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Biological activity of neurotrophins is dependent on recruitment of Rac1 to lipid rafts

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Abstract

The Rho family of small GTPases, key regulators of the actin cytoskeleton in eukaryotic cells, is implicated in the control of neuronal morphology. Here, we report that neurotrophin dependent cytoskeletal changes, characteristic of the phenotype of Rac1, in the hippocampal neurons or PC12 cells are inhibited by the disruption of lipid raft integrity. Activation of Rac1 induced by NGF is impaired in cholesterol-depleted PC12 cells. Pretreatment with γGTP shifted significant amount of Rac1, presumably in a GTP-bound form, from non-raft to raft fractions. Proper recruitment of activated Rac1 to lipid rafts, structures that represent specialized signaling organelles, is of fundamental importance in determining neurotrophins' bioactivity.

Keywords: Rac; Neurotrophin; Lipid rafts; neuron; Cholesterol; Growth cone

Neurotrophins mediate the survival, differentiation, growth, and apoptosis of neurons by binding to two types of cell surface receptors, the Trk tyrosine kinases and the p75 neurotrophin receptor [1,2]. One of the most prominent biological functions of neurotrophins may be that they enhance neurite outgrowth, as neurite extension and retraction are important processes in the formation of neuronal networks [3]. These biological changes are vitally regulated by the actin cytoskeleton organization. The Rho GTPases are key regulators of the actin cytoskeleton in eukaryotic cells from yeast to humans [4], and mediate the morphological changes that can be observed during neuronal development and plasticity, such as growth of neurites, axonal guidance, and dendrite elaboration [5–8]. Each member of the archetypal trio of the Rho GTPases, RhoA, Rac1, and Cdc42, has been found to regulate distinct actin filament containing structures. The potential of the Rho GTPases to function as signaling switches resides in their ability to cycle between active, GTP-bound states and inactive, GDP-bound states. The Rho GTPases in the GTP-bound form are recruited to the plasma membrane where they function as molecular switches in the transduction of extracellular signals to the cytoplasm and the nucleus. In fact, neurotrophins were shown to activate Rac1 and inactivate Rho through their receptors in the neuronal cells [7,9–11]. As the reorganization of the actin cytoskeleton of the neurons by neurotrophins begins at specific sites on the growth cones or the cell surface, activated Rho GTPases may exhibit also a polarized distribution.

Lipid rafts are cholesterol and sphingolipid-rich lipid microdomains in eukaryotic cell membranes [12]. Recent evidence suggests that these rafts act in signal transduction in immunocytes such as T lymphocytes

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and basophils [13–15]. Rafts are believed to function in cellular signaling by concentrating or separating specific molecules in a unique lipid environment, not only in immunocytes, but also in neurons [16]. For example, GDNF transduces its signal through RET, a transmembrane receptor tyrosine kinase, and a GPI-anchored co-receptor GFRα1 which localizes in lipid rafts. GFRα1 recruits RET to lipid rafts after GDNF stimulation and results in strong and continuous signal transduction [17].

Here, we report that the effects of neurotrophins on the morphology of the neurons were dependent on the lipid raft integrity. Activation of Rac1, which was induced by neurotrophins, is impaired by the disruption of lipid rafts, where activated Rac1 accumulates.

Materials and methods

Materials. Agents obtained and commercial sources were as follows: NGF, Upstate Biotechnology; polyclonal anti-rac1 antibody, Santa Cruz Biotechnology; and GTP-γS, Texas Red-conjugated phalloidin and methyl-β-cyclodextrin, Sigma. Wild-type Rac1 in pEF-BOS vector was kindly provided by Dr. S. Narumiya.

Cell culture. PC12 cells were cultured in Dulbecco's modified Eagle's medium containing 10% horse serum, 5% fetal bovine serum, 70 mg/ml, penicillin, and 100 mg/ml streptomycin. Cells were serum-starved for serum more than 4 h before experiments.

