Ex vivo BIN1/Amphiphysin 2-induced tubulation of membranes, and visualization of Dynamin 2 recruitment to the membrane tubules

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Method Article

Keywords: amphiphysin, BIN1, dynamin, membrane, tubulation, fluorescence and confocal microscopy, disease-causing mutation, centronuclear myopathy

Posted Date: August 8th, 2007

DOI: https://doi.org/10.1038/nprot.2007.344

Abstract

Introduction

Membrane remodeling is an important process implicated in several cellular functions including compartmentalization and membrane and protein trafficking. Amphiphysin 2 \(BIN1) and dynamin 2 \(DNM2) are membrane tubulating proteins, that are mutated in recessive and dominant centronuclear myopathy, respectively \(refs 1-4). The overexpression of a muscle isoform of BIN1 \(iso 8) in COS-1 cells provokes a striking rearrangement of membranes into long tubules. The large GTPase DNM2 is recruited to these membrane tubules through interaction between its proline-rich domain and the SH3 domain of BIN1. This protocol allows to create and visualise BIN1-induced membrane tubules in cultured cells, and to test the recruitment of DNM2 to the tubules.

Reagents

· COS-1 cells · Dulbecco's modified Eagle's medium \(DMEM) + 1g/L glucose \(Invitrogen) · Fetal Calf Serum \(FCS) \(PAN Biotech) · Gentamicin sulfate \(Kalys) · Fugene-6 transfection reagent \(Roche) · Paraformaldehyde · Phosphate Buffered Saline \(PBS) \(Sigma) · NH_4Cl · Triton X-100 · Anti-Myc 9E10 monoclonal antibody \(IGBMC, Illkirch, France) · Goat anti Mouse Cy3 antibody \(Beckman Coulter France SA) · Anti-fading solution: propylgalate 5% and glycerol 80% in PBS

Equipment

· Tissue culture flasks 75 cm^2 \(Falcon) · Tissue culture 6 wells plate \(Corning Incorporated) · 22 mm^2 cover glasses \(Labonord) · Parafilm \(Pechiney) · Microscope slides 25x75x1.0 mm \(Menzel-Gläser) · Leica SP2-AOBS confocal microscope or similar confocal microscope

Procedure

Transfection 1. Inoculate COS-1 cells in a 75 cm^2 cell culture flask and grow in DMEM + 1g/L glucose + 5% FCS to 100% confluence. Dilute the cells 1:4 in same medium and spread on 22 mm^2 glasses into 6 wells tissue culture plate. Let them adhere and grow 5 hrs at 37°C. 2. Mix in this order 100 microl. DMEM \(without glucose nor FCS) with 3 microl. Fugene-6 transfection reagent and 1 microg. of plasmids DNA encoding the proteins of interest, pEGFP-BIN1 iso 8 and pSG5 Myc-tagged-DNM2 in this study. Incubate 10 min at room temperature, then add to cells in 1 ml DMEM + 1g/L glucose + 5% FCS. Incubate overnight at 37°C. **Immunofluorescence** 3. Wash cells with PBS once, then fix the cells with 4% paraformaldehyde 20 min to overnight at 4°C. 4. Quench with NH_4Cl 50mM 15 min. 5. Wash cells with PBS 5 min before permeabilisation with PBS + 0.2% Triton 10 min. Wash cells with PBS 5 min. 6. Saturate non specific immunoglobulin binding sites with PBS + 0.1% Triton + 10% FCS for 1 hr. Wash cells with PBS 5 min. 7. Dilute primary antibody \(anti-Myc) 1:400 in PBS + 0.1% Triton + 3% FCS.

Transfer the 22 mm^2 glasses carrying cells into a humid chamber \(parafilm surrounded by water drops is enough) to avoid drying of the small volumes during the following incubations. This procedure allows the use of a small amount of antibodies, as a drop covering the cells on the glasses. Incubate cells on 22 mm^2 glasses with primary antibody dilution for 1 hr at room temperature. Wash cells three times with PBS + 0.1% Triton for 5-10 min. 8. Dilute secondary antibody \(Goat anti Mouse Cy3) 1:200 in PBS + 0.1% Triton + 3% FCS, and incubate on cells for 45 min at room temperature. 9. Wash cells three times with PBS + 0.1% Triton for 5-10 min, then once with PBS for 5-10 min. Mount glasses on microscope slides \ (cells facing the slides) with a drop of anti-fading solution, that keeps cells from drying and protect from fluorescence fading. 10. Use a confocal microscope with an Argon 488 excitation to visualize the GFP-BIN1 positive membrane tubules and a HeNe 543 excitation for the red Myc-DNM2 localization.

Timing

Complete procedure takes 5 days Cell Culture: 30 minutes Transfection: 30 minutes Immunolabeling: 5 hours Confocal microscopy: 2-4 hours

Critical Steps

1. Use of other cell types is possible but formation of membrane tubules following overexpression of BIN1 iso 8 should be tested first. This procedure is also working at least for mouse C2C12 muscle cells. 2. When adding the Fugene-6 reagent to the transfection mix, do not touch the plastic surface of the tube. 10. For the best visualization of membrane tubules, the use of confocal imaging is recommended.

Troubleshooting

2. Sequence and length of the N-terminal protein tag and linker added to the BIN1 cDNA sequence may impact the formation of membrane tubules \(refs 1, 2). Try several constructs or use untagged proteins, that need to be detected with specific antibodies in step 7.

Anticipated Results

Following this procedure, we were able to produce membrane tubules ex vivo through overexpression of BIN1 iso 8 cDNA. The formation of these tubules depends on a functional N-BAR domain, and missense mutations of this domain, found in patients with autosomal recessive centronuclear myopathy, abrogate their formation \(ref. 4). The recruitment of dynamin 2 to these tubules could be monitored by confocal microscopy; while overexpressed dynamin 2 is diffuse in the cytoplasm, it is recruited to and colocalizes with BIN1 on the membrane tubules. This recruitment depends on the presence of a functional SH3 domain in BIN1, as either deletion of this domain, or a premature stop codon reproducing a mutation found in a patient with autosomal recessive centronuclear myopathy, do not allow the recruitment of dynamin 2, while membrane tubules are still formed. This procedure may be applied to test the impact of

other mutations in BIN1 and/or dynamin 2, and to test the tubulation and dynamin-recruitment properties of other proteins.

References

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Acknowledgements

We thank Pietro De Camilli for providing the BIN1 iso 8 cDNA.

Figures

