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Research paper

Rab20, a novel Rab small GTPase that negatively regulates neurite outgrowth of PC12 cells

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ABSTRACT

The Rab family small GTPases are key players in the membrane traffic that underlies various cellular phenomena. Neurite outgrowth, which is a prerequisite for neuronal network formation, also requires membrane traffic from the cell body to the tips of neurites. Although several Rabs have been shown to promote neurite outgrowth, very little is known about Rab involvement in the negative regulation of neurite outgrowth. Here we used nerve growth factor-stimulated PC12 cells to perform siRNA-based comprehensive knockdown screenings for Rabs that negatively regulate neurite outgrowth and succeeded in identifying Rab20 as a novel negative regulator of neurite outgrowth. Our findings showed that knockdown of endogenous Rab20 in PC12 cells promoted neurite outgrowth, whereas overexpression of active Rab20 inhibited it. We also found that the presence of Gly-64 and Cys-70, both of which are conserved only in the switch II region, a putative effector binding domain, of Rab20 is required for the inhibitory effect of Rab20 on neurite outgrowth. These findings indicated that active Rab20 suppresses neurite outgrowth of PC12 cells, possibly through interaction with an unidentified effector molecule that specifically recognizes certain amino acids in the switch II region of Rab20.

1. Introduction

The central nervous system contains numerous neurons that form complicated neuronal networks within the brain. Proper formation of neuronal networks requires that each neuron extend several neurites to form synapses with other neurons. It is well known that both neurite extension and regression require the rearrangement of cytoskeletal structures. Moreover, it is now widely accepted that membrane traffic, *e.g.*, the surface expansion of neurites and transport of adhesion molecules or receptors to the tips of neurites, is also required for neurite outgrowth, and several membrane traffic regulators have been shown to control neurite outgrowth [1].

Rab family small GTPases, which belong to the Ras superfamily and are thought to be key players in membrane traffic in all eukaryotic cells [2–4], are important regulators of neurite outgrowth [5]. Like other small GTPases, Rab cycles between two guanine nucleotide binding states, a GTP-bound active state and a GDP-bound inactive state. In its active state, each Rab interacts with a specific effector molecule and controls a specific membrane traffic pathway. It has been reported that several Rabs participate in neurite outgrowth of PC12 cells or primary cultured neurons (reviewed in [5]). Most of the reported Rabs, *e.g.*, Rab8, Rab11, Rab13, Rab17, Rab33A, and Rab35, positively regulate neurite outgrowth, *i.e.*, promote neurite outgrowth, when in their active state [6–12], but very little is known about the involvement of "active Rabs" in the negative regulation of neurite outgrowth, *i.e.*, the inhibition of neurite outgrowth. Moreover, no attempt has ever been made to comprehensively analyze Rab proteins during neurite outgrowth.

In this study, we used specific small interfering RNAs (siRNAs) against rat Rabs to comprehensively perform screen for Rabs that negatively regulate neurite outgrowth of nerve growth factor (NGF)-stimulated PC12 cells. The results identified Rab20 as a novel negative regulator of neurite outgrowth: knockdown of endogenous Rab20 and overexpression of active Rab20 in PC12 cells promoted and inhibited, respectively, neurite outgrowth. A possible mechanism of Rab20-mediated neurite outgrowth is discussed on the basis of our findings.

2. Materials and methods

2.1. Antibodies and siRNAs

Anti-EEA1 mouse monoclonal antibody (#610457; BD Biosciences, San Jose, CA), anti-EEA1 rabbit monoclonal antibody (#3288S; Cell

Abbreviations: CA, constitutively active; EE, early endosome; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; HRP, horseradish peroxidase; KD, knockdown; LE, late endosome; mStr, monomeric Strawberry; NGF, nerve growth factor; NS, not significant; PAGE, polyacrylamide gel electrophoresis; siRNA, small interfering RNA; SR, siRNA-resistant

