Biochemical and Biophysical Research Communications xxx (2018) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Use of CK-548 and CK-869 as Arp2/3 complex inhibitors directly suppresses microtubule assembly both in vitro and in vivo

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ARTICLE INFO

Article history:
Received 19 January 2018
Accepted 23 January 2018
Available online xxx

Keywords:
Arp2/3 complex
CK-869
CK-548
Microtubule

ABSTRACT

Two types of Arp2/3 complex inhibitors, CK-666/636 and CK-548/869, are commonly used to study Arp2/3 complex-dependent actin assembly both in vitro and in vivo. However, we found that CK-548 and CK-869 directly suppress microtubule (MT) assembly independent of the actin cytoskeleton. Treatment of cultured mammalian cells with 50 μM CK-869 dramatically decreased MT networks and, instead, accumulated tubulin at the cell periphery, as did nocodazole that inhibits MT assembly. An in vitro MT-sedimentation assay revealed that CK-548 and CK-869 significantly suppressed MT polymerization. In budding yeast, although CK-548 and CK-869 are reported to lack binding abilities in the yeast Arp3, CK-548 treatment decreased cytoplasmic MT at several tens of micromolar concentrations. In addition, we found that the effects of CK-548 and CK-869 on MT assembly varied according to species. We propose that CK-548 and CK-869 are not suitable for studying the cytoskeleton in living cells.

1. Introduction

Actin-related protein 2/3 (Arp2/3) complex functions as a key regulator for actin assembly by promoting branched actin nucleation. The Arp2/3 complex is composed of seven highly conserved subunits (Arp2, Arp3, and Arpc1–5) and requires interaction with the preformed actin filament, monomer actin, ATP, and nucleation promoting factors (NPFs) for activation [1–3]. Wiskott–Aldrich Syndrome (WAS) protein family is a well-characterized NPF comprising WAVE/SCAR, WASP/N-WASP, WASH, WHAMM, and JMY, and possesses a conserved C-terminal domain called Verprolin homology or WH2, Connector and Acidic regions (VCA) that binds to both monomeric actin and the Arp2/3 complex to promote the formation of branched actin filaments [2–6]. Arp2/3 complex-dependent actin nucleation contributes to multiple cellular functions such as directional cell migration, exocytosis, endocytosis, and vesicle trafficking [7].

To study Arp2/3 complex-dependent cellular functions, two types of small molecules are generally used in vitro and in vivo: Arp2/3 complex inhibitor I, CK-666 and CK-636, and inhibitor II, CK-548 and CK-869 [8–14]. CK-666/636 binds between Arp2 and Arp3 and blocks conformational changes, leading to activation of the Arp2/3 complex, whereas CK-548/869 binds to the hydrophobic pocket of Arp3 and alters Arp2/3 complex conformation [8,15]. Although both types of inhibitor suppress Arp2/3 complex-dependent actin nucleation most efficiently in vertebrates, CK-548/869 has no effect on invertebrate Arp2/3 complex because of the non-conserved hydrophobic pocket of Arp3 [8,15]. Despite these inhibitors being commonly used for cytoskeletal research, their specificities are scarcely mentioned [16].

In the present study, we show that CK-548 and CK-869 directly suppress microtubule (MT) assembly independent of the Arp2/3 complex. Furthermore, CK-869 exhibits a much stronger effect on mammalian MT than CK-548 and only CK-548 causes disassembly of yeast cytoplasmic MT.

Abbreviations: Arp2/3 complex, Actin-related protein 2/3 complex; MT, microtubule; MTOC-TMA, microtubule organizing center and transient microtubule array; NPFs, nucleation promoting factors; NOC, Nocodazole; Lat B, latrunculin B.

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https://doi.org/10.1016/j.bbrc.2018.01.143
0006-291X/

2. Results

2.1. CK-869 disrupts MT assembly in cultured cells

First, we confirmed the effects of Arp2/3 complex inhibitors, CK-666, CK-548, CK-869, and CK-312 (an inactive form of drug used as control for CK-548/869) as well as nocodazole (NOC, a MT-depolymerizing drug) on MT networks in rat fibroblastic 3Y1 cells. Arp2/3 complex inhibitors suppressed the formation of lamellipodia (Fig. 1), and no significant morphological effects, such as stress fibers, were detected in actin filaments [13,17,18]. On the other hand, while CK-666 and CK-312 had no effect on MT assembly in 3Y1 cells (Fig. 1A–C), CK-869 markedly abolished MT assembly (Fig. 1E). Accumulated tubulin staining was also observed around the cell edge of CK-869-treated as well as NOC-treated cells (Fig. 1E, F, E′, and F′, arrowheads). The same results were obtained in mouse fibroblastic NIH3T3 cells as well as with two different lots of CK-869 (purchased from Sigma and Tocris, respectively; data not shown). CK-548 scarcely affected MT assembly at 50 μM (Fig. 1D) but decreased it to the same level with CK-869 in the presence of 200 μM CK-548 (data not shown). In addition, spindles were formed normally at 6 h after thymidine release in dimethyl sulfoxide (DMSO, control) or CK-666-treated cells (Fig. 1G), whereas some chromosome masses were observed in a single CK-869-treated cell that showed spherical morphology characteristic of the mitotic phase (Fig. 1H–J).

2.2. CK-548 and CK-869 directly inhibit MT polymerization

Cross-linkers that couple Arp2/3 complex-dependent actin polymerization with MT networks have previously been reported, including WASH, WHAMM, and ARPC2 in human, *Drosophila*, and plants [19–23]. Furthermore, WASH and the Arp2/3 complex localize to the centrosome [24,25]. To clarify whether the effect of CK-548 and CK-869 on MT assembly depends on actin networks, we disrupted actin filaments using an actin monomer-sequestering drug, latrunculin B (Lat B). Although MT straightened and bundled with actin disassembly in control cells as previously reported [26], Lat B did not impede MT disassembly and tubulin accumulation at the cell periphery when induced by CK-869 (Fig. 2A).

