Spatio-temporal Regulation of Rac1 and Cdc42 Activity during Nerve Growth Factor-induced Neurite Outgrowth in PC12 Cells*

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Neurite outgrowth is an important process in the formation of neuronal networks. It is widely accepted that Rac1 and Cdc42, members of the Rho GTPase family, positively regulate neurite extension through reorganization of the actin cytoskeleton; however, it remains largely unknown when and where Rac1 and Cdc42 are activated during neuritogenesis. This study visualized the spatio-temporal regulation of Rac1 and Cdc42 activities during nerve growth factor (NGF)-induced neurite outgrowth in living PC12 cells by using probes based on the principle of fluorescence resonance energy transfer (FRET). Immediately after the addition of NGF, Rac1 and Cdc42 were transiently activated in broad areas of the cell periphery; a repetitive activation and inactivation cycle was then observed at the motile tips of protrusions. This localized activation, which was more evident in PC12 cells treated with NGF for more than 24 h, might facilitate neurite extension, because the expression of constitutively active mutants of Rac1 and Cdc42 abrogated NGF-induced neurite outgrowth. FRET imaging also delineated a difference between the localization of activated Rac1 and that of Cdc42 within the neurite tips. Experiments with dominant-negative mutants suggested that Rac1 and Cdc42 were activated by a common guanine nucleotide exchange factor(s) in an early stage of the activation phase. Therefore, the difference between Rac1- and Cdc42-activated areas possibly came from the differential localization between Rac1-specific GTPase-activating proteins (GAPs) and Cdc42-specific GAPs. It was concluded that the localized activation of Rac1 and Cdc42 was caused by both guanine nucleotide exchange factors and GAPs, and was important for neurite extension.

Neuronal morphogenesis, including axon growth and dendrite elaboration, is required for the basic function of neurons to communicate with each other and with effector cells. Rho GTPases (RhoA, Rac1, and Cdc42), which control actin dynamics in a diversity of cellular functions (1–3), also play key roles in neuronal morphogenesis during the development of the neuronal network (4, 5). Rac1 and Cdc42, implicated in the formation of lamellipodia and filopodia in non-neuronal cells, respectively (2), have been accepted as positive regulators of neurite outgrowth (4). However, much less is known about the mechanism of the spatio-temporal regulation of their respective activities.

Rho GTPases function as molecular switches, shuttling between a GDP-bound inactive state and a GTP-bound active state; the GTP-bound Rho GTPases can bind to various effectors to elicit different biological activities. Rho GTPases are regulated by two classes of enzymes, guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs act to enhance the exchange of bound GDP for GTP and thus activate the Rho GTPases; whereas GAPs inhibit Rho family members by potentiating their intrinsic GTPase activity (3). In addition, guanine nucleotide dissociation inhibitors inhibit the exchange of GDP for GTP and also hold Rho GTPases in the cytoplasm (6). Recent studies have unraveled an increasing number of regulators and downstream targets of Rho GTPases (1, 7, 8). To understand the signaling pathway in neurite outgrowth, the particular subset of regulators and effectors working with Rac1 and Cdc42 during this process needs to be clarified. As an initial step, determining when and where Rac1 and Cdc42 are activated during neuritogenesis is essential.

Rat pheochromocytoma PC12 cells have been frequently used as a model system for neurite outgrowth induced by nerve growth factor (NGF). In this study, we visualized Rac1 and Cdc42 activities in this process by using in vivo probes based on the principle of fluorescence resonance energy transfer (FRET) (9). Immediately after NGF addition, Rac1 and Cdc42 were broadly activated in peripheral areas. However, this widespread activation was transient, and then recurrent activation was observed at the tips of protrusions. The localized activity of Rac1 and Cdc42 may be required for neurite extension, because the expression of constitutively active mutants of Rac1 and Cdc42 in the whole cell area inhibited NGF-induced neurite outgrowth. Furthermore, at the neurite tips, Rac1 and Cdc42 exhibited different modes of activation with respect to their distribution. Our data indicated that Rac1 and Cdc42 were activated by a common GEF(s) during an early stage of the activation phase. Given that, the difference between Rac1- and Cdc42-activated areas could be attributed to the differential localization between Rac1-specific and Cdc42-specific GAPs.