For hippocampal neurons, hippocampi were removed from E18 rats and incubated with 40 U/ml papain and 200 U/ml DNase1 for 30 min at 37 °C, and the treatment was stopped by adding DMEM containing 10% FCS. The cells were centrifuged at 1000 rpm for 5 min and resuspended in 10% FCS/DMEM. Glass coverslips were coated with 10 μg/ml poly-L-lysine (Sigma) for 1 h at room temperature [18]. Excess poly-L-lysine was washed off with H_2O, and the dishes were washed one more time. Isolated neurons were plated onto the poly-L-lysine-coated dishes for phalloidin staining. The medium was replaced with DMEM including B27 supplement.

Pull-down GTPase assay. In vivo Rac1/Cdc42 activation assay was performed according to the method described previously [19]. Cells were lysed in 20 mM Hepes, pH 7.4, 150 mM NaCl, 2% Nonidet P-40, 20% glycerol, 8 mM EGTA, 8 mM EDTA, 80 μM *p*-amidinophenylmethanesulfonyl fluoride (hydrochloride), 100 μg/ml aprotinin, and 200 μg/ml each of leupeptin, chymostatin, and pepstatin A. Cell lysates were clarified by centrifugation, and the supernatant was incubated with 20 μg GST-PAK2 protein immobilized on glutathione–Sepharose beads for 3 min. Beads were washed with washing buffer (20 mM Hepes, pH 7.4, 142.5 mM NaCl, 1% Nonidet P-40, 10% glycerol, 4 mM EGTA, and 4 mM EDTA), and bound Rac proteins were detected by Western blotting with the anti Rac1 polyclonal antibody (1:1000, Santa Cruz Biotech.).

Transient transfection of 293T cells and raft fractionation. 293T cells were transfected with BOS vector containing wild-type Rac1 using Lipofectamine (Gibco-BRL) and incubated for 24 h on 10 cm tissue culture plates. GTP-γS at the concentration of 100 μM was added for 1 h. Cells were then lysed on ice with 0.5 ml extraction buffer (10 mM Tris–HCl [pH 7.5], 150 mM NaCl, and 5 mM EDTA) containing 0.5% Brij-58 (Sigma), 100 μg/ml orthovanadate, and protease inhibitor cocktail (Roche). Extracts were adjusted to 40% sucrose by adding 0.5 ml of 80% sucrose in extraction buffer, then placed in a SW40Ti ultracentrifuge tube (Beckman), and overlayered with 8 ml of 30% sucrose in extraction buffer and 1 ml distilled water. All of these steps were performed in a 4 °C cold room and on ice. After centrifugation (16 h, 200,000*g*, 4 °C), 12 fractions of 0.83 ml each were collected from the top, and equal volumes of them were prepared for SDS–PAGE and immunoblot analysis. For detection of Rac1, polyclonal anti-Rac1 antibody was used.

F-actin staining. Cells were grown on glass coverslips and treated with various reagents. Cells on coverslips were fixed with 2% paraformaldehyde for 1 h at room temperature. The fixed cells were washed three times with PBS and permeabilized with 0.2% Triton X-100 in PBS for 10 min. The cells were then incubated with Texas Red-conjugated Phalloidin for 30 min at room temperature and washed three times with PBS. Samples were examined under confocal laser scanning microscope (Carl Zeiss).

Results

The effects of neurotrophins on neuronal cells are sensitive to perturbations of plasma membrane cholesterol

We first assessed whether disruption of lipid rafts would specifically affect the actin cytoskeleton reorganization induced by neurotrophins. A cholesterol-sequestering reagent, methyl-β-cyclodextrin (MβCD) which extracts cholesterol from the cells, was used for the disruption of lipid rafts [17]. Dissociated hippocampal neurons from rat E18 embryos, incubated for three to five days after plating, were treated with or without 5 mM MβCD for 5 min, followed by the addition of BDNF at the final concentration of 100 ng/ml for 10 min. These neurons expressed TrkB as well as the pan-neurotrophin p75 receptor (data not shown). The neurons were fixed and their F-actin-based structures were examined by staining with Texas Red-conjugated phalloidin. As shown in Fig. 1, the neurons stimulated with BDNF for 10 min displayed significantly more spreading growth cones in comparison with the control cells not treated with BDNF. A large fraction of BDNF treated neurons had filopodial and lamellipodial structures in their growth cones. On the other hand, when the cells were pre-treated with MβCD, collapse of the growth cones was predominantly observed after BDNF treatment, demonstrating no significant change in the actin cytoskeleton structures between BDNF treated and non-treated cells (Figs. 1A and B). These results show that MβCD treatment inhibits the effects of BDNF on the morphology of the hippocampal neurons.