Next, we performed an in vitro sedimentation assay using purified porcine tubulin in the presence of inhibitors (Fig. 2B). CK-666 and CK-312 did not affect MT polymerization. Surprisingly, however, CK-869 significantly inhibited MT polymerization even at a concentration of 25 μM. CK-548 also inhibited MT polymerization, although its ability to inhibit was weaker than that of CK-869. These results clearly indicate that CK-548 and CK-869 directly inhibit MT polymerization, independent of the Arp2/3 complex.

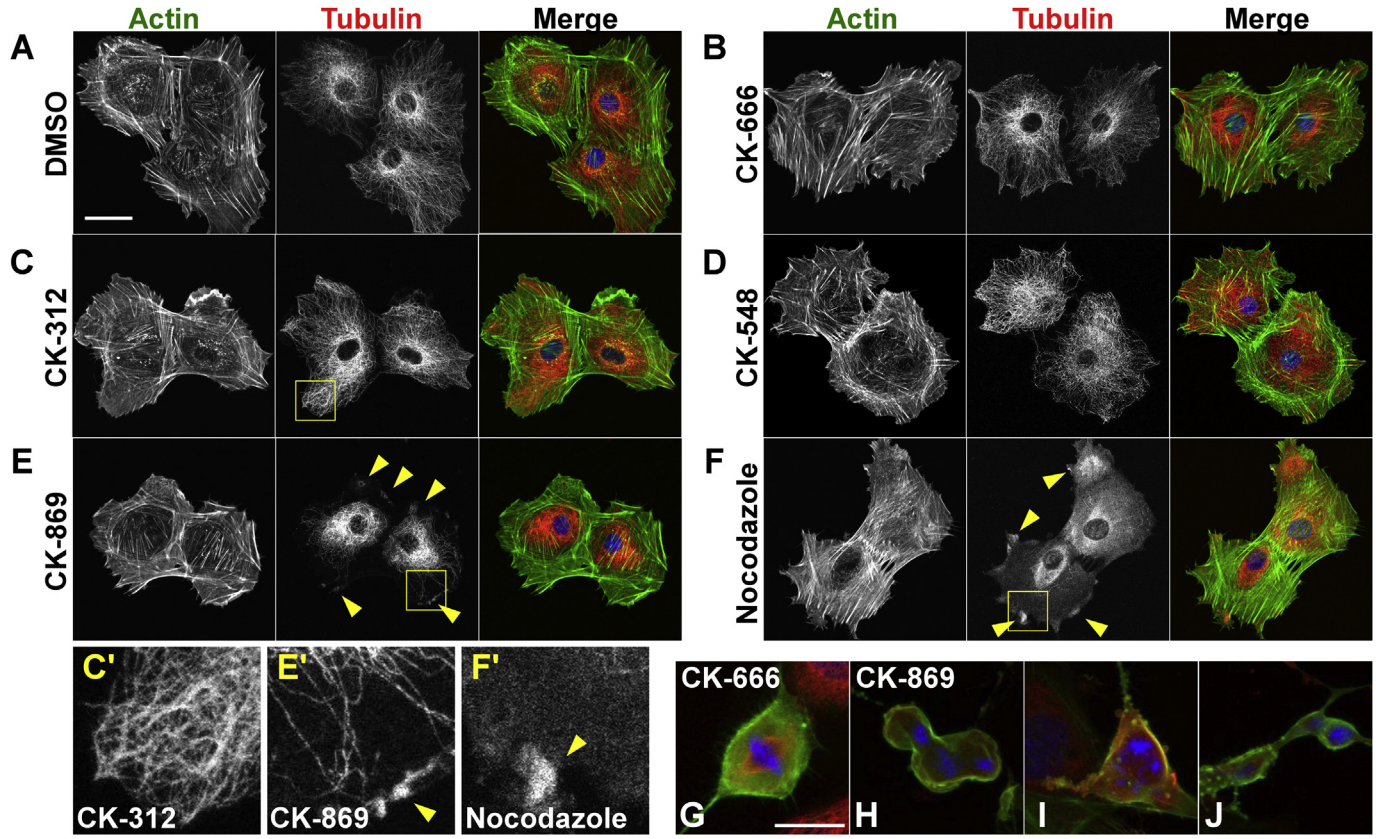

Fig. 1. Effects of Arp2/3 complex inhibitors on 3Y1 cells. (A–F) Cells were incubated with 0.1% DMSO (A), 50 μM CK-666 (B), 50 μM CK-312 (C), 50 μM CK-548 (D), 50 μM CK-869 (E), and 10 nM nocodazole (F) for 1 h. C′, E′, and F′ show and enlarged view from the boxed region in C, E, and F. Arrowheads indicate accumulation of tubulin staining at the cell periphery. (G–J) Treatment of cells with CK-666 (G) or CK-869 (H–J), which were synchronized at S phase using 2 mM thymidine treatment for 18 h, were released from thymidine block and incubated for 6 h. Samples were triple-stained with Alexa 488-phalloidin (actin; green), anti-tubulin antibody (tubulin; red), and DAPI (DNA; blue). Bars represent 30 μm (A) and 10 μm (G). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Please cite this article in press as: Y. Yamagishi, et al., Use of CK-548 and CK-869 as Arp2/3 complex inhibitors directly suppresses microtubule assembly both in vitro and in vivo, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.01.143