Cellular Signalling 27 (2015) 630–637

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Non-prenylatable, cytosolic Rac1 alters neurite outgrowth while retaining the ability to be activated

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ARTICLE INFO

Article history:
Received 13 October 2014
Received in revised form 26 November 2014
Accepted 27 November 2014
Available online 2 December 2014

Keywords:
Geranylgeranylation
Neurite outgrowth
Prenylation
Rho GTPases
Statins
Subcellular localization

ABSTRACT

Rac1 is an important regulator of axon extension, cell migration and actin reorganization. Like all Rho guanine triphosphatases (GTPases), Rac1 is targeted to the membrane by the addition of a geranylgeranyl moiety, an action thought to result in Rac1 guanosine triphosphate (GTP) binding. However, the role that Rac1 localization plays in its activation (GTP loading) and subsequent activation of effectors is not completely clear. To address this, we developed a non-prenylatable emerald green fluorescent protein (EmGFP)-Rac1 fusion protein (EmGFP-Rac1^{C189A}) and assessed how expressing this construct affected neurite outgrowth, Rac1 localization and activation in neuroblastoma cells. Expression of EmGFP-Rac1^{C189A} increased localization to the cytosol and induced cell clustering while increasing neurite initiation. EmGFP-Rac1^{C189A} expression also increased Rac1 activation in the cytosol, compared to cells expressing wild-type Rac1 (EmGFP-Rac1). These results suggest that activation of Rac1 may not require plasma membrane localization, potentially leading to differential activation of cytosolic signaling pathways that alter cell morphology. Understanding the consequences of differential localization and activation of Rho GTPases, including Rac1, could lead to new therapeutic targets for treating neurological disorders.

1. Introduction

The Rho family small guanosine triphosphatase (GTPase), Rac1, is an important regulator of axon and dendrite extension [1,2], actin filament organization [3,4], growth cone morphology [5], and plasticity and synapse formation [6]. Rac1 dysfunction is implicated in several human neurological disorders, including amyotrophic lateral sclerosis [7], ischemic stroke [8] and Alzheimer's disease [9]. For instance, aberrant Rac1 activity promotes degeneration in post-ischemic mice and inhibits recovery of spatial memory [10]. Therefore, it is imperative to understand the mechanisms regulating Rac1 activation to facilitate the development of effective therapies for disorders associated with altered Rac1 signaling.

Rac1 is considered active when bound to guanosine triphosphate (GTP) and able to interact with its effectors, whereas Rac1 is inactive when bound to guanosine diphosphate (GDP; [2]). GTP loading of Rac1 is facilitated by guanine exchange factors (GEFs). Since many Rac1 GEFs (e.g. Dbl, Vav, Tiam-1) need to associate with the plasma membrane for activation, it is widely held that Rac1 translocation to the plasma membrane is required for its activation [11,12]. This is accomplished through post-translational geranylgeranylation via geranylgeranyl transferase I (GGTaseI; [13,14]), which attaches a geranylgeranyl moiety to the cysteine in the C terminal CaaX (where a is an aliphatic amino acid and X is any amino acid) motif in Rac1 (which is CLLL), followed by cleavage of the terminal three amino acids and methylation [15]. The geranylgeranylation of Rac1 is specifically implicated in neuronal plasticity and protection [16], formation of lamellipodia, cell adhesion and motility [17].

Geranylgeranylation is pharmacologically blocked by statin inhibitors of 3-hydroxyl-3-methylglutaryl coenzyme A (HMG CoA) reductase, the rate-limiting enzyme of the mevalonate pathway that produces cholesterol and isoprenoids like geranylgeranyl pyrophosphate and farnesyl pyrophosphate [18]. In our prior work, we found that treatment with lovastatin increases neurite branching and decreases neurite initiation, while affecting both cofilin activity and actin filament content in growth cones [19]. Since lovastatin prevents geranylgeranylation of Rho GTPases, this discovery led us to hypothesize that a cytosolic pool of activated, GTP-bound Rac1 might be causing the observed effects. However, it is difficult to link diverse effects of statin treatment to alteration of Rac1, specifically. Thus, we have constructed a non-prenylatable Rac1 mutant (EmGFP-Rac1^{C189A}) by mutating the cysteine in the CaaX motif to an alanine, which should decrease Rac1 membrane association and signal propagation. Here, we assess how expressing EmGFP-Rac1^{C189A} in neuroblastoma cells affects cell morphology, neurite outgrowth and GTP loading of Rac1.