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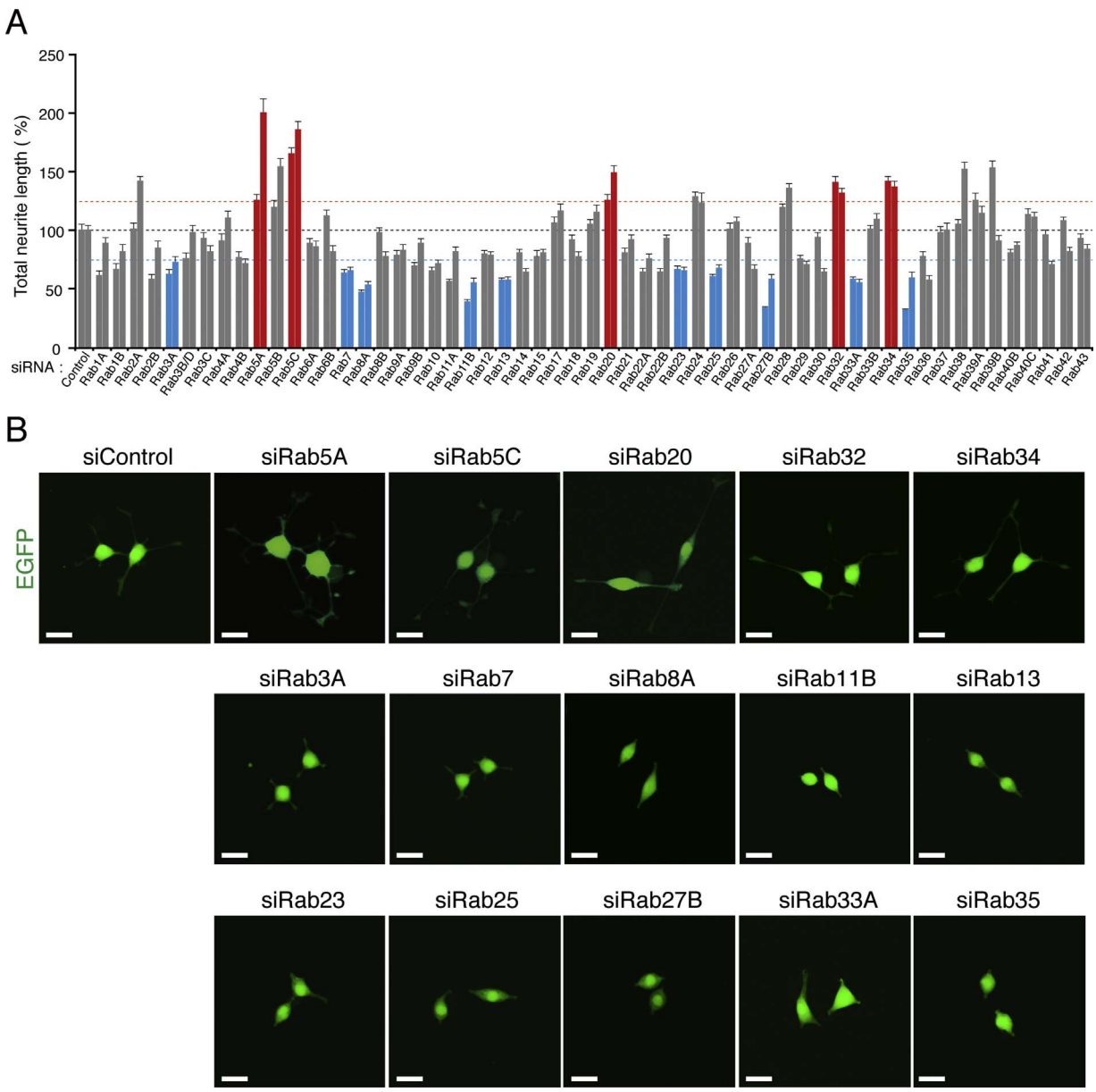

Fig. 1. Screening for Rabs whose knockdown promoted or inhibited neurite outgrowth of PC12 cells. (A) The total neurite length of each Rab-KD cell after NGF stimulation for 36 h was measured with MetaMorph software (n > 50). The total neurite length of each sample was normalized to that of the control cells. Error bars indicate the SEMs of the data from > 50 cells. The red, black, and blue dotted lines indicate 125%, 100%, and 75%, respectively, of the total neurite length of the control cells. The red and blue bars indicate candidate Rabs whose total neurite length was more than 125% and less than 75%, respectively, of the total neurite length of the control cells in two independent experiments. (B) Typical images of PC12 cells expressing EGFP together with either siControl or an siRNA of a candidate Rab. After NGF stimulation for 36 h the cells were fixed and examined with a confocal fluorescence microscope. Scale bars, 40 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Signaling Technology, Danvers, MA), anti-Rab7 rabbit polyclonal antibody (#2094S; Cell Signaling Technology), anti-GM130 mouse monoclonal antibody (#610822, BD Biosciences), anti-NOGOA rabbit polyclonal antibody (#AHP1799; Bio Rad Laboratories, Hercules, CA), horseradish peroxidase (HRP)-conjugated anti-GFP rabbit polyclonal antibody (#598-7; MBL, Nagoya, Japan), and Alexa Fluor 488/594-conjugated secondary antibodies (Thermo Fisher Scientific Corp., Hudson, NH) were obtained commercially. Anti-Rab32 rabbit polyclonal antibody was affinity-purified as described previously [13]. Anti-Rab20 rabbit polyclonal antibody was generated by using purified glutathione *S*-transferase-tagged Rab20, as the antigen, and it was affinity-purified as described previously [14].

Sequence information regarding the siRNAs against mouse/rat Rab1–43 (*e.g.*, siRab20#1; the first siRNA site in Rab20) except Rab6B, Rab24, Rab37, Rab39B, and Rab43, is provided elsewhere [15]. Because of the high sequence similarity between Rab3B and Rab3D, the target sequence of these Rabs was the same (named siRab3B/D in Fig. 1A). The siRNAs specific for rat Rab20 (target site: 5′-ACGTGGACCTCCTCTTTGA-3′ [siRab20#2; the second siRNA site in Rab20]), rat Rab37 (target site: 5′-CCTGTTTCCTGATCCAATT-3′), and rat Rab43 (target site: 5′-CCAGATCCTTCTACCGGAA-3′) were chemically synthesized by Nippon Gene (Toyama, Japan). The Stealth RNAi™ siRNAs against rat Rab6B (#RSS325891), Rab24 (#RSS360029), and Rab39B (#RSS369246) were purchased from Thermo Fisher Scientific Corp .

2.2. cDNA cloning and plasmid construction

Constitutively active (CA) forms of mouse Rabs, *i.e.*, Rab5A(Q79L), Rab5C(Q80L), Rab20(R59L), Rab32(Q83L), and Rab34(Q111L), were prepared and subcloned into the pEGFP-C1 vector (Clontech-Takara Bio