line at http://www.jbc.org
invasion-inducing gene product (24). Overexpression of Tiam1 induces membrane ruffling in NIH3T3 cells, invasion in T-lymphoma cells, and neurite-like processes in N1E-115 neuroblastoma cells in a Rac1-dependent manner (12, 25).

We attempted to characterize the fundamental functions of STEF using KB epidermoid and N1E-115 neuroblastoma cells. Deletion analyses were used to determine the function of individual STEF domains. Ectopic expression of STEF and establishment of a dominant-negative form of STEF allowed us to further elucidate its in vivo role. These results, in conjunction with the predominant expression of stef and Tiam1 in the brain, particularly in regions where neurite extension is believed to occur during development (19, 26), implicate STEF/Tiam1 members to be potential key regulators for Rac1 in the context of neurite growth during the development of the mammalian nervous system.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs**—cDNAs encoding full-length (FL) and N-terminal truncated (ΔN; amino acids 464–1715) STEF (19) were engineered to contain an artificial Kozak initiation and subcloned into a pcDNA3 expression vector (Invitrogen). Oligonucleotides encoding FLAG, Myc, or hemagglutinin (HA) epitopes flanked by stop codons were introduced into the vectors. ΔPHc, ΔTSS, AΔPZ, ΔDHc, ΔPHc, and DHPhc mutants were constructed by removing amino acids 540–595, 620–792, 824–1078, 1115–1315, 1409–1480, and 464–1076, respectively, from the ΔN STEF construct. Oligonucleotides encoding the N-terminal 20 residues of the mouse c-Src signal for myristoylation were inserted to construct Myr-DHPHc STEF. Myr-DHc was derived from Myr-DHPHc by deleting the PHc domain. The PHnTSS fragment of STEF (amino acids 464–780) was generated by PCR and cloned into pcDNA3 containing C-terminal FLAG or HA epitope tags.

pEF-BOS-HA plasmids encoding wild-type and mutant Rac1 (27) were generous gifts from K. Kaibuchi (Nagoya University, Japan). To construct pcDNA3-Myc-V12Rac1 and pcDNA3-Myc-N17Rac1, respective cDNAs were inserted in a pcDNA3 vector containing an N-terminal Myc epitope. ΔN Tiam1 cDNA, originally provided by J. G. Collard (The Netherlands Cancer Institute), was subcloned into a pcDNA3 vector containing a C-terminal Myc, FLAG, or HA epitope. βPix cDNA was isolated using Rapid-Screen cDNA library panels from mouse E19 miRNA (OriGene Technologies) and subcloned into pFLAG-CMV-2 vector (Kodak). pEGFP plasmid was from CLONTECH.

**Preparation of Anti-STEF Antibody**—Rabbit polyclonal antisera was generated with glutathione S-transferase (GST) fusion STEF protein corresponding to the middle region of STEF (amino acids 812–1003). Antiserum was purified by affinity chromatography.

**Transient transfection in KB and N1E-115 Cells**—KB and N1E-115 cells were maintained in Dulbecco’s modified Eagle medium (Invitrogen) and transfected with our Northern analyses showing that PHn and TSS domains are necessary and sufficient for membrane localization of STEF in KB cells. **Production of Anti-STEF Antibody**—Polyclonal antibody against the central region of the STEF protein (amino acids 812–1003) was generated and used for Western blot analyses. The affinity-purified antibody revealed a band of ~190 kDa, the predicted molecular mass of STEF, in extracts from fetal mouse brain and N1E-115 neuroblastoma cells but not KB epidermoid carcinoma cells (Fig. 1A, lanes 3–5), which correlates with our Northern analyses showing that stef transcripts are expressed predominantly in the nervous system (19). The antibody recognized a 190-kDa band in KB cell lysates transfected with full-length STEF expression plasmid and exhibited no cross-reactivity with exogenously expressed Tiam1 (Fig. 1A, lanes 1 and 2). The specificity of anti-Tiam1 antibody was also confirmed against STEF (Fig. 1B).

**PHn and TSS Domains Are Necessary and Sufficient for Membrane Localization of STEF in KB Cells**—Ectopic expression of full-length and constitutively active N-terminal truncated (ΔN) STEF (Fig. 2) induced prominent ruffling of the membrane in KB cells above the sites of colocalized STEF.
protein and F-actin (Fig. 3A, a and b), although the number and intensity of FL STEF-expressing cells were lower, likely because of instability of the FL STEF (data not shown and Fig. 3C). This effect was dependent on Rac1 activity, because induction of membrane ruffling was suppressed by the coexpression of N17Rac1, a dominant-negative mutant of Rac1 (Fig. 4A, a and B).