We next used PC12 cells, a model system for neuronal differentiation and neurite outgrowth, to assess this issue. NGF is known to induce differentiation and neurite outgrowth from PC12 cells that express two kinds of NGF receptors, TrkA and p75. Importantly, it was shown that the ligand binding to p75 activates Rac1 and inactivates RhoA in a rapid time-course (~5 min) [7,10]. Therefore, we assessed the actin cytoskeleton of PC12 cells 10 min after the addition of NGF. NGF-induced morphological changes in the presence or absence of 10 mM MβCD are shown in Fig. 2. The cells treated with NGF showed F-actin redistribution and the

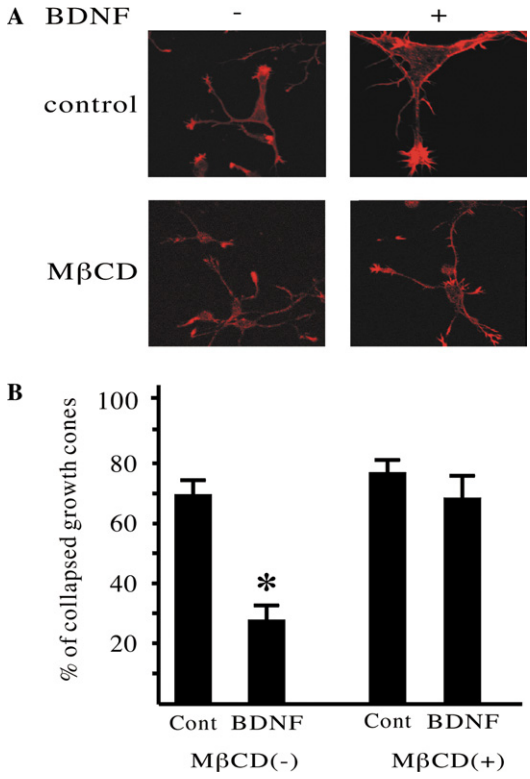

Fig. 1. The effects of BDNF on the hippocampal neurons pretreated with or without MβCD. (A) Dissociated hippocampal neurons were incubated for 10 min with or without MβCD and then were stimulated with or without BDNF (100 ng/ml). F-actin structures are shown by phalloidin staining. (B) Quantitative analysis of growth cone collapse induced by neurotrophins. A growth cone is considered to exhibit collapse when it withdraws at least 10 μm. Data are means ± SEM. An asterisk indicates statistical significance, $^{*}p < 0.01$ (Student's t test).

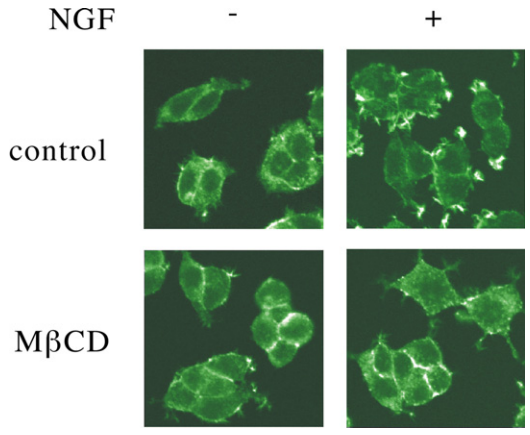

Fig. 2. Alteration of actin cytoskeleton organization induced by NGF in PC12 cells. PC12 cells were incubated for 10 min with or without MβCD and then were stimulated with or without NGF (100 ng/ml). F-actin structures are shown by phalloidin staining.

protrusion formation, where F-actin accumulated. This phenotype change is consistent with the previous report [9], which showed Rac1 activation in ligand stimulated PC12 cells. Consistent with the findings in the hippocampal neurons, pretreatment with MβCD completely inhibited the effects of NGF. Our observations in PC12 cells and the hippocampal neurons suggest that lipid raft integrity is required for the effects of the neurotrophins on the actin cytoskeleton reorganization.