EXPERIMENTAL PROCEDURES

Plasmids—The prototype probes for Rac1 and Cdc42, used in our previous study (9), consisted of the Cdc42/Rac1 interacting-binding (CRIB) domain of Pak, Rac1 and Cdc42, a pair of green fluorescent protein mutants, and the carboxyl-terminal region of Ki-Ras4B, respec-

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† The abbreviations used are: GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; NGF, nerve growth factor; FRET, fluorescence resonance energy transfer; CRIB, Cdc42/Rac1 interacting-binding; PI 3-kinase, phosphatidylinositol 3-kinase.
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We refer to these probes as Raichu-Rac1/Ki-Ras-CT(1011x) and Raichu-Cdc42/Ki-Ras-CT(192) (12). We also subcloned into the pRM21-FLAG vector, which contained the FLAG tag and an internal ribosomal entry site followed by the cDNA of a red fluorescent protein, dsFP593 (9), at the 5′- and 3′-side of the cloning site, respectively. The CRIB domain of N-WASP (amino acids 124 to 274) (10) was also subcloned into the pRM21-FLAG vector. pRM21-CdGAP has been described previously (9).

Cell Culture and Reagents—PC12 cells were grown in RPMI medium containing 10% horse serum and 5% fetal bovine serum. NGF and U73122 were purchased from Calbiochem. LY294002 and U0126 were obtained from Sigma.

Imaging—PC12 cells were plated on polyethyleneimine- (Fig. 1) or collagen- (except for Fig. 1) coated 35-mm glass-base dishes (Asahi Techno Glass Co.), and then transfected with the Raichu vectors using Lipofectamine 2000 (Invitrogen). After recovery, the medium was changed to phenol red-free Dulbecco’s modified eagle’s medium/F-12 containing 0.1% bovine serum albumin for imaging. Cells were starved for 6 h when necessary, and then treated with 50 ng/ml NGF. For long-term observation (Fig. 1), the medium was covered with mineral oil (Sigma) to preclude evaporation. In some experiments, cells were pretreated with 20 μM LY294002, 20 μM U0126, or 2.5 μM U73122 for 30 min prior to the addition of NGF. Thereafter, cells were imaged with an IX70 inverted microscope (Olympus) equipped with a Cool SNAP HQ cooled CCD camera (Roper Scientific) controlled by MetaMorph software (Universal Imaging), as described previously (13). Filters used for the dual-emission imaging studies were obtained from Omega Optical, Inc.: an XF1071 (440A/F21) excitation filter, an XF2034 (455DRLP) dichroic mirror, and two emission filters (XF3075 (480AF30) for CFP and XF3079 (535AF26) for YFP) were used. Cells were illuminated with a 75-W xenon lamp through a 12%/80% dichroic mirror and a ×40 or ×100 oil immersion objective lens. Exposure times for 4 × 4 binning were 400 ms to obtain images of CFP and YFP, and 100 ms to obtain a differential interference contrast image. After background subtraction, the YFP/CFP ratio image was created with MetaMorph software and the image was used to represent FRET efficiency. In the cotransfection experiments, pRM21-Flag-Rac1, pRM21-Flag-Cdc42, pRM21-Flag-N-WASP-CRIB were introduced along with the Raichu vectors. The expression levels of the dominant-negative mutants and CdGAP were monitored by the red fluorescence of FP593, which was translated from the internal ribosomal entry site on the mRNAs of the dominant negative mutants and CdGAP. Thus, we were able to choose cells in a range from one-half to 2-fold of the averaged efficiency of these Raichu probes correlates with the GTP/GDP ratio of Rac1 or Cdc42 (9), their activities can be visualized by this YFP/CFP ratio image. Undifferentiated PC12 cells having round shapes showed only slight activity changes of Rac1 and Cdc42 (Fig. 1, A and B, 0 h). Upon NGF treatment, these cells began to spread and exhibit multiple protrusions with a rapid cycling of extension and retraction; 12 h later, some protrusions became stable neurites and continued to extend. During this outgrowth process, activation of Rac1 and Cdc42 was observed mostly at the newly protruding tips of neurites (Fig. 1, A and B).