To investigate the role of each STEF domain, we performed a series of transfections with deletion mutant constructs containing C-terminal FLAG epitope tags (Fig. 2). Mutant proteins and F-actin were detected with anti-FLAG antibody and rhodamine-phalloidin, respectively, to analyze protein localization and cell shape using confocal laser scanning microscopy. Initially, we focused on the membrane localization ability of each deletion mutant protein. Horizontal xy axis images as well as vertical xz axis images showed that mutants lacking the PDZ (ΔPDZ), DH (ΔDH), or C-terminal PH (ΔPHc) domains were localized at the plasma membrane, whereas those lacking the N-terminal PH (ΔPHn) or TSS (ΔTSS) domains appeared mainly cytoplasmic (Fig. 3A, c–g). We found that a fragment encompassing the PHn and the adjacent TSS domain (PHnTSS) was capable of localizing to the plasma membrane (Fig. 3A, h). These results indicate that both PHn and TSS domains are necessary and sufficient for the membrane localization of STEF in KB cells.

To further examine subcellular localization of the mutant proteins, transfected cell lysates were biochemically fractionated into Triton X-100-soluble and -insoluble fractions and subjected to Western blot analyses (Fig. 3B). Triton X-100-insoluble fractions have previously been reported to contain components of plasma membrane and cytoskeleton (28). We found that FL, ΔN, ΔPDZ, ΔDH, ΔPHc, and PHnTSS mutant proteins were strongly detected in the insoluble fraction, whereas the ΔTSS mutant protein was barely detectable, consistent with our confocal observations. Interestingly, the ΔPHn protein was abundantly detected in the insoluble fraction, although it seemed mainly cytoplasmic in confocal images, implying that STEF may associate with cytoskeletal components apart from cell membrane in the absence of the PHn domain.

Membrane Association of DHPHe Domain Is Sufficient for the Induction of Ruffling Membrane in KB Cells—We next investigated the ability of the deletion mutants to drive membrane ruffling in KB cells. Although both ΔPDZ and ΔPHc mutants were able to induce ruffling membranes (Fig. 3A, e and g), ΔPHc exhibited a much weaker phenotype than ΔN and ΔPDZ. ΔDH was unable to induce membrane ruffling (Fig. 3A, f), supporting the consensus that the DH domain is a catalytic domain for the exchange reaction (13). Notably, ΔPHn and ΔTSS mutants that do not localize, also failed to elicit ruffling (Fig. 3A, c and d), despite the presence of the catalytic DH domain, suggesting that membrane association is crucial for the membrane ruffling ability. To examine this possibility, we tested a C-terminal mutant containing DH and PHc domains (DHPHe) and found that it could neither localize to the plasma membrane nor evoke membrane ruffling (Fig. 3A, i and B). To enable membrane localization, a myristoylation site derived from c-Src (30) was added at the N terminus of DHPHe (Myr-DHPHe). The resulting mutant protein successfully localized to the membrane and induced prominent membrane ruffling in KB cells (Fig. 3A, j and B), demonstrating that membrane localization of the DHPHe region is sufficient to induce ruffling membrane in KB cells; it suggests further that the PHnTSS region functions mainly for STEF localization. In addition, the Myr-DH mutant, which lacks the PHc domain, exhibited an apparently weaker ability to induce ruffling membrane than Myr-DHPHe, although it contains the catalytic DH domain and can localize to the membrane (Fig. 3A, k and B).

Domains Required for Rac1 Activation in KB Cells—We also investigated the capacity of each mutant to activate Rac1 in KB cells. To measure Rac1 activation, we performed affinity pull-down assays using the PAK1 CRIB domain that specifically binds to GTP-bound active forms of Rac1 and Cdc42 (29). After cotransfection of a STEF mutant and HA-tagged Rac1 (HA-Rac1) in KB cells, GTP-bound HA-Rac1 was precipitated with GST-PAR CRIB conjugated with glutathione-Sepharose beads and immunodetected with anti-HA antibody (Fig. 3C). ΔN STEF was found to significantly increase the amount of GTP-bound HA-Rac1 compared with control, consistent with the fact that STEF serves as a Rac1 GEF in vitro (19), although expression levels of FL STEF were too low to enhance Rac1 activity, possibly because of protein instability. Deletion of the entire PDZ domain (ΔPDZ) did not impair the ability of STEF to activate Rac1, in contrast with mutants lacking PHn, TSS, or DH domains (ΔPHn, ΔTSS, or ΔDH). DHPHe mutants were unable to enhance the Rac1 activity in KB cells, albeit our previous findings that the DHPHe portion of STEF alone was
sufficient to exhibit GDP/GTP exchange activity for Rac1 in vitro (19). Furthermore, forced targeting of DHPHc to the membrane (Myr-DHPHc) restored the exchange activity, suggesting a direct correlation between the ability to activate Rac1 and the ability to induce membrane ruffling and further indicating that STEF works as an exchange factor only when it is localized at the membrane in KB cells. It is also notable that deletion of the PHc domain from /H9004N or Myr-DHPHc (/H9004PHc or Myr-DH, respectively) resulted in a remarkable reduction of Rac1 activation compared with their parental constructs (Fig. 3A, g and k). These results suggest that the PHc domain may be involved in enhancing the enzymatic activity of the adjacent DH domain and are supported by the fact that both ΔPHc and Myr-DH mutants induced much weaker membrane ruffling than their parental constructs (ΔN and Myr-DHPHc) (Fig. 3A, h, g, j, and k).

