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Nuclear Rac1 regulates the bFGF-induced neurite outgrowth in PC12 cells

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Rac1 plays a key role in neurite outgrowth via reorganization of the actin cytoskeleton. The molecular mechanisms underlying Rac1-mediated actin dynamics in the cytosol and plasma membrane have been intensively studied, but the nuclear function of Rac1 in neurite outgrowth has not yet been addressed. Using subcellular fractionation and immunocytochemistry, we sought to explore the role of nuclear Rac1 in neurite outgrowth. bFGF, a strong agonist for neurite outgrowth in PC12 cells, stimulated the nuclear accumulation of an active form of Rac1. Rac1-PBR (Q) mutant, in which six basic residues in the polybasic region at the C-terminus were replaced by glutamine, didn't accumulate in the nucleus. In comparison with control cells, cells expressing this mutant form of Rac1 displayed a marked defect in extending neurites that was concomitant with reduced expression of MAP2 and MEK-1. These results suggest that Rac1 translocation to the nucleus functionally correlates with bFGF-induced neurite outgrowth. [BMB Reports 2013; 46(12): 617-622]

INTRODUCTION

Rat pheochromocytoma PC12 cells have been used as a model system to increase our understanding of the molecular mechanisms underlying neuronal differentiation (1). In response to stimulation with basic fibroblast growth factor (bFGF) or nerve growth factor (NGF), PC12 cells stop proliferation and begin to undergo neuronal differentiation; they develop electrical excitability and neurite extension (2). Neurite outgrowth involves rearrangement of the actin cytoskeleton, with formation of lamellipodia and filopodia (3). Neurite outgrowth is subject to tight regulation by activation of diverse signal transduction pathways, including Rho GTPases, guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (4). Rho GTPase Rac1 activity is particularly important for neurite extension because of its ability to form lamellipodial actin structures (5).

Rac1 also contributes to a wide range of biological responses, such as cell migration, gene transcription and cell cycle progression (6). Thus, the temporal and spatial activation of Rac1 is controlled precisely by upstream regulators: GEFs, GAPs and RhoGDIs (6). In connection with neurite outgrowth and migration, most studies to date have focused on the roles of Rac1 in the neurite tip and at the leading edge of migratory cells, respectively (7-9). Rac1 has a canonical nuclear localization signal (NLS), which consists of the polybasic amino acids KKRKRK, in its C-terminal polybasic region (PBR) (10, 11). Accordingly, several lines of evidence have indicated that Rac1 localizes to the nucleus and have suggested potential roles in the nucleus. All of these studies addressed the function of Rac1 in connection with the cell cycle and cell division (12, 13). Consistent with this, some cancer cells showed nuclear staining for Rac1 (14). However, whether nuclear Rac1 is functionally associated with cellular differentiation has not yet been addressed.

In this study, we sought to explore the nuclear function of Rac1 in neuronal differentiation using the well-established PC12 cell model. We found that Rac1 was translocated to the nucleus and that its nuclear accumulation increased gradually during neurite outgrowth. Furthermore, PC12 cells expressing a Rac1 mutant that was defective in nuclear translocation displayed shortened neurites compared with control cells.

RESULTS

Translocation of Rac1 to the nucleus during bFGF-induced neurite outgrowth

To determine whether Rac1 is targeted to the nucleus during neuronal differentiation, we investigated its localization by Western blot analysis and by immunocytochemistry. PC12 cells were stimulated with bFGF for the indicated times, and lysates were separated into cytosolic and nuclear fractions before being subjected to Western blotting. Fractionation was verified using the specific markers GAPDH and histone H1 for the cytosolic and nuclear fractions, respectively. Rac1 targeting to the nucleus increased gradually over time during bFGF-induced differentiation in PC12 cells (Fig. 1A). However,

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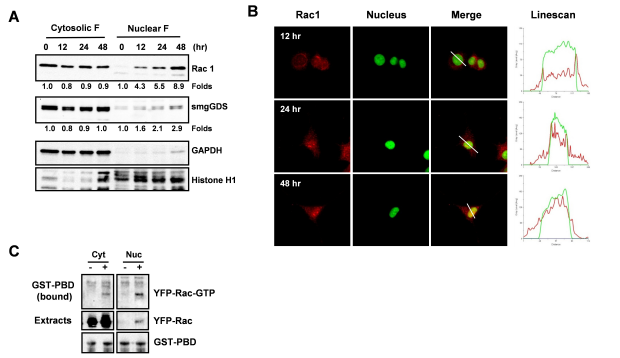

