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Research article

Expression of a dynamin 2 mutant associated with Charcot-Marie-Tooth disease leads to aberrant actin dynamics and lamellipodia formation

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HIGHLIGHTS

- The expression of dynamin mutants 555Δ3 and K562E decreased lamellipodia formation.
- The K562E mutation caused the disappearance of radially aligned actin bundles.
- The K562E mutation caused the appearance of F-actin clusters.
- Short F-actin assembled into immobile F-actin clusters in K562E-expressing cells.

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ABSTRACT

Specific mutations in dynamin 2 are linked to Charcot-Marie-Tooth disease (CMT), an inherited peripheral neuropathy. However, the effects of these mutations on dynamin function, particularly in relation to the regulation of the actin cytoskeleton remain unclear. Here, selected CMT-associated dynamin mutants were expressed to examine their role in the pathogenesis of CMT in U2OS cells. Ectopic expression of the dynamin CMT mutants 555Δ3 and K562E caused an approximately 50% decrease in serum stimulation-dependent lamellipodia formation; however, only K562E caused aberrations in the actin cytoskeleton. Immunofluorescence analysis showed that the K562E mutation resulted in the disappearance of radially aligned actin bundles and the simultaneous appearance of F-actin clusters. Live-cell imaging analyses showed F-actin polymers of decreased length assembled into immobile clusters in K562E-expressing cells. The K562E dynamin mutant colocalized with the F-actin clusters, whereas its colocalization with clathrin-coated pit marker proteins was decreased. Essentially the same results were obtained using another cell line, HeLa and NG108-15 cells. The present study is the first to show the association of dynamin CMT mutations with aberrant actin dynamics and lamellipodia, which may contribute to defective endocytosis and myelination in Schwann cells in CMT.

1. Introduction

Charcot-Marie-Tooth disease (CMT) is a hereditary motor and sensory neuropathy [1]. Two types of CMT show an autosomal dominant inheritance pattern, namely CMT1 and CMT2. CMT1 is characterized by demyelination and nerve conduction deficits, whereas CMT2 results from axonal abnormalities leading to decreased amplitude of neuronal transmission. There are four kinds of intermediate subtypes, namely, dominant intermediate (DI)-CMTA, DI-CMTB, DI-CMTC and DI-CMTD, which show

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demyelination and aberrant axonal forms. Dynamin 2 is one of the disease-causing genes in DI-CMTB [2].

Dynamin in mammalian cells is classified into three isoforms designated as dynamin 1, 2, and 3 [3]. Dynamin 1 is mainly localized in neurons, whereas dynamin 3 is highly expressed in neurons, lung and testis. Dynamin 2 is ubiquitously expressed. Dynamins have common functional domains including a GTPase domain at the N-terminus, a middle domain, a Pleckstrin homology (PH) domain, a GTPase effector domain (GED), and a proline/arginine rich domain (PRD) located at the C-terminus [4]. Dynamins are involved in the process of membrane fission during endocytosis [4].

Dynamins also regulate dynamics of cytoskeleton including actin [4] and microtubule [5]. Dynamin participates in the formation of actin-rich structures, including lamellipodia and dorsal membrane ruffles, invadopodia, podosomes, growth cones, and phagocytic cups [4]. Dynamins directly and indirectly control actin dynamics. Actin bundling mediated by dynamin and cortactin, an F-actin binding protein, stabilizes growth cone filopodia [6]. Crosslinking of F-actin by dynamin and cortactin is involved in intracellular F-actin organization [7]. Furthermore, dynamin alone directly binds to actin filaments and changes the higher order structure of F-actin [8].

Eight CMT-associated mutations in dynamin 2 have been reported to date [9]. These mutations occur mainly at the PH domain, which is a binding motif for phosphoinositide. Mutations have also been detected in the middle and PRD domains [9]. Analysis of fibroblasts derived from CMT patients or cells expressing CMT mutant dynamin 2 showed that mutations in the PH domain lead to defective endocytosis of surface receptors, including EGF and transferrin receptors [10,11]. In particular, the dynamin 2 K562E mutant severely inhibits endocytosis [11]. The dynamin 2 CMT mutation 555Δ3 results in alterations in the microtubule cytoskeleton [2], which in turn affect microtubule-dependent membrane transport [5]. Considering physiological significance of dynamin in the regulation of actin, dynamin CMT mutants remain to be analyzed focusing on actin dynamics.