**PHnTSS STEF Specifically Interferes with Functions of STEF in KB Cells—**As described above, we found a functional significance for the PHnTSS region of STEF. Taken together with the demonstration by Stam et al. (31) that polypeptides containing the PHn and TSS (or CC-Ex) regions of Tiam1 effectively suppressed Tiam1-induced membrane ruffling in COS-7 cells, we postulated that the PHnTSS region of STEF might act as a dominant-negative form. To test this idea, we cotransfected PHnTSS STEF with /H9004N STEF into KB cells to see whether the /H9004N STEF-induced membrane ruffling would be abolished by the presence of PHnTSS STEF. Although 93% of /H9004N STEF-expressing cells exhibited prominent membrane ruffling, cotransfection of PHnTSS STEF reduced the proportion of cells with ruffles to 29% (Fig. 4A, b and B), demonstrating the ability of PHnTSS STEF to significantly suppress STEF-induced ruffling. Likewise, cotransfection of PHnTSS STEF with ΔN Tiam1 was examined. Surprisingly, PHnTSS STEF partially, albeit mildly, suppressed Tiam1-induced membrane ruffling (Fig. 4B), possibly because of the highly conserved PHnTSS region (66.9% amino acid identity). PHnTSS STEF had no marked effect on the membrane ruffling induced
Critical Role for STEF in Neurite Growth

Fig. 4. PHnTSS STEF specifically interferes with the function of STEF in KB cells. A, a–c, Myc-tagged ΔN STEF was cotransfected with HA-tagged N17Rac1 (a) or HA-tagged PHnTSS STEF (b), and Myc-tagged V12Rac1 was cotransfected with HA-tagged PHnTSS STEF (c) at a ratio of 1:4. Cells were stained with an anti-Myc antibody (green), an anti-HA antibody (blue), and phalloidin (red). PHnTSS STEF suppressed membrane ruffles induced by ΔN STEF but did not suppress those induced by V12Rac1. d and e, KB cells were transiently transfected with HA-tagged N17Rac1 (d) or HA-tagged PHnTSS STEF (e). After incubating for 24 h in serum-containing medium, cells were serum-starved for 24 h and were then treated with 5 ng/ml EGF for 5 min and fixed and stained with anti-HA antibody (green) and phalloidin (red). PHnTSS STEF did not suppress ruffling membrane formation induced by EGF, whereas N17Rac1 did. Arrowheads indicate regions with ruffling membranes. Scale bars, 10 μm. B, quantification of the inhibitory effect of PHnTSS STEF on ruffling membrane formation induced by ΔN STEF, ΔN Tiam1, or V12Rac1 in KB cells. Cells were cotransfected with indicated plasmids and stained with phalloidin and anti-Myc antibody to detect ΔN STEF, ΔN Tiam1, or V12Rac1. At least 100 cells positively stained with anti-Myc antibody were scored as to whether they exhibited membrane ruffling. Each bar represents the mean ± S.E. from three independent experiments. **, p < 0.005; *, p < 0.01; t test, compared with the control vector. C, PHnTSS STEF inhibits the GEF activity of STEF. KB cells were cotransfected with HA-tagged wild-type Rac1 and the indicated plasmids and were subjected to pull-down GTPase activity assay followed by Western blotting using an anti-HA antibody. The amount of expressed protein was determined by Western blotting with anti-FLAG antibody (bottom two panels). Similar results were obtained from three independent experiments.

Critical Role for STEF in Neurite Growth

To clarify whether these effects of PHnTSS STEF are due to the inhibition of the GEF activities of STEF and Tiam1, we directly assessed the amount of GTP-bound HA-Rac1 in PHnTSS STEF-expressing KB cells using pull-down GTPase assays (Fig. 4C). Both ΔN STEF and ΔN Tiam1 increased GTP-bound HA-Rac1 levels in KB cells (lanes 2 and 7), and this could be significantly suppressed by coexpression of PHnTSS STEF (lanes 3 and 8), indicating the ability of PHnTSS STEF to inhibit the GEF activity of STEF and Tiam1. On the other hand, ΔDH STEF did not suppress the Rac1 activation induced by ΔN STEF, despite the presence of an intact PHnTSS region (lane 4).