Activation of Rac1 by neurotrophins is dependent on the integrity of cholesterol-rich microdomains

The Rho family of small GTPases, including Rho, Rac, and Cdc42, regulates various aspects of the actin cytoskeleton, and these GTPases are therefore good candidates for mediating the signals that regulate neurite outgrowth. The activated Rac1 promotes ruffling and the lamellipodial formation at the plasma membrane, while RhoA regulates the formation of stress fibers. The neurotrophins binding to p75 receptor elicit Rac activation and Rho inactivation [7,10], and the addition of NGF to PC12 cells, expressing Trk and p75 receptors, resulted in Rac1 activation [9]. Given the data presented above, morphological changes in the hippocampal neurons as well as PC12 cells may be attributable to Rac1 activation by the activated neurotrophin receptors by their ligands. If it is the case, proper Rac1 activation might be inhibited by MβCD treatment.

To examine this hypothesis, we measured in vivo activity of Rac1 by the pull-down assay. Since Rho GTPases in the GTP-bound state bind to their downstream effectors, GST fusions of these effectors can be used to capture active Rho GTPases from cell lysates. Thus, a GST fusion to the Rac1/Cdc42 binding domain of PAK (GST-PAK2) was used to specifically precipitate GTP-bound Rac1 or Cdc42 from cell extracts [19]. PC12 cells were treated with or without 10 mM MβCD and then stimulated with 100 ng/ml NGF for 3 min. This assay revealed that extracts of PC12 cells treated with NGF contained increased amount of GTP-Rac1 compared to the control cells (Fig. 3A), although the levels of expression of Rac1 were comparable. However, no increase of the activity could be observed in the cells pretreated with MβCD after NGF stimulation (Fig. 3A). Expression of Rac1 in the lysates was comparable between MβCD treated cells and non-treated control cells. These results demonstrate that activation of Rac1 by NGF was impaired by the treatment with MβCD.

GTP-dependent recruitment of Rac1 to lipid rafts

Rac1 cycles between active and inactive states. Rac1 in an inactivated form localizes in the cytosolic fraction of the cell, whereas activated Rac1 moves to the plasma membrane. These findings prompted us to hypothesize that activated Rac1 utilizes a compartment in which