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http://dx.doi.org/10.1016/j.cellsig.2014.11.033
0898-6568/

2. Materials and methods

2.1. Generation of expression vectors

Mammalian expression vectors containing the open reading frame (ORF) for human Rac1 in frame with an N-terminal Emerald Green Fluorescent Protein (EmGFP) tag were constructed by homologous recombination of the gene supplied in entry vector, pENTR221 (Open Biosystems human, OHS4559-99868817, now GE), with the mammalian expression vector, pcDNA6.2/EmGFP-DEST (Vivid Colors, Invitrogen). Non-prenylatable Rac1 mutants were constructed using site-directed mutagenesis, according to the manufacturer's instructions (Quick Change II, Stratagene) using a mutagenizing primer (Biosynthesis) that mutated cysteine 189 (C189) to alanine (EmGFP-Rac1^{C189A}). After amplification in *Escherichia coli*, proper insertion and orientation of EmGFP-Rac1 and EmGFP-Rac1^{C189A} ORFs were assessed by restriction digestion and sequencing (Genewiz). Plasmids were purified using mini- or midi-prep spin columns (Qiagen) and DNA was incubated for 1 h with appropriate concentrations of Stu1 (Invitrogen) at 37 °C. Undigested and digested plasmids were electrophoresed in a 1.0% Tris-acetate-EDTA (TAE) agarose gel at 100 V for 1 h and incubated with 0.5 μg/ml ethidium bromide for 20 min at room temperature. Gels were visualized using an ultraviolet transilluminator and images were captured using an HD Fluorochem gel imaging system (Alpha Innotech). Stu1 was predicted to cut the plasmid at two locations, one of which was located within the Rac1 ORF, with expected fragments of 1253 bp and 5207 bp.

2.2. Cell culture, treatment and transfection

B35 rat neuroblastoma cells were routinely cultured in DMEM/F12 with 10% fetal bovine serum and passed when 90% confluent. Cells were seeded in 6 well plates at 20,000 cells/cm^2 for western blotting or on 12 mm coverslips at a density of 5000 cells/cm^2 for microscopy. For pharmacological experiments, cells were either maintained in serum-containing medium (SCM) or treated with 10 μM lovastatin (LOV) or a combination of 10 μM LOV and 20 μM geranylgeraniol (GGOH) for 24 h at 37 °C. Cells were fractionated and assessed by western blotting or activation assays as described below. For all other experiments, cells were transfected with EmGFP (without Rac1), EmGFP-Rac1 or EmGFP-Rac1^{C189A} using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Untransfected cells were maintained in serum-free medium (SFM). After 48 h, cells were fixed in 4.0% paraformaldehyde and transfection was observed by direct fluorescence or by immunolabeling with rabbit anti-GFP (1:200, overnight at 4.0 °C, Sigma) and Alexafluor 488 goat anti-rabbit (1:200, 1 h at room temperature, Invitrogen) antibodies to assess transfection efficiency and localization.

2.3. Subcellular fractionation

In some experiments, control and transfected cells were fractionated into membrane and cytosolic fractions. Cells were grown to 98% confluent and transfected as described above. After two days, untransfected and transfected cells were fractionated using the S-PEK proteolysis kit (Pierce) into cytosolic, membrane, nuclear and cytoskeletal fractions.