To evaluate the specificity of this dominant-negative-like effect of PHnTSS STEF on Rac1 GEFs, we tested whether PHnTSS STEF could suppress the activity of another Rac1 GEF, βPIX, which lacks a PHnTSS domain (32). Pull-down assays showed that ectopically expressed βPIX caused substantial increase of GTP-bound HA-Rac1 levels in KB cells (lane 5). However, this elevation of Rac1 activity was not suppressed by the coexpression of PHnTSS STEF (lane 6).

The specificity of this inhibitory effect of PHnTSS STEF was further investigated. It has been reported that KB cells form ruffling membranes at the cell periphery within minutes in response to growth factors such as EGF, insulin, and insulin-like growth factor I in a Rac1-dependent manner (33). These phenomena are believed to involve a Rac1 GEF other than STEF or Tiam1, because the extremely low endogenous STEF and Tiam1 levels in KB cells (see Fig. 1) are barely detectable by reverse transcriptase-PCR (data not shown). We transfected N17Rac1 or PHnTSS STEF into KB cells and subsequently treated the cells with 5 ng/ml EGF for 5 min. Although the cells expressing N17Rac1 did not display ruffling (Fig. 4A, d), cells expressing PHnTSS STEF exhibited EGF-stimulated ruffling (Fig. 4A, e), suggesting that PHnTSS STEF does not interfere with the function of endogenous Rac1 GEF in KB cells. Together these results indicate that PHnTSS STEF has the specific property to inhibit the function of STEF and that of Tiam1, at least partially, in KB cells.

Localization of Endogenous STEF Protein in N1E-115 Neuroblastoma Cells—Previous work has revealed that stef transcripts are strongly detected in the developing brain, especially in cortical neurons (19). STEF protein was also detected in the fetal brain as well as in N1E-115 neuroblastoma cells by immunoblotting (see Fig. 1A), suggesting that STEF may play a physiological role in neuronal cells. Therefore, we aimed to analyze the role of STEF in N1E-115 cells. Initially, immunocytochemical analysis was performed to examine the subcellular localization of endogenous STEF protein under serum-starved conditions that induce neurite growth. Anti-STEF antibody yielded significant signals throughout the soma and neurites including the growth cones (Fig. 5A). Confocal images illustrated that STEF was present at the plasma membrane as well as in the cytoplasm. Anti-Tiam1 antibody gave a staining...
To examine the domains essential for neurite induction, we transfected a series of STEF mutant constructs into N1E-115 cells (Figs. 2 and 6B). Transfected cells were evaluated in terms of their morphological features under phase-contrast fluorescence microscopy (see Fig. 6). Although the ΔPDZ mutant was as efficient in inducing neurite-like processes and lamellipodia as ΔN STEF, the ΔPHn, ΔTSS, or ΔDH mutants did not induce prominent changes. In addition, deletion of the PHc domain from ΔN STEF (ΔPHc) resulted in considerable reduction of those morphological changes, indicating that the PHn, TSS, and DH domains are essential and the PHc domain is required to some extent for neurite extension. Interestingly, the ability of STEF mutants to induce neurite-like structures in N1E-115 cells appeared to be closely correlated to their ability to induce membrane ruffling in KB cells (Fig. 2).

**PHnΔTSS STEF Suppresses the Morphological Changes Induced by STEF and Tiam1 in N1E-115 Cells**—To test whether PHnΔTSS STEF can suppress the morphological changes induced by ectopically expressed STEF in N1E-115 cells, we cotransfected ΔN STEF and PHnΔTSS STEF. ΔN STEF-induced phenotypes were strikingly suppressed by the presence of PHnΔTSS STEF (Fig. 6C). PHnΔTSS STEF also moderately blocked ΔN Tiam1-induced phenotypes but had no effect on V12Rac1-induced morphological changes (Fig. 6C). These results show that PHnΔTSS STEF suppresses the function of exogenously expressed STEF and Tiam1 and suggest that it may act similarly on endogenous STEF and Tiam1 in N1E-115 cells.

**PHnΔTSS STEF Blocks Neurite Outgrowth of N1E-115 Cells**—The pattern of *ste* and *tiam1* expression have implicated the involvement of STEF and Tiam1 in neuritogenesis (19, 26). As shown in Fig. 6 and by van Leeuwen et al. (12), exogenously expressed STEF and Tiam1 have the ability to induce neurite-like structures in N1E-115 cells. High levels of STEF and Tiam1 are present endogenously in N1E-115 cells (see Figs. 1 and 5), raising the possibility that they are involved in N1E-115 neurite growth. To address this possibility, we transfected PHnΔTSS STEF to suppress the function of both endogenous STEF and Tiam1 in N1E-115 cells, observing the transfected cells after 24 h in serum-free medium, which induces neurite extension (34). 75% of control cells transfected with EGFP and mock vectors exhibited neurites (Fig. 7A, a and B), whereas only 38% of those transfected with PHnΔTSS STEF possessed neurites (Fig. 7A, a and B). Likewise, N17Rac1 also suppressed the neurite outgrowth of N1E-115 cells (Fig. 7B) as described previously (9).