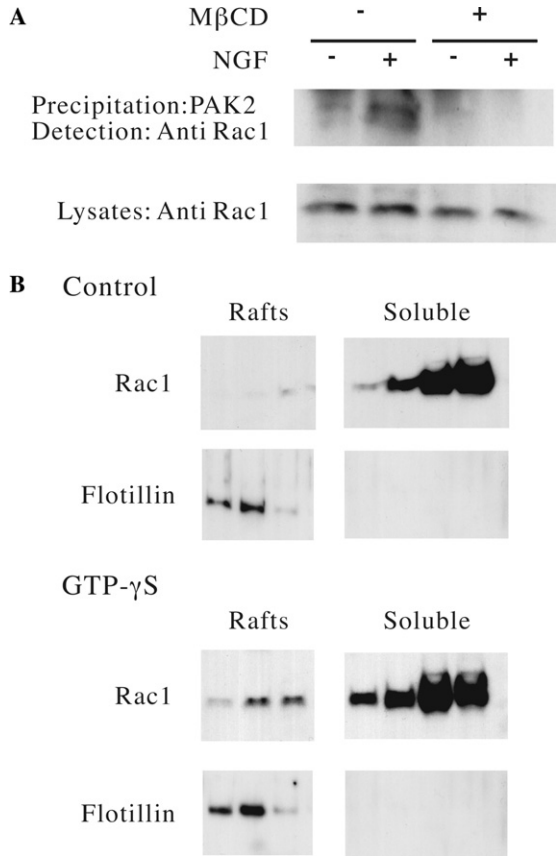

Fig. 3. Association of activated Rac1 with lipid rafts. (A) Pull-down Rac1 activity assays. The activity for Rac1 in PC12 cells was detected by affinity precipitation using GST fusions of its effector. The amounts of Rac1 in the lysates are shown in the lower panel. Rac1 activity was increased after the addition of NGF (100 μg/ml). (B) Rac1 becomes associated with detergent resistant membrane fractions after γGTP stimulation. Transfected 293 cells were treated with or without γGTP (100 μM/ml) for 60 min. The cells were then detergent extracted and the supernatant fraction was subjected to sucrose density gradient centrifugation. Fractions of the gradient were collected from the top to the bottom (#1 and #12). Equal volumes of insoluble raft fractions (#2,3) and soluble high-density fractions (#11,12) were applied to SDS–PAGE. Western blots for Rac1 are shown. In unstimulated cells, Rac1 mainly fractionates to soluble fractions. After γGTP stimulation, a significant portion of Rac1, preferentially in a GTP-bound form, floats to the lipid raft fraction, where Flotillin (raft marker) fractionates.

particular downstream signal molecules assemble to regulate a wide range of signals. We tested whether active form of Rac1 clustered into lipid rafts. HEK293 cells, transfected with Rac1, was used to evaluate the membrane localization. We separated Brij 58-insoluble, low-density membranes (raft-associated proteins) from fully soluble, high-density membranes (non-raft-associated proteins) and cytoskeletal proteins using flotation gradients and analyzed them by SDS–PAGE for Rac1 immunoreactivity (Fig. 3B). Stimulation with GTP-γS (100 μM/ml), unhydrolyzable analog of GTP, resulted in movement of a significant amount of Rac1 to the top of the flotation gradient, confirming its association with low-density lipids, whereas without any treatment, Rac1 was present mainly in the soluble fraction in the cell. These data suggest that Rac1 is targetted to lipid rafts when activated, but can migrate from this domain unless retained.

Discussion

A growing body of evidence indicates that the tips of lamellipodia and filopodia serve an analogous function of localizing and harnessing actin polymerization for cell motility. The assembly of actin-based membrane projections is regulated by Rho GTPases [4]. Two members of this family, Rac1 and Cdc42, play roles in the formation of lamellipodia and filopodia, respectively [20]. The activation of Rac1 and Cdc42 can be mediated by stimulation of both growth factor and integrin receptors, and requires GDP/GTP exchange factors, many of which have been described [21,22]. Rho GTPases are synthesized as cytosolic proteins but can be targeted to membranes by a series of posttranslational modifications [23]. General membrane localization cannot explain the focal induction of lamellipodia or filopodia at the cell periphery, and so we speculated that Rac1 and Cdc42 might be locally activated to induce these protrusions. In this context, our findings demonstrating that neurotrophin-mediated morphological changes and Rac1 activation are dependent on lipid raft integrity, suggesting that the reorganization of the actin cytoskeleton may begin at specific sites of the cell surface, are intriguing. Recently, a fluorescence resonance energy transfer approach to visualizing GTP-bound Rac1 in live cells revealed an accumulation of the activated state of this GTPase in membrane ruffles upon growth factor stimulation [24]. Therefore, membrane domains where motility begins might be lipid rafts themselves or functionally related with them. This hypothesis may have been raised also by the previous reports showing that cholesterol depletion using the inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase caused the inhibition of the dendrite outgrowth in primary cortical neurons [25].

In addition, we show that activation of Rac1 facilitates its translocation to lipid rafts. Polarized distribution of the neurotrophin receptors or the active form of Rho GTPases in lipid rafts may be required for effective signal transduction, which would contribute to morphological changes. Thus, interesting hypothesis that should be assessed in the future is that lipid rafts are the sites where signal transduction of the neurotrophins takes place. With regard to trk receptors for neurotrophins, MβCD treatment of NBL-S neuroblastoma cells, which express trkA, had no effect on Akt or MAP kinase phosphorylation by the addition of NGF [17]. The

authors concluded that trk function was not disturbed by the disruption of lipid rafts. As the neurotrophins binding to p75 receptor were reported to elicit Rac activation as well as Rho inactivation [7,10], neurotrophin-induced Rac1 activation, which was inhibited by MβCD treatment, might be dependent on p75 receptor. Further studies are required to clearly elucidate which receptors are responsible for our observations.

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