2.4. Western blotting

Lysates were electrophoresed through 12% SDS-PAGE gels and electrotransferred to nitrocellulose. Membranes were blocked in 5% nonfat dry milk in Tris buffered saline containing 0.1% Tween-20 (TBST). Blots were probed with rabbit anti-Rac1 (1:500, Cell Signaling Technologies) or rabbit anti-GFP (1:1000, Sigma) antibodies overnight at 4 °C. Blots were incubated in appropriately conjugated goat anti-rabbit secondary antibodies overnight at 4 °C and immunoreactive bands were visualized by enhanced chemiluminescence or near infrared detection (Licor Odyssey system).

2.5. Neurite outgrowth

Digital images were captured through 40× and 100× objectives using either phase contrast optics and a Zeiss Axiovert 200M interfaced with the Axiovision image analysis system or differential interference contrast (DIC) imaging using a Nikon Ti confocal system. Neurite outgrowth was measured using the number of neurites/cell, the number of branch points/neurite, the length of longest neurite/cell, the total neurite length/cell and percentage of neurite bearing cells as previously described [19].

2.6. Measurement of Rac1 activation

Cells were grown to 98% confluence and transfected with EmGFP, EmGFP-Rac1 or EmGFP-Rac1^{C189A}. After two days, cells were fractionated as described above and snap frozen and stored in liquid nitrogen. Rac1 activation was assayed using either a Rac1 G-LISA kit (Cytoskeleton), according to the manufacturer's instructions, or pull-down assays [20]. For pull-downs, cell lysates were incubated with agarose beads coated with the PAK protein binding domain overnight at 4.0 °C, washed in lysis buffer and total and activated Rac1 was visualized by western blotting for GFP or Rac1.

2.7. Statistical analysis

For all experiments, the sampling units were individual cultures. The number of sample replicates in each condition is indicated in the results for individual experiments. Data are reported as average values and were analyzed for differences across groups using univariant analyses of variance (ANOVA) with treatment as a fixed factor at a significance level of $\alpha = 0.05$. When there was a significant main effect for treatment, differences from control cultures were determined using the Least Significant Difference (LSD) post hoc test at $\alpha = 0.05$ (SPSS).

3. Results

3.1. EmGFP-Rac1^{C189A} localizes primarily to the cytosol

The effects of statin treatment on neurite outgrowth vary with the particular statin and system employed [14,16,19,21–28]. However, most studies suggest that observed effects on outgrowth are through inhibiting geranylgeranylation leading to inhibition of Rho GTPases [16,21–23]. In prior experiments, we found that inhibiting geranylgeranylation using the statin, lovastatin (LOV), leads to decreased neurite initiation and increased branching in B35 neuroblastoma cells [19]. These effects are likely elicited through altered localization of and signaling from Rac1. To assess the effects of LOV treatment on Rac1 localization, we separated treated cells into membrane/organelle and cytosol fractions and assessed the total amount of Rac1 in each fraction. As expected, treatment with 10 or 25 μM LOV significantly decreased the amount of Rac1 associated with the membrane, as assessed by the ratio of membranous to cytosolic Rac1 (Fig. 1A; $F_{3,10} = 6.940$, $p = 0.017$, $n = 4$ for SCM, 2 for 5 μM LOV, 3 for 10 μM LOV and 2 for 25 μM LOV). Efficient separation of the membrane/organelle and cytosol fractions was demonstrated by immunoblotting for the β2 subunit of the GABA-A receptor and ERK1/2. GABA-$A_{\beta 2}$ segregated only to the membrane fraction, while ERK1/2, a cytosolic cellular signaling protein that can associate with membrane-bound receptors, was found in approximately equal amounts in the cytosol and membrane fractions (Fig. 1B). We next assessed whether cytosol-localized Rac1 is active using commercially available plate-based activation and pull-down assays. Overall, Rac1 activation was greater in the membrane/organelle fraction (Fig. 1C) than in the cytosol fraction (Fig. 1D). As expected, treatment with lovastatin caused a consistent,