The characteristics of this localized activation of Rac1 and Cdc42 was further examined by another set of time-lapse experiments conducted at shorter intervals (Fig. 1, C and D). At 24 h after NGF addition, PC12 cells expressing the Raichu probes were imaged every 2 to 3 min to precisely trace the movement of the neurite tips; which showed prominent activation of Rac1 and Cdc42 when they were protruding (arrowheads in Fig. 1, C and D), although some differences in the activated areas were observed between Rac1 and Cdc42 (described below in detail). In contrast, the retracting protrusion (arrow) and non-motile areas of the cell bodies exhibited no remarkable activation of Rac1 and Cdc42.

Rac1 and Cdc42 Activation Patterns Were Spatially Different in NGF-induced Neurites—Differences in the spatial patterns of activation were reproducibly observed between Rac1 and Cdc42 when neurites with similar shapes were analyzed (Fig. 2, A and B). Highly activated areas of Rac1 tended to spread over the distal half of the neurite tips (Fig. 2A). On the other hand, marked activation of Cdc42 was mostly concentrated at the distal end of the neurite tips; in many cases, potent Cdc42 activation was confined to microspikes projecting from the neurite tips (Fig. 2B). In Fig. 2C, the mode of Rac1/Cdc42 activation at the neurite tips is classified into four categories. A histogram plotting the neurite number in each category showed that the Cdc42 activation mode was significantly different from that of Rac1. This difference between Rac1- and Cdc42-activated neurites at the protrusions was already observed at 4 h after the addition of NGF (Fig. 2, D and E).

The localization of activated Rho GTPases were primarily determined by their carboxyl-terminal regions (16). Thus, the Raichu-Rac1 and Raichu-Cdc42 probes used here were fused to the authentic carboxyl termini to bring the monitor to the membrane compartments where the endogenous Rac1 and Cdc42 should be activated. Although both Raichu-Rac1 and Raichu-Cdc42 were localized at the plasma membrane and endomembrane, Raichu-Cdc42 were preferentially localized at the endomembrane compartment (10). This raised the possibility that the different modes of activation described above resulted from the differences in subcellular localization of these FRET probes. To exclude this possibility, another type of probes were used for comparison: Raichu-Rac1/Ki-Ras-CT and Raichu-Cdc42/Ki-Ras-CT harbor the carboxyl-terminal region of Ki-Ras, which delivers probes primarily to the plasma membrane of HeLa cells (10). No significant difference in localization was observed between these two probes having the Ki-Ras carboxyl termini in PC12 cells (data not shown). With these probes fused to Ki-Ras-CT, we obtained essentially the same results as with the probes with the authentic carboxyl termini; there were clear differences between the Rac1- and Cdc42-activated areas (Fig. 2, F and G).

Early Response of Rac1 and Cdc42 Activities upon NGF Stimulation—Using the Raichu probes, we further examined...
the early phase of Rac1/Cdc42 activation in PC12 cells immediately after NGF addition. The representative time sequences of the FRET images are shown in Fig. 3, A and B. Around 3–5 min after NGF addition, both Rac1 and Cdc42 were diffusely activated in broad areas at the cell peripheries (Fig. 3, A and B, 2–4 min); this contrasted with their localized activation during the outgrowth process depicted in Figs. 1 and 2. It should be noted that this widespread activation was transient, after which recurrent activation was observed at the tips of the protrusions (Fig. 3, A and B, 10–30 min). To demonstrate the time course of the activity changes more quantitatively, we calculated the YFP/CFP ratios for Rac1 and Cdc42 averaged over the whole cell, and plotted them for three samples (Fig. 3, C and E). The first peak in Rac1/Cdc42 activation appeared primarily 3–5 min after NGF stimulation, whereas the succeeding peaks appeared stochastically in each cell. This observation was compared with the result of the pull-down assay, which showed that Rac1 and Cdc42 peaked −2.5 min after the addition of NGF (Fig. 4); we thus concluded that this peak was most likely produced by widespread and synchronized activation in numerous cells. However, as shown by FRET imaging, the peaks following the first peak were more localized (Fig. 3, A and B) and unsynchronized (Fig. 3, D and E), and thus were not detected by the pull-down method (Fig. 4 and Refs. 14, 17, and 18). In contrast to the localized and intermittent activation of Rac1 and Cdc42, Ras activation was observed throughout the cells and was sustained with a gradual decrease over 30 min (Fig. 3, C and F).

