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A mutation associated with centronuclear myopathy enhances the size and stability of dynamin 2 complexes in cells

Nicholas G. James [a], Michelle A. Digman [b], Justin A. Ross [a,1], Barbara Barylko [c], Lei Wang [c], Jinhui Li [d], Yan Chen [d], Joachim D. Mueller [d], Enrico Gratton [b], Joseph P. Albanesi [c], David M. Jameson [a,*]

[a] Department of Cell and Molecular Biology, John A. Burns School of Medicine, University of Hawaii, 651 Ilalo Street, Biosciences 222, Honolulu, HI 96813, USA
[b] Laboratory for Fluorescence Dynamics, Department of Biomedical Engineering, University of California, Irvine, CA 92697, USA
[c] Department of Pharmacology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9041, USA
[d] School of Physics and Astronomy, University of Minnesota, Minneapolis, MN 55455, USA

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ABSTRACT

Background: Dynamin 2 (Dyn2) is a ~100 kDa GTPase that assembles around the necks of nascent endocytic and Golgi vesicles and catalyzes membrane scission. Mutations in Dyn2 that cause centronuclear myopathy (CNM) have been shown to stabilize Dyn2 polymers against GTP-dependent disassembly in vitro. Precisely timed regulation of assembly and disassembly is believed to be critical for Dyn2 function in membrane vesiculation, and the CNM mutations interfere with this regulation by shifting the equilibrium toward the assembled state.
Methods: In this study we use two fluorescence fluctuation spectroscopy (FFS) approaches to show that a CNM mutant form of Dyn2 also has a greater propensity to self-assemble in the cytosol and on the plasma membrane of living cells.
Results: Results obtained using brightness analysis indicate that unassembled wild-type Dyn2 is predominantly tetrameric in the cytosol, although different oligomeric species are observed, depending on the concentration of expressed protein. In contrast, an R369W mutant identified in CNM patients forms higher-order oligomers at concentrations above 1 μM. Investigation of Dyn2-R369W by Total Internal Reflection Fluorescence (TIRF) FFS reveals that this mutant forms larger and more stable clathrin-containing structures on the plasma membrane than wild-type Dyn2.
Conclusions and general significance: These observations may explain defects in membrane trafficking reported in CNM patient cells and in heterologous systems expressing CNM-associated Dyn2 mutants.

1. Introduction

Centronuclear myopathies (CNM) are congenital disorders characterized clinically by muscle weakness and wasting and morphologically by the presence of chains of centralized nuclei in muscle fibers (reviewed in [1,2]). The majority of CNM cases have been linked to mutations in proteins implicated in membrane trafficking pathways [3]. These proteins include myotubularin, associated with an X-linked form of the disease [4], Bin1/amphiphysin 2, associated with an autosomal recessive form [5], and dynamin 2 (Dyn2), the protein investigated in this study and responsible for most known autosomal dominant forms of CNM [6].

Dynamins (Dyns) are ~100 kDa GTPases involved in membrane fission during vesicle formation (recently reviewed in [7–10]). They have been most extensively characterized as mediators of endocytosis, a role first described by van der Bliek and Meyerowitz [11], but they also participate in Golgi budding [12], phagocytosis [13], and organization of the actin cytoskeleton [14]. Three dynamin isoforms, Dyn1, Dyn2, and Dyn3, are expressed in mammals, with each isoform containing multiple splice variants [15]. Dyn1 is expressed primarily in presynaptic nerve terminals, where it functions in synaptic vesicle recycling [16]. Dyn2 is ubiquitously expressed and accounts for most of the dynamin functions that are not specifically related to synaptic vesicle retrieval after exocytosis; and Dyn3 is enriched in the testes, lung and brain, where its precise functions remain to be elucidated. All three dynamin isoforms contain five functional domains: an N-terminal GTPase domain; a middle domain that participates in dynamin self-association; a Pleckstrin