To confirm the dominant-negative effect of PHnΔTSS STEF on the exchange activity from the biochemical point of view, N1E-115 cells were transfected with PHnΔTSS STEF and HA-Rac1, and then Rac1 activity was monitored by pull-down GTPase assays under serum-free conditions. This experiment revealed that GTP-bound HA-Rac1 was significantly reduced by PHnΔTSS STEF (Fig. 7C), suggesting that the exchange activities of endogenous STEF and Tiam1 were suppressed in the transfected cells. Similar results were obtained with N17Rac1 (Fig. 7C).

To further confirm that the effect of PHnΔTSS was really due to the functional blockage of STEF and/or Tiam1, we carried out rescue experiments (Fig. 7D). N1E-115 cells grown in reduced-serum media were transfected with PHnΔTSS and control vector, ΔN STEF, or ΔN Tiam1. Coexpression of ΔN STEF with PHnΔTSS STEF restored the proportion of neurite-bearing cells. ΔN Tiam1 had a similar effect, suggesting that the PHnΔTSS STEF inhibits the function of both STEF and Tiam1 in N1E-115 cells. These results indicate that STEF and Tiam1 are critically involved in the neurite outgrowth induced by serum

---

**Critical Role for STEF in Neurite Growth**

**Fig. 5.** Subcellular localization of endogenous STEF and Tiam1 in N1E-115 neuroblastoma cells. N1E-115 cells cultured in serum-free medium for 24 h were fixed and stained with anti-STEF antibody (green in A) or anti-Tiam1 antibody (green in B). Cell morphology was visualized by F-actin staining using rhodamine-phalloidin (red). Localization of STEF and Tiam1 in the growth cone is indicated by arrows. Scale bar, 20 μm. C, fractionation of endogenous STEF protein in N1E-115 cells cultured in serum-containing or deprived medium. N1E-115 cell lysates were fractionated into Triton X-100-soluble (S) and -insoluble (P) fractions.
periphery were regarded as “shape. Cells with processes longer than their cell bodies were regarded as medium. The morphology of at least 150 EGFP-positive cells was scored as to whether they exhibit neurite-like processes, lamellipodia, or a round shape. Cells with processes longer than their cell bodies were regarded as “neurite-like processes.” Cells exhibiting lamellipodia around their periphery were regarded as “lamellipodia.” The graph represents the mean percentage ± S.E. from three independent experiments. *, p < 0.05, t test, compared with the control vector. **, p < 0.005, *p < 0.05, t test, compared with the control vector.

depletion through the activation of Rac1 in N1E-115 neuroblastoma cells.

**DISCUSSION**

Functional Significance of Domains of the STEF Protein in KB Cells—GEFs are supposed to be activated at appropriate subcellular regions within cells in response to various extracellular cues. Such a property of GEFs possibly resides in its multiple protein motifs, which may function to interact with various signaling molecules. Deletion analyses of STEF, a GEF specific for Rac1, revealed that both the PHn and TSS domains are necessary for membrane localization because mutants lacking these domains (∆PHn and ∆TSS) could not localize to the plasma membrane. Interestingly, the ∆PHn and ∆TSS proteins were preferentially detected in different subcellular fractions, ∆TSS in the Triton X-100-soluble fraction and ∆PHn in the insoluble fraction containing membrane and cytoskeletal proteins, suggesting the involvement of the TSS domain in association with cytoskeletal components.

Dbl family GEFs invariably contain a single PH domain located immediately C-terminal to the DH domain. Two distinct roles have been proposed for the GEF PH domains. One role involves recruitment of GEFs to the plasma membrane. The PH domain of Lfc (Lbc’s first cousin) is thought to be a good candidate for this role, because this domain can be substituted with a membrane targeting signal (13). The other role of the PH domain is to enhance the catalytic activity of the DH domain. In the case of Trio, deletion of the PH domain resulted in reduction of the Rac1 nucleotide exchange activity by 100-fold in vitro (35). Our deletion analyses of STEF revealed that the PHn domain is necessary for the membrane localization of STEF and that the second PH domain (PHc) is engaged in the enhancement of the catalytic activity of the adjacent DH domain. From this point, the dual PH domains should make STEF a unique protein because of the distinct roles assigned to each of the two domains. In contrast to the significant role of the PHc domain of STEF, previous studies have reported that truncation of the PHc domain did not affect Tiam1 function in NIH3T3 cells (36).