Next, we asked whether or not these localized and repeated activation-inactivation cycles of Rac1/Cdc42 at neurite tips might have any role in the induction of neurite outgrowth. To answer this question, PC12 cells were transfected with constitutively active or dominant-negative mutants of Rac1 and Cdc42, followed by NGF treatment for 2.5 days. The cells transfected with green fluorescent protein alone had efficiently developed neurites by 2.5 days of treatment with NGF (Fig. 5 A); the proportion of neurite-bearing cells in green fluorescent protein-transfected cells (control) in the presence of NGF was 50% (Fig. 5 D). The dominant-negative mutants of Rac1 and Cdc42 were potent inhibitors of NGF-induced neurite extension, as shown previously (19). Of note, only 8% of the Rac1-V12 expressing cells, and only 7% of the Cdc42-V12 expressing cells, bore neurites in the presence of NGF. Cells expressing Rac1-V12 became flattened and had membrane ruffles (Fig. 5 B), whereas Cdc42-V12 expressing cells produced a large number of microspikes around the cell periphery (Fig. 5 C). These morphologies were essentially the same as those observed in the absence of NGF (20). Therefore, the presence of constitutively
active Rac1 and Cdc42 throughout the cells also abrogated NGF-induced neurite extension, as did dominant-negative Rac1 and Cdc42. These results, taken together, suggest that localized activation of both Rac1 and Cdc42 is required for neurite outgrowth in NGF-treated PC12 cells.

**PI 3-Kinase Was Required for the Early Phase Activation of Rac1 and Cdc42 upon NGF Stimulation**—To investigate the mechanism of Rac1/Cdc42 activation using these probes, we developed a sensitive method to quantify Rac1/Cdc42 activation. As shown in Fig. 3, A and B, the Rac1/Cdc42 probes near the center of the cells basically did not respond to NGF stimulation, and thus lowered the YFP/CFP ratios when they were averaged over the whole cell. First, in each sample, we determined the average ratio before NGF stimulation and used that ratio as the reference value. Then, we measured the percentage of the area in which the local YFP/CFP ratio exceeded the reference value by at least 10%. Fig. 6, A and B, represents the results of this procedure for the samples analyzed in Fig. 3, D and E, respectively. The values of the first peaks in Fig. 6, A and B, were increased by 10–20% over the reference value, although the corresponding increases in the first peaks in Fig. 3, D and E, were only 3–6%.

By using this procedure of quantification (i.e., the percentage of activated area), we searched for an upstream regulator of Rac1 and Cdc42, and found that PI 3-kinase regulated the early phase of Rac1/Cdc42 activation in NGF-stimulated PC12 cells. In the control cells, the activated area was increased by 15% (Rac1) or 10% (Cdc42) upon NGF stimulation (Fig. 6, C and D, lane 1). In contrast, in the PC12 cells pretreated with LY294002, the percentage of Rac1- and Cdc42-activated area was significantly reduced (lane 2). This reduction indicates that the widespread activation corresponding to the first peak was largely dependent on PI 3-kinase, in agreement with the results of biochemical studies (14, 18).

Notably, the localized Rac1/Cdc42 activation corresponding to the recurrent peaks shown in Fig. 6, A and B, was also inhibited by the PI 3-kinase inhibitor, although there remained some degree of Rac1 activation independent of the PI 3-kinase activity (Fig. 6, E and F). The other NGF/Trk signaling cascades, i.e., the Ras/MEK/Erk pathway and the phospholipase C-γ1 pathway, have also been shown to contribute to NGF-induced neurite outgrowth (21, 22). However, in cells pretreated with a MEK inhibitor (U0126) or a phospholipase C-γ1 inhibitor (U73122), the observed increases in Rac1/Cdc42 activation were comparable with those in the control cells (Fig. 6, C and D, lanes 3 and 4).