Fig. 1. Translocation of Rac1 to the nucleus during bFGF-induced neurite outgrowth in PC12 cells. (A) PC12 cells were stimulated with 20 ng/ml bFGF for the indicated times. Cells were fractionated as described in the Materials and Methods. Proteins (40 μg) were separated on 10-12% SDS-PAGE and were immunoblotted for Rac1 and smgGDS. To verify correct fractionation, GAPDH and histone H1 were blotted as markers. (B) PC12 cells were seeded on 12-mm cover slips and incubated with 20 ng/ml bFGF for the indicated times. To localize endogenous Rac1, cells were stained using an anti-Rac1 antibody (red) and Hoechst 33342 for the nucleus (green). Linescans for cytosolic Rac1 (red) and nuclear Rac1 (green) were performed using MetaMorph. (C) PC12 cells were transfected with Rac1-wild type (WT)-YFP and incubated in the presence or absence of bFGF (20 ng/ml) for 48 h. The cytosolic and nuclear fractions were incubated with GST-PBD-bound agarose. Bound Rac1 was detected by immunoblotting with an anti-GFP antibody.

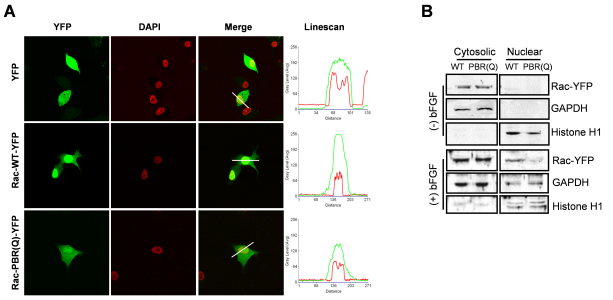

Fig. 2. The Rac1-PBR (Q) mutant does not translocate to the nucleus during bFGF-induced neurite outgrowth in PC12 cells. (A) PC12 cells were transfected with pYFP, pYFP-Rac1-WT or pYFP-Rac1-PBR (Q) plasmids and stimulated with 20 ng/ml bFGF for 48 h. The nucleus was DAPI stained (red). Linescans for YFP (green) and DAPI (red) were performed using MetaMorph. (B) PC12 cells were transfected with pYFP-Rac1-WT or pYFP-Rac1-PBR (Q) plasmids and incubated without (upper) or with (lower) 20 ng/ml bFGF for 48 h. Cells were fractionated as described in Fig. 1A. Lysates were immunoblotted for GFP, GAPDH or histone H1 as indicated.

smgGDS GEF did not substantially co-translocate with Rac1, as Lanning *et al*. reported (11). A previous study revealed that the βPIX, PAK2 and GIT proteins were associated with Rac1 in the cytosol (15). Translocation of these proteins was also negligible (data not shown). We further analyzed the localization of Rac1 by staining cells with an anti-Rac1 antibody and using a linescan to detect fluorescence in the nucleus. Rac1 staining in the nucleus showed a marked increase 24 h and 48 h after bFGF treatment (Fig. 1B, 2nd and 3rd rows). NGF is also a strong inducer of neuronal differentiation in PC12 cells. Thus, we asked whether NGF stimulation is capable of translocating Rac1 to the nucleus. A similar nuclear accumulation of Rac1 occurred in response to NGF for 48 h (Fig. S1). Our next question was whether nuclear Rac1 was activated. We performed a PBD pull-down assay to assess Rac1 activation. Both the cytosolic and nuclear fractions showed an active GTP-bound form of Rac1 only in response to bFGF stimulation (Fig. 1C). Interestingly, the nuclear fraction contained much higher levels of active Rac1 than the cytosolic fraction. These results suggest that active Rac1 in the nucleus might be functionally correlated with bFGF-stimulated neuronal differentiation.

A Rac1-PBR (Q) mutant shows defective nuclear targeting in differentiated PC12 cells

To determine whether the PBR at the C-terminus was required for the nuclear targeting of Rac1, we generated and assessed the localization of a Rac1-PBR (Q) mutant, in which the KKRKRK sequence in the C-terminal PBR of Rac1 was replaced by six glutamines (Q). PC12 cells were transfected with the YFP vector (as a control), Rac1-WT-YFP or Rac1-PBR (Q)-YFP and stimulated with bFGF for 48 h to induce neuronal differentiation. Under these conditions, the nuclear YFP fluorescence of the Rac1-PBR (Q) mutant was markedly reduced compared to Rac1-WT and YFP alone as monitored by a linescan (Fig. 2A). Consistent with this, a fractionation assay revealed increased nuclear targeting of Rac1-WT, but not of Rac1-PBR (Q), in bFGF-treated cells (Fig. 2B). These results suggest that the PBR of Rac1 is important in regulating the bFGF-stimulated nuclear targeting of Rac1.