**Neurite-like Structure-inducing Ability of STEF in N1E-115 Cells**—In overexpression experiments using N1E-115 cells, STEF showed a remarkable potential to induce lamellipodia and neurite-like processes in a Rac1-dependent manner under serum-containing, neurite-inhibiting conditions. Immunocytochemical studies revealed that these STEF-induced processes contain F-actin, neuron-specific β tubulin, MAP2, and GAP43. MAP2 and GAP43 are often used as markers for neurites. These data indicate that activation of the STEF-Rac1 signaling pathway induces neurite-like processes in N1E-115 cells and suggest that the STEF-mediated signaling pathway may influence both F-actin and microtubule dynamics. This notion is consistent with the recent report that Rac1 may control not only actin polymerization but also microtubule dynamics.
through the phosphorylation of stathmin, a depolymerizing factor of microtubules (37). The morphology of the STEF-induced neurite-like processes often differed slightly from neurites induced by serum depletion, which may be caused by the widespread unregulated STEF overactivation in the cells.

Under our conditions, process formation in N1E-115 cells could be induced by the overexpression of STEF, Tiam1, or a constitutively active Rac1 even on non-substrate plastic dishes. In contrast to our results, van Leeuwen et al. (12) found that overexpression of Tiam1 did not induce process formation in N1E-115 cells when dishes without laminin coating. These discordant results may be due to the nature of the different sublines of N1E-115 cells. Alternatively, differences in plastic dishes may account for the discrepancy because the proportion of process-bearing cells was altered considerably depending on the source of the plastic dishes.2

Analyses of a series of deletion mutants revealed that the PHn, TSS, and DH domains were absolutely essential and the PHc domain was required to some extent to induce neurite-like structures in N1E-115 cells. Because these results are basically identical to those obtained in the experiments using KB epidermoid cells, we believe that these STEF domains exhibit similar functions in neurons as well.

The PHnTSS Fragment as a Dominant-Negative—Previous reports implicated the region encompassing the PHn and the neighboring TSS (or CC-Ex) domains of Tiam1 as a potential dominant-negative form (31, 38). We showed that the PHnTSS STEF fragment effectively blocked the function of ectopically expressed STEF and unexpectedly also was able to partially suppress the function of Tiam1. A plausible hypothesis may be that the PHnTSS fragment competes with STEF and Tiam1 to interact with similar or the same cofactor(s), because the PHnTSS region is highly conserved between the two proteins.

Considering that in vertebrates, the TSS domain is found only in STEF and Tiam1, it is likely that the inhibitory effect of the STEF PHnTSS fragment is highly specific. This is supported by the fact that PHnTSS STEF did not affect the activities of other Rac1 GEFs, such as βPIX nor that of the proposed endogenous, but yet unidentified Rac1-GEF believed involved in EGF-dependent ruffling membrane formation in KB cells. Introduction of the PHnTSS fragment into N1E-115 cells in which STEF and Tiam1 were presumably the major Rac1 GEFs resulted in suppression of Rac1 activity, demonstrating that PHnTSS STEF suppresses the function of endogenous STEF and partially suppresses that of Tiam1. From these data, we concluded that PHnTSS STEF is a specific dominant-negative form of STEF and a partial inhibitor of Tiam1.

Functions of STEF and Tiam1 in Mammalian Nervous System—Although overexpression experiments performed in this report and in the Collard group (12) showed the abilities of STEF and Tiam1 to induce neurite-like processes in N1E-115 cells, there is still no direct evidence for the requirement of STEF and Tiam1 for neurite formation. To address this question, we performed loss-of-function experiments using a dominant-negative form in N1E-115 cells and showed that a functional blockade of STEF and Tiam1 resulted in the suppression of Rac1 activation and neurite formation, both of which are induced by serum depletion in these cells. This result indicates that STEF and/or Tiam1 are required for neurite formation of N1E-115 cells through the activation of Rac1 and successive cytoskeletal reorganization. In addition, our experiment showed that these dominant-negative effects were rescued by exogenous expression of either STEF or Tiam1 protein. Considering that both STEF and Tiam1 were clearly present in neurites of N1E-115 cells, this fact implies that the functions of STEF and Tiam1 are likely to be cooperatively and redundantly involved in neurite formation of these cells.