**Relationship between the Activation Mechanisms of Rac1 and Cdc42 upon NGF Stimulation**—To further analyze the mechanism of Rac1/Cdc42 activation during the early phase of NGF-induced morphological change, we used dominant negative mutants of Rac1 and Cdc42. FRET imaging with Raichu-Rac1 showed that Cdc42-N17 inhibited Rac1 activation upon NGF stimulation, as did Rac1-N17 (Fig. 7A). Also, NGF-induced Cdc42 activation was significantly reduced by both Rac1-N17 and Cdc42-N17 (Fig. 7B). The most likely explanations for these observations are as follows. The dominant-negative mutants used here sequester their cognate GEFs (23), and many GEFs activate both Rac1 and Cdc42 (7); thus, one possibility is that Rac1 and Cdc42 were activated by the same GEF following NGF stimulation. A similar possibility was pointed out in the case of the bradykinin treatment of PC12 cells (24). Alternatively, there might be a positive feedback loop between Rac1 and Cdc42, and thus the inhibition of either Rac1 or Cdc42 would block the activation of both. We tried to distinguish between these two possibilities by specifically inhibiting Cdc42 activation. C3GAP (25), which has been shown to act on Cdc42 but not on Rac1 in cultured cells (9), effectively inhibited Cdc42 activation upon NGF stimulation in PC12 cells (Fig. 7C). However, in the presence or absence of C3GAP, a comparable level of Rac1 activation was observed following NGF stimulation (Fig. 7D). Furthermore, NGF-induced Rac1 activation was not inhibited at all by the expression of the CRIB domain of N-WASP (Fig. 7, D and E), which has been shown to specifically bind to GTP-Cdc42 (26) and inhibit the downstream signaling of Cdc42 in fibroblasts (12). The ability of N-WASP CRIB to block the downstream signaling of Cdc42 in PC12 cells was confirmed by neurite extension assay; the expres-
NGF stimulation can be attributed to the same GEF. The early phase activation of both Rac1 and Cdc42 upon NGF addition are shown for Rac1 (A), Cdc42 (B), and Ras (C). Scale bars, 10 µm. D–F, YFP/CFP ratios of three representative data sets were expressed by measuring the increase over the basal activity, which was averaged over 10 min before NGF addition.

**DISCUSSION**

FRET imaging of Rac1 and Cdc42 activity possesses a unique advantage over the conventional biochemical methods in that time-dependent change and spatial distribution can be traced in individual cells. A biochemical study of PC12 cells has already detected a single peak of Rac1 activation immediately after the addition of NGF (14). However, in single-cell recordings using the FRET probes, intermittent activation of Rac1 and Cdc42 following NGF treatment was also observed. Immediately after NGF addition, Rac1 and Cdc42 were transiently activated in broad areas at the cell periphery, and thereafter, activation localized at the neurite tips was recurrently observed. This finding of localized activation during the extension process may help to explain why expression of Rac1-V12 and Cdc42-V12 inhibited NGF-induced neurite outgrowth (Fig. 5). A similar failure of the constitutively active mutants of Rac1 and Cdc42 to induce neurites was reported in the absence of ligands in PC12 cells (20, 27) and other neuronal cells (28). We propose that localization cues, including the localized Rac1/Cdc42 activity shown here, are necessary for neurite formation; as was discussed recently (27). Another possible explanation is that the Rho GTPase signaling pathway has a cyclic mode of action, and constitutively active mutants may block this cycle (4), although the underlying mechanisms have yet to be described.

The Raichu-Cdc42 probe detected significant activation of Cdc42 upon NGF stimulation; this finding is consistent with the essential role of N-WASP, a Cdc42-specific effector, in NGF-dependent neurite extension in PC12 cells (29). However, a study using a pull-down analysis has reported that Cdc42 is not significantly activated upon NGF treatment (14). This is probably because the activation of Cdc42 was more localized than...
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Fig. 6. Requirement of PI 3-kinase for the early phase activation of Rac1 and Cdc42 following NGF treatment. A and B, the time-dependent changes of Rac1/Cdc42 activity in the samples used in Fig. 3, D and E, were analyzed by another procedure (percentage of activated area), described under “Results.” C and D, serum-starved PC12 cells expressing Raichu-Rac1 (C) or Raichu-Cdc42 (D) were mock-treated or treated with 20 μM LY294002 (14), 20 μM U0126 (39), or 2.5 μM U73122 (24) for 30 min, and then were stimulated with NGF. Cell images were taken as described in the legend to Fig. 1. Rac1/Cdc42 activation was quantified as described above. For each treatment, more than five cells were analyzed, and the average of the highest values within 10 min after NGF addition is shown with standard deviations. The asterisks indicate the results of the t test analysis; *, p < 0.01 compared with the cells treated with NGF alone. E and F, three representative time courses of changes in the activity of Rac1 (E) and Cdc42 (F) upon NGF stimulation in the presence of 20 μM LY294002.

that of Rac1. The pull-down assay, which monitors averaged changes in GTP-Cdc42, may not be sensitive enough to detect Cdc42 activation localized at the periphery of the cell body.