In the present study, we analyzed the effects of two selected dynamin 2 CMT mutants, 555Δ3 and K562E, on intracellular actin dynamics by immunofluorescence and live imaging with Total Internal Reflection Fluorescence Microscopy (TIRFM).

2. Materials and methods

2.1. Antibodies

Polyclonal rabbit anti-V5 antibody (AB3792) and mouse monoclonal antibody against α-adaptin (AP-2) (CP46) were purchased from Life Technologies (Carlsbad, CA). Mouse monoclonal anti-clathrin heavy chain antibody (MA1-065) was from Thermo Fisher Scientific (Waltham, MA). Alexa 488- or Rhodamine Red X-conjugated anti-rabbit IgG or anti-mouse IgG, and Alexa Fluor 488- or Rhodamine-phalloidin were purchased from Life Technologies (Carlsbad, CA).

2.2. cDNA constructs

Rat dynamin 2 wild-type (WT), 555Δ3 or K562E were cloned into pcDNA4 V5/His as previously described by Tanabe and Takei [5]. Rat dynamin 2 WT was subcloned into pIRES2-DsRed2 as an EcoRI-SmaI fragment (Clontech Laboratories, Inc., Santa Clara, CA). Mutations were introduced with QuikChange II XL (Agilent Technologies, Santa Clara, CA) in accordance with the manufacturer's instructions.

2.3. Cell culture and transfection

U2OS (ATCC No; HTB96) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS; Life Technologies) at 37 °C in humidified air with a 5% CO_2 atmosphere. For transfection, U2OS cells were cultured in a six-well plate at a density of 0.5×10^5/well and transfected with 0.5 μg of cDNA of dynamin 2 WT, 555Δ3 or K562E cloned into a pIRES2-DsRed2 expression vector (Clontech Laboratories, Inc.) using Lipofectamine 2000, and then cultured for 32 h. The cells were cultured with 0.2% FBS/DMEM for more than 16 h and stimulated with 10% serum for 40 min, and the cells were fixed and stained as described below. To examine colocalization of dynamin with F-actin cluster, AP-2 or clathrin, U2OS cells were transfected with 0.5–1.5 μg pcDNA4 V5/His harboring dynamin 2 WT or mutants. Then, serum-stimulated cells were fixed and stained.

2.4. Fluorescent microscopy

Cells were fixed with 4% paraformaldehyde and stained by immunofluorescence as described previously [6]. Transfected cells were identified by DsRed fluorescence. Samples were examined using a spinning disc confocal microscope system (CSU10, Yokogawa Electric Co., Tokyo, Japan) combined with an inverted microscope (IX-71, Olympus Optical Co., Ltd., Tokyo, Japan) and a CoolSNAP-HQ camera (Roper Industries, Sarasota, FL). The confocal system was controlled by MetaMorph Software (Molecular Devices, Sunnyvale, CA). Images were processed using Adobe Photoshop CS3 or Illustrator CS3 software when it is necessary.

2.5. Live imaging

U2OS cells were co-transfected with 0.5 μg LifeAct-GFP2 (ibidi GmbH, Planegg, Germany) and 0.5 μg pIRES2-DsRed2 harboring dynamin 2 WT or K562E. For live imaging, the transfected cells were cultured and stimulated with 10% FBS as described above. The stimulated cells were captured using TIRFM. Dynamin 2 WT or mutant-expressing cells were identified by DsRed fluorescence. Time-lapsed images were acquired with an inverted microscope (IX-71) and a CoolSNAP-Pro camera. Images were automatically captured every 10 s and processed by MetaMorph software.

2.6. Morphometry

To investigate lamellipodia formation, cells were stained with Alexa 488 Fluor-phalloidin, and analyzed by fluorescence confocal microscopy. F-Actin-rich protrusive membrane sheets at the leading edge were defined as lamellipodia [12]. Cells with lamellipodia were counted. Ratio value of the number of cells with lamellipodia to total number of counted cells was expressed.

F-actin clusters were identified by visual inspection, and the number of F-actin clusters was counted from images of cells stained with Alexa Fluor 488-phalloidin. Average number of F-actin clusters per cell was expressed. Data were analyzed in 33–53 cells from three experiments. All morphometric data were expressed as the mean ± S.E.M.

2.7. Statistical analysis

Data were analyzed for statistical significance using KaleidaGraph software for the Macintosh (version 4.1) (Synergy Software Inc., Essex Junction, VT, USA). Analysis of variance and Tukey's honest significant difference post hoc test were applied for more than