In developing neurons, a variety of extracellular cues such as cell adhesion molecules, components of extracellular matrix, and trophic factors evoke intracellular signaling cascades that eventually influence the behaviors of neurites (39). Considering the fact that STEF and Tiam1 are involved in conveying the extracellular signals of serum depletion to activate intracellular Rac1 in N1E-115 cells, it is possible that STEF and Tiam1 work as key molecules to integrate environmental information in the modulation of local activation of Rac1 within neurons. Although the functional blockade experiment was performed only in a cultured cell line, N1E-115, the results further implicate the involvement of STEF and Tiam1 in neurite formation in the developing brain. Both genes are dominantly expressed in the brain during development, and their expression patterns in the nervous system are overlapping in many regions (19, 26). In addition, the role of each

---

2 N. Matsuo, M. Hoshino, and Y. Nabeshima, unpublished data.
domain is fundamentally conserved between STEF and Tiam1. These facts imply overlapping functions for the two proteins in similar molecular environments and in influencing the same cellular events, such as neurite extension.

However, there are also distinct differences between STEF and Tiam1. With respect to their protein structure, regions outside the PHnTSS and DHPHc domains are not highly conserved. For example, an ankyrin-binding region that is found in Tiam1 just C-terminal to the TSS (CC-Ex) domain (40) is not found in STEF. Concerning the expression patterns, stef expression is relatively confined to the telencephalon in the nervous system (19), whereas Tiam1 transcripts are more widely distributed (26). These distinct expression patterns as well as their divergent amino acid sequences suggest different functions for STEF and Tiam1, with a more limited role in the development of the nervous system for STEF.

Among over 30 Rho family GEFs currently known in vertebrates, STEF and Tiam1 are the first GEFs that have been shown to be actually involved in the promotion of neurite growth, although a recent report suggested the involvement of Tiam1 in the polarization of cultured hippocampal neurons (27). Further analyses of STEF and Tiam1, including in vitro and in vivo genetic approaches, would provide important clues as to how extracellular cues ultimately lead to the proper behavior of neurons during the formation of mammalian neuronal network.

Acknowledgments—We are grateful to M. Fukata, S. Kuroda, K. Kaibuchi, J. G. Collard, and A. Nakai for providing us with cell lines, plasmids, and antibody. We also thank M. Sone, T. Kawauchi, T. Fujimori, S. Yoshida, R. T. Yu, and C. Hama for helpful discussions and comments on this work.