Another unexpected feature of NGF-induced Rac1/Cdc42 activation revealed in this study was its intermittent activation, although this finding agrees with a previous observation that neurites do not grow continuously, but often pause before restarting extension (30). This intermittent activation at the motile tips was dependent on PI 3-kinase as well as the initial robust activation immediately after NGF treatment. Neurite tips are accumulation sites for both F-actin (14) and PI 3-kinase activity (31), the latter detected by anti-phospho-Akt staining. Recent studies have shown that positive feedback loops linking increases in PI(3,4,5)P3, activation of Rac1/Cdc42, and actin polymerization are important for neutrophil or hippocampal neuron polarity (31–33). In NGF-induced neurite outgrowth, a similar positive feedback loop, which might frequently be interrupted by a shortage of monomeric actin or the turnover of lipid products of PI 3-kinase, might be a driving force localizing morphological changes and regulatory signals.

In contrast to the localized and intermittent activation of Rac1 and Cdc42, Ras activation in NGF-treated PC12 cells was detected diffusely at the plasma membrane and had a longer duration (Fig. 3 and Ref. 13). In line with diffuse Ras activation, it has been shown that the expression of constitutively active Ras does not inhibit, but rather promotes neurite outgrowth in PC12 cells (34). This concurs with a bulk of evidence implicating the Ras/Raf/Erk pathway in the activation of transcription/translation of proteins required for neurite extension (35). In this case, the site of Ras activation is remote from the site of action, whereas we assume that the sites of activation and action of Rac1/Cdc42 are regionally confined by the positive-feedback loop described above.

Raichu-Rac1 and Raichu-Cdc42 monitor the balance between GEF and GAP activities at the membranes, but they are insensitive to Rho guanine nucleotide dissociation inhibitor
activity (9). Another type of FRET probe, Raichu-CRIB-X, in which FRET inversely correlates with the local concentration of endogenous GTP-Rac1 and GTP-Cdc42, enables Rho guanine nucleotide dissociation inhibitor activity to be considered (9). Observations using Raichu-CRIB-X in NGF-treated PC12 cells (data not shown) were consistent with the findings obtained with Raichu-Rac1 and Raichu-Cdc42 suggesting that Rho guanine nucleotide dissociation inhibitor did not contribute significantly to the regulation of Rac1/Cdc42 activity upon NGF stimulation in PC12 cells.

As shown above, FRET imaging is a promising tool for unraveling linkages between neuronal morphogenesis and intracellular signaling. However, many of these neuronal events take a day or more, and at present, long-term imaging remains difficult to perform. In our experience, neurite formation could be frequently impaired by accumulated photo-damage because of repeated exposure to intense light, as described previously (36). In addition, substantial increases in the basal fluorescent intensity emitted by YFP and CFP, possibly because of slow folding of the proteins, were sometimes observed during the long-term recordings; these drifts might be accompanied by unreliable changes in the YFP/CFP ratios. For wider application in neuroscience, further improvements of the current methods will be desirable.

Experiments with dominant-negative mutants strongly suggested that Rac1 and Cdc42 were activated by a common GEF(s) during early phase activation. Rac1/Cdc42 activation was inhibited by LY294002, thus this putative GEF should be PI 3-kinase-dependent. This conclusion is in line with the findings from previous biochemical studies demonstrating the PI 3-kinase dependence of Rac1 activation (14, 18) and neurite outgrowth (37, 38) in NGF-treated PC12 cells. Assuming that the same GEF or GEFs activate(s) Rac1 and Cdc42 upon NGF stimulation, the difference in Rac1- and Cdc42-activated areas observed in this study could be attributable to differential localization between one or more Rac1-specific GAPS and Cdc42-specific GAPS. Our previous study using Raichu-Rac1/ Cdc42 in HT-1080 fibroblasts showed a similar difference in the distributions of active Rac1 and Cdc42 during cell migration; Rac1 activity was highest immediately behind the leading edge, whereas Cdc42 activity was most prominent at the tip of the leading edge (9). If this type of difference between Rac1 and Cdc42-activated areas is generally observed in the morphological changes of various cells, it is an intriguing question whether or not there exists a common underlying mechanism. A future study combining FRET imaging and function-interference techniques should help clarify the molecular network of spatio-temporal regulation of cellular morphogenesis.

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