Defective nuclear targeting of Rac1 hampers bFGF-stimulated neurite outgrowth

To investigate a potential role for nuclear Rac1, we analyzed the ability of Rac1-WT and Rac1-PBR (Q) to mediate bFGF-stimulated neurite outgrowth. PC12 cells were transfected with YFP, Rac1-WT-YFP or Rac1-PBR (Q)-YFP, and neurite outgrowth was then induced by bFGF stimulation. PC12 cells with neurite extension of approximately twice the cell body length were counted as positive for differentiation. Many cells expressing Rac1-WT elaborated neuronal processes (Fig. 3A). In contrast, neurite extension was minimal in cells expressing YFP or Rac1-PBR (Q). Quantification of cells revealed that 50% of Rac1-WT expressing cells were differentiation positive, whereas YFP and the Rac1-PBR (Q) mutant showed only 20% and 18% positivity, respectively (Fig. 3B). This result strongly suggested that the nuclear translocation of Rac1 was functionally linked to bFGF-induced neurite outgrowth. This finding led us to consider that nuclear Rac1 might regulate the

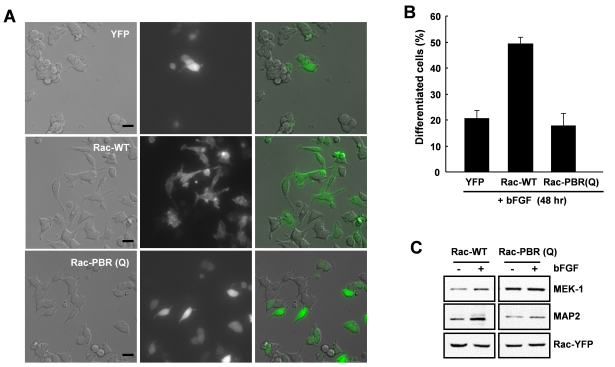

Fig. 3. Nuclear Rac1 functionally correlates with neurite outgrowth. (A) PC12 cells were transfected with pYFP, pYFP-Rac1-WT or pYFP-Rac1-PBR (Q) plasmids. To induce neurite outgrowth, PC12 cells were stimulated with 20 ng/ml bFGF for 48 h. Representative images are shown for PC12 cells expressing a Rac1-YFP construct. Scale bar, 50 μm. (B) Quantification of the differentiation rate is shown. (C) PC12 cells were transfected with pYFP-Rac1-WT or pYFP-Rac1-PBR (Q) plasmids and incubated with or without 20 ng/ml bFGF for 48 h. Cell lysates were immunoblotted for MEK-1, MAP2 or GFP as indicated.

expression of several genes required for bFGF-stimulated differentiation. We postulated MEK-1 and MAP2 as potential targets, as MEK-1 is a component of the Ras-ERK pathway whose persistent activation is critical for differentiation of PC12 cells and MAP2 plays an important role in microtubule polymerization for neurite extension. As illustrated in Fig. 3C, MEK-1 was up-regulated in Rac1-WT-expressing cells during differentiation. Similarly, MAP2 showed a significant increase in its expression levels in response to bFGF stimulation. In contrast, the expression levels of these two proteins remained unaltered in Rac1-PBR (Q)-expressing cells during differentiation. Collectively, these results suggest that bFGF-induced nuclear translocation of Rac1 might promote neuronal differentiation by up-regulating MEK-1 and MAP2.

DISCUSSION

In the present study, we demonstrated that Rac1 accumulated in the nucleus during neurite outgrowth and that nuclear Rac1 contributed to bFGF-induced neuronal differentiation in PC12 cells. Previous studies reported that nuclear Rac1 appeared in a cell cycle-dependent manner and played a role in promoting cell division (12, 16, 17). Consistent with a proliferative function, tumor tissues and colorectal cancer cells displayed strong immunoreactivity to Rac1 in the nucleus (14). This study is the first to suggest a cell differentiation-related role for nuclear Rac1.