REFERENCES

1. Hall, A. (1998) Science 279, 509–514
2. Luo, L. (2000) Nat. Rev. Neurosci. 1, 173–180
3. Luo, L., Liao, Y. J., Jan, L. Y., and Jan, Y. N. (1994) Genes Dev. 8, 1787–1802
4. Luo, L., Hensch, T. K., Ackerman, L., Barbel, S., Jan, L. Y., and Jan, Y. N. (1996) Nature 379, 837–840
5. Zipkin, I. D., Kindi, R. M., and Kenyon, C. J. (1997) Cell 90, 883–894
6. Kaufmann, N., Wills, Z. P., and Van Vactor, D. (1998) Development 125, 453–461
7. Ruchhoft, M. L., Ohnuma, S., McNeill, L., Holt, C. E., and Harris, W. A. (1999) J. Neurosci. 19, 8454–8463
8. Li, Z., Van Aelst, L., and Cline, H. T. (2000) Nat. Neurosci. 3, 217–225
9. Kozma, R., Sarner, S., Ahmed, S., and Lim, L. (1997) Mol. Cell. Biol. 17, 1201–1211
10. Threadgill, R., Bohb, K., and Ghosh, A. (1997) Neuron 19, 625–634
11. Daniels, R. H., Hall, P. C., and Bokoch, G. M. (1998) EMBO J. 17, 754–764
12. van Leeuwen, F. N., Kain, H. E., van der Kamen, R. A., Michiels, F., Kranenburg, O. W., and Collard, J. G. (1997) J. Cell Biol. 138, 787–807
13. Whitehead, J. P., Campbell, S., Rossman, K. L., and Der, C. J. (1997) Biochim. Biophys. Acta 1332, F1–F23
14. Steen, R., Kubiesi, T. J., Zheng, H., Kulkarni, S., Manzilis, J., Ruiz Morales, A., Hogue, C. W., Lawson, T., and Culotti, J. (1998) Cell 92, 785–795
15. Awasaki, T., Saito, M., Sone, M., Suzuki, E., Sakai, R., Itu, K., and Hama, C. (2000) Neuron 26, 119–131
16. Bateman, J., Shu, H., and Van Vactor, D. (2000) Neuron 26, 93–106
17. Liebl, E. C., Forsthoefel, D. J., Franco, L. S., Sample, S. H., Hess, J. E., Cowger, J. A., Chandler, M. P., Shupert, A. M., and Seeger, M. A. (2000) Neuron 26, 107–118
18. Newsome, T. P., Schmidt, S., Dietzl, G., Keleman, K., Asling, B., DeBant, A., and Dickson, B. J. (2000) Cell 101, 283–294
19. Hoshino, M., Sone, M., Fukata, M., Kuroda, S., Kaibuchi, K., Nabeshima, Y., and Hama, C. (1999) J. Biol. Chem. 274, 17837–17844
20. Sone, M., Hoshino, M., Suzuki, E., Kuroda, K., Kaibuchi, K., Nakagoshi, H., Saigo, K., Nabeshima, Y., and Hama, C. (1997) Science 275, 543–547
21. Sone, M., Suzuki, E., Hoshino, M., Hou, D., Kuroki, H., Fukata, M., Kuroda, S., Kaibuchi, K., Nabeshima, Y., and Hama, C. (2000) Development 127, 4157–4168
22. Lemonou, M. A., Falasca, M. F., Kurnus, M. G., and Schlessinger, J. (1997) Trends in Cell Biol. 7, 237–242
23. Craven, S., and Bredt, D. S. (1998) Cell 93, 495–498
24. Habels, G. L., Scholtès, E. H., Zaydlycev, D., van der Kamen, R. A., Stam, J. C., Berns, A., and Collard, J. G. (1994) Cell 77, 557–549
25. Michiels, F., Habels, G. B., Stam, J. C., van der Kamen, R. A., and Collard, J. G. (1995) Nature 375, 338–340
26. Ehler, E., van Leeuwen, F. N., Collard, J. G., and Salinas, P. C. (1997) Mol. Cell. Neurosci. 9, 1–12
27. Kuroda, S., Fukata, M., Kobayashi, K., Nakafuku, M., Nomura, N., Iwamatsu, A., and Kaibuchi, K. (1996) J. Biol. Chem. 271, 23633–23637
28. Payrastre, B., van Bergen en Henegouwen, P. M., Breton, M., den Hartigh, J. C., Plantavin, M., Verkleij, A. J., and Boonstra, J. (1991) J. Cell Biol. 115, 121–128
29. Sander, E. E., van Delft, S., ten Klooster, J. P., Reid, T., van der Kamen, R. A., Michiels, F., and Collard, J. G. (1998) J. Cell Biol. 143, 1385–1398
30. Resh, M. D. (1994) Cell 76, 411–413
31. Stam, J. C., Sander, E. E., Michiels, F., van Leeuwen, F. N., Kain, H. E., van der Kamen, R. A., and Collard, J. G. (1997) J. Biol. Chem. 272, 28447–28454
32. Manser, E., Loo, T. H., Koh, C. G., Zhao, Z. S., Chen, X. Q., Tan, L., Tan, I., Leung, T., and Lim, L. (1998) Mol. Cell 1, 183–192
33. Niehjiyama, T., Sasaki, T., Takaishi, K., Kato, M., Yaku, H., Araki, K., Matsura, Y., and Taki, Y. (1994) Mol. Cell. Biol. 14, 2447–2456
34. Jalink, K., van Corven, E. J., Hengeveld, T., Mori, N., Narumiya, S., and Moolenaar, W. H. (1994) J. Cell Biol. 126, 801–810
35. Liu, X., Wang, H., Eberstadt, M., Schinzel, A., Ofen, T., Lernert, R. P., Schkerlyantz, J. M., Janowick, D. A., Harlan, J. E., Harris, E. A., Staunton, D. E., and Fesik, S. W. (1998) Cell 95, 269–277
36. Michiels, F., Stam, J. C., Nordli, P. L., van der Kamen, R. A., Ruuske-Van Stalle, L., Feil, C., and Collard, J. G. (1997) J. Cell Biol. 137, 387–398
37. Daub, H., Gevaert, K., Van den Bergh, J., Sobel, A., and Hall, A. (2001) J. Biol. Chem. 276, 1677–1680
38. Bourguignon, L. Y., Zhu, H., Shao, L., and Chen, Y. W. (2000) J. Biol. Chem. 275, 1829–1838
39. Song, H., and Poo, M. (2001) Nat. Cell Biol. 3, 81–88
40. Bourguignon, L. Y., Zhu, H., Shao, L., and Chen, Y. W. (2000) J. Cell Biol. 150, 177–191
41. Kunda, P., Puglisi, G., Quiroga, S., Kasik, R., and Caceres, A. (2001) J. Neurosci. 21, 2061–2072

Copyright © 2001 by The Rockefeller University Press
25 July 2018
Characterization of STEF, a Guanine Nucleotide Exchange Factor for Rac1, Required for Neurite Growth
Naoki Matsuo, Mikio Hoshino, Masato Yoshizawa and Yo-ichi Nabeshima

J. Biol. Chem. 2002, 277:2860-2868.
doi: 10.1074/jbc.M106186200 originally published online November 13, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M106186200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 41 references, 21 of which can be accessed free at http://www.jbc.org/content/277/4/2860.full.html#ref-list-1