Abbreviations: Dyn, Dynamin; R369W, rat dynamin 2 isoform 2ba construct containing an Arg to Trp mutation at residue 369; Dyn2-EGFP, rat dynamin 2 isoform 2ba with a C- terminal EGFP and terminal hexa-histidine tag; MEF, Mouse embryo fibroblasts; CNM, centronuclear myopathy; FFS, fluorescence fluctuation spectroscopy; TIRF, Total Internal Reflection Fluorescence; PCH, Photon Counting Histogram; PH, Pleckstrin Homology; GED, GTPase effector domain; wt, wild-type; ICS, Image Correlation Spectroscopy; N&B, Number and Brightness; PM, plasma membrane; CME, Clathrin-mediated endocytosis; EMCCD, electron multiplying charge-coupled device; ACF, autocorrelation function; PSF, point spread function

* Corresponding author. Tel.: +1 808 956 8332; fax: +1 808 692 1968, +1 808 692 1970.
E-mail address: djameson@hawaii.edu (D.M. Jameson).
[1] Present address: PerkinElmer, Level 2 Building 5, Brandon Business Park, 530-540 Springvale Road, Glen Waverley, Victoria, Australia 3150.

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Homology (PH) domain involved in phosphoinositide binding; a GTPase effector domain (GED), which interacts with the GTPase domain [17] in a manner that stimulates catalytic activity [18]; and a C-terminal proline/arginine-rich domain (PRD), which mediates most interactions between dynamins and other proteins. Four mutations (E368K, R369W, R369Q, and R465W) affecting three middle domain residues were originally linked to Dyn2-dependent CNM [6]. Additional mutations have been localized to residues in the PH domain (R522C/H, R523G, E560K, D614N, A618D/T, S619L/W, L621P), in the PH domain-GED linker region (V625del and P627H/R), and in the GED (E650K) [19] (Fig. 1). In addition to these mutations, a non-overlapping set of mutations in dynamin 2 have been linked to Charcot-Marie-Tooth neuropathies [19].

Dynamins catalyze membrane fission by assembling around the necks of budding vesicles, thereby constricting and severing these necks in a process that is tightly coupled to GTP hydrolysis. The self-assembly and GTP-dependent disassembly of Dyn polymers have been characterized in vitro, primarily by monitoring changes in turbidity and/or sedimentation. Using these methods, we showed that CNM mutations in the middle domain (E368K, R369W, and 465 W) and PH domain (A618T) enhance Dyn2 polymerization, stabilize Dyn2 polymers against GTP-dependent disassembly and, as a result of this stabilization, increase Dyn2 GTPase activity [20]. Enhanced Dyn1 GTPase activity due to CNM-linked PH domain mutations was also reported by Kenniston and Lemmon [21]. In the present study we use fluorescence fluctuation spectroscopy (FFS) approaches to examine how the R369W mutation affects the behavior of Dyn2 in the cytosol and plasma membrane of living cells. This mutation accounts for ~10% of known cases of autosomal dominant CNM [19], and was recently shown to induce histopathological changes in extraocular muscles [22].

2. Materials and methods

2.1. Materials

NaCl, HEPES, PIPES, $MgCl_2$, NADH, EDTA, GTP (sodium salt) and phenylmethylsulfonyl fluoride (PMSF) were all from Sigma-Aldrich (St. Louis, MO, USA). KCl was from Fluka (St. Louis, MO, USA). Amicon Ultracell-4 10 kDa MWCO filters were from Millipore (Billerica, MA, USA). Male African green monkey cells (CV1), human osteosarcoma (U2OS) cells, Eagle's Minimum Essential Medium (EMEM) and Fetal bovine serum (FBS) were purchased from ATCC (Manassas, VA, USA). Lipofectamine 2000, trypsin and mouse embryo fibroblast (MEF) cells were from Invitrogen (Carlsbad, CA, USA). TransFectin reagent was from Bio-Rad (Hercules, CA). DTT was from Fisher (Pittsburgh, PA, USA). Dulbecco's modified Eagle's medium (DMEM) and penicillin/streptomycin were from Gibco (Carlbad, CA, USA).

2.2. Constructs and protein expression

Wild-type (wt) Dyn2-EGFP and the CNM-associated Dyn2 mutant, R369W-EGFP were generated by inserting the corresponding constructs with C-terminal His_6 tags [20], into the pEGFP-N1 vector (Clontech). This EGFP construct contains F64L and S65T mutations to reduce self-association. Clathrin light chain a (LCa)-mCherry was constructed by introducing the LCa into the pmCherry-N1 vector (Clontech).

2.3. Cell culture

CV1 cells were cultured in EMEM supplemented with 10% FBS. MEFs and U2OS cells were cultured in high glucose DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 20 mM HEPES buffer. For imaging experiments, cells were grown at 37 °C in 5% CO_2 and transfected with wt-Dyn2-EGFP or R369W-EGFP alone, or together with LCb-mCherry, using Lipofectamine 2000 according to the manufacturer's protocol. After 24 h of transfection, cells were trypsinized, and plated alone (CV1) or on 2 μg/ml fibronectin-coated dishes (MEF) 2–3 h prior to imaging. For brightness analysis, U2OS cells were subcultured into eight-well coverglass chamber slides (Nalge Nunc International, Rochester, NY) two days before measurement. Transfection was carried out using TransFectin reagent according to the manufacturer's instructions 24 h before measurements.

2.4. Transmission Electron Microscopy (TEM)

WT-Dyn2 and the R369W mutant containing C-terminal His_6 tags were expressed in Sf9 cells and purified over Ni^{2+}-nitriloacetic acid resin [23]. Purified proteins were dialyzed against 20 mM HEPES (pH 7.5), 300 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF. Aliquots were frozen and stored at −80 °C. Immediately before use, samples were centrifuged at 213,000 ×g for 20 min to remove aggregated protein. For electron microscopy, the cleared Dyn2 and R369W samples were diluted into 20 mM HEPES, pH 7.5 and 50 mM NaCl to a final protein concentration of 1 μM. After 30 s, Dyns were added to Formvar-coated 200 mesh copper grids, incubated for a further 30 s, and stained with 2% uranyl acetate for 60 s. To examine the effect of GTP on the stability of Dyn2 polymers, proteins were incubated with 1 mM MgGTP for 5 min prior to addition to grids. The grids were washed and dried before viewing on an LEO 912 energy filtering TEM (EFTEM) (Ziess, Oberkochen, Germany) at an accelerating voltage of 100 kV. Images were captured with a Proscan slow-scan fast-transfer 1 k × 1 k CCD.

2.5. Confocal images

Images where recorded on an Olympus Fluoview FV1000 confocal laser scanning microscope mounted on an Olympus IX-81 inverted microscope using a 60× 1.35 NA oil objective. Both Dyn2-EGFP and R369W-EGFP were excited at 488 nm with an Ar-Ion laser (Melles Griot, Carlsbad, CA) and fluorescence intensity was collected through a Q500LP dichroic mirror in front of the PMT.

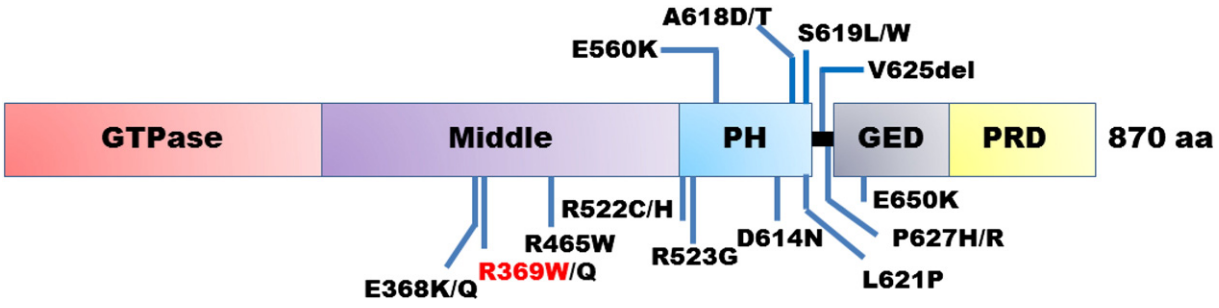

Fig. 1. Domain map of Dyn2 showing sites of CNM mutations; the R369W mutation is highlighted in red. Numbering refers to the dynamin 2 isoform 1, 870 aa, GI:56549121 variant.