Rac1 contains a nuclear localization signal (NLS) in its C-terminal polybasic region. Lanning et al. suggested that smgGDS, a GEF for a wide variety of small GTPases, including K-Ras, Rap1, Rac1, Cdc42 and RhoA, had a role in the translocation of Rac1 (11, 18). However, our observation that nuclear targeting of smgGDS did not significantly occur during the differentiation of PC12 cells would seem to exclude the involvement of smgGDS. More recently, it has been shown that Karyopherin alpha 2 (KPNA2) mediates the nuclear import of Rac1 by directly binding the NLS (19). Although this process required Rac1 activation, it still remains controversial whether Rac1 activation is necessary for nuclear transport, as it has been observed that nuclear localization of Rac1 did not require its activation in cells such as C33A and SiHa cervical cancer cell lines (14). Our PBD pull-down assay revealed the presence of a higher proportion of active GTP-bound Rac1 in the nuclear compartment. This might have been due to cytosolic or nuclear activation of Rac1. Basic FGF stimulates cytosolic Rac1 downstream of PI3K; this activated Rac1 could then be targeted to the nucleus. Alternatively, a Rac1-specific GEF, such as Tiam1, could be translocated to the nucleus, where it would convert inactive Rac1 to active Rac1 (20). The presence of Rac1, activated by either pathway, suggests a somewhat active role for Rac1 in the nucleus.

How nuclear Rac1 contributes to neuronal differentiation remains to be established. First, nuclear Rac1 may promote neuronal differentiation in a transcription-dependent manner. Because the progressive outgrowth of neuronal processes requires a supply of cytoskeletal components, such as microtubule-associated proteins and signaling proteins, that must be transported to the neurite tip, transcriptional up-regulation is necessary (21). In this study, MAP2 and MEK-1 were up-regulated in Rac1-WT-expressing cells, but not in Rac1-PBR (Q)-expressing cells, during differentiation (Fig. 3C). Accumulating evidence indicates that actin is present in the nucleus and has roles ranging from chromatin remodeling and transcription to maintenance of the nuclear architecture. Consistent with these roles, disintegration of epithelial cell-cell contact following Ca^{2+} withdrawal stimulated SRF-mediated transcription via a Rac1-actin-MAL signaling pathway (22). Although that study did not explicitly indicate the localization of Rac1, it appears that nuclear Rac1 was at least partially involved. It is well established that cytosolic Rac1 reorganizes the actin cytoskeleton, resulting in the formation of lamellipodia for cell migration and neurite extension. Rac1 signals to the actin cytoskeleton via the PAK-LIMK pathway. We reported previously that PAK2 is a major isoform in PC12 cells (15), but we did not observe a substantial amount of PAK2 in the nucleus (data not shown). Moreover, many actin-binding proteins have been identified in the nucleus. It is thus tempting to suggest that nuclear Rac1 may stimulate transcription by regulating actin dynamics. It will be of interest to identify the transcription factor(s) mediating the effects of nuclear Rac1.

Rac1 may also regulate nucleocytoplasmic shuttling of transcription factors, including such armadillo (ARM) family proteins as APC, plakoglobin and β-catenin (23-25). Because APC and β-catenin are reportedly involved in neurite outgrowth

(26), nucleocytoplasmic shuttling of these proteins may constitute a role for nuclear Rac1 in neuronal differentiation. Moreover, Rac1 may cooperate with an NLS-containing MgcRacGAP, as seen in its activation and the nuclear transport of the STAT transcription factor (16).

Furthermore, proteasomal degradation of Rho GTPases via the ubiquitin-dependent pathway is important in many cellular functions (27, 28). It was initially suggested that nucleus-targeted Rac1 was destined for proteasomal degradation (10, 19). However, no specific E3 ubiquitin ligase was identified. Recently, it was reported that the E3 ubiquitin ligases HACE1 and the IAPs XIAP and c-IAP1 are responsible for the ubiquitination of GTP-bound Rac1 (29, 30). However, as these ubiquitin ligases function mostly in the cytosol, it remains unclear how nuclear Rac1 would be ubiquitinated and degraded. Moreover, as evidenced by the gradual increase in nuclear Rac1 accumulation, it seems unlikely that degradation of nuclear Rac1 correlates with neuronal differentiation.

We provided evidence that nuclear Rac1 functionally correlates with neuronal differentiation in PC12 cells. N17Rac1, a dominant negative form of Rac1, has an intact PBR in the C-terminus and is thus translocated to the nucleus in response to bFGF stimulation (data not shown). A number of studies revealed a negative role for cytosolic N17Rac1 in actin dynamics, such as disruption of lamellipodial actin structures that led to inhibition of neurite outgrowth. Because nuclear actin is also dynamic, nuclear N17Rac1 might also suppress nuclear actin dynamics and thereby block a transcriptional event, such as up-regulation of MEK-1 and MAP2, required for neuronal differentiation. Rac1-PBR (Q)-expressing cells exhibited a marked defect in extension of neurites (Fig. 3A, B). This result was somewhat unexpected, as Rac1-PBR (Q) was considered to retain its cytosolic function in the regulation of actin dynamics despite being defective in nuclear translocation. These findings suggest that a solely cytosolic role of Rac1 might be insufficient for neuronal differentiation, highlighting a previously unidentified complementary role for nuclear Rac1.

MATERIALS AND METHODS

Antibodies and reagents

Human recombinant bFGF, Lipofectamine 2000, FBS and DMEM were obtained from Invitrogen (Carlsbad, CA, USA). Anti-Rac1, smgGDS, GAPDH, GFP and histone H1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The QuikChange site-directed mutagenesis kit and fractionation kit were purchased from Stratagene (La Jolla, CA, USA) and BioVsion (Milipitas, CA, USA), respectively.

Cell culture and differentiation

PC12 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1 x antibiotics (31). To induce differentiation, PC12 cells were incubated in DMEM with 2% FBS and 20 ng/ml bFGF (Invitrogen) for 48 h. After 48 h of bFGF treatment, transfected cells expressing yellow fluorescent protein (YFP) were monitored with a fluorescence microscope (Olympus IX81-ZDC; Olympus Optical Co., Tokyo, Japan), equipped with a cooled CCD camera (Cascade 512B; Photometrics, Tucson, AZ, USA). Basic FGF-induced differentiation was measured by scoring as positive those green cells with one or more growth cone-tipped neurites longer than two cell bodies in length (32). Cells from at least 10 different fields of view were counted using a fluorescence microscope.

Transient transfection

PC12 cells were seeded on sterilized glass coverslips that had been pre-coated with 20 μg/ml poly-L-lysine (Sigma, St. Louis, MO, USA) and were inserted into 24-well plates. For transfection, PC12 cells were transfected with pYFP, pYFP-Rac1-WT (wild-type) or pYFP-Rac1-PBR (Q) (mutant) constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfected cells were incubated in DMEM containing 2% FBS in the presence or absence of 20 ng/ml bFGF for 48 h.

Nuclear and cytosolic fractionation

Nuclear and cytosolic fractionation was performed using a fractionation kit (BioVision) according to the manufacturer's instructions. Briefly, cells were collected by scraping from culture dishes, centrifuged and washed twice with cold PBS. Cells were added to cytosol extraction buffer with protease inhibitors, and the tubes were incubated on ice for 10 min and centrifuged at 16,000 x g for 5 min. The supernatant was transferred to a new tube as the cytosolic fraction. Next, the pellet was resuspended in cold nuclear extraction buffer and centrifuged at 16,000 x g. The second supernatant was collected in a new tube as the nuclear fraction. Each fraction was separated by SDS-PAGE and analyzed by immunoblotting with specific antibodies.

Western blot analysis

Cells were washed twice with PBS and lysed in lysis buffer [125 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2% SDS] for 1 h at 4°C (33). Lysates were fractionated by SDS-PAGE and transferred to a PVDF membrane in a Tris-glycine-methanol buffer (25 mM Tris base, 200 mM glycine, 20% methanol). Membranes were blocked with 3% skim milk in PBS for 1 h, incubated with primary antibodies for 1 h at RT and then washed three times (10 min each) with PBS containing 0.1% Tween 20. Membranes were blotted with horseradish peroxidase-conjugated secondary antibodies for 1 h at RT. After five washes with PBS containing 0.1% Tween 20, signals were detected using enhanced chemiluminescence (ECL) reagents (Amersham Bioscience, Piscataway, NJ). In some cases, membranes were stripped and re-probed with different antibodies.

GST-PBD pull-down assay

GST-PBD was expressed in *E. coli* (DH5α) and purified with glutathione-Sepharose affinity chromatography. Cells were stimulated with or without 10 ng/ml bFGF for 48 h, fractionated as described above, further incubated with purified soluble GST-PBD (1 μg) at 4°C for 2 h in binding buffer (25 mM Tris HCl pH 7.5, 1 mM DTT, 30 mM MgCl2, 40 mM NaCl, 0.5% NP-40) and washed five times. Beads were boiled for 5 min, resolved by 12% SDS-PAGE and transferred to a PVDF membrane. Membranes were immunoblotted with an anti-Rac1 antibody and then re-probed with an anti-GST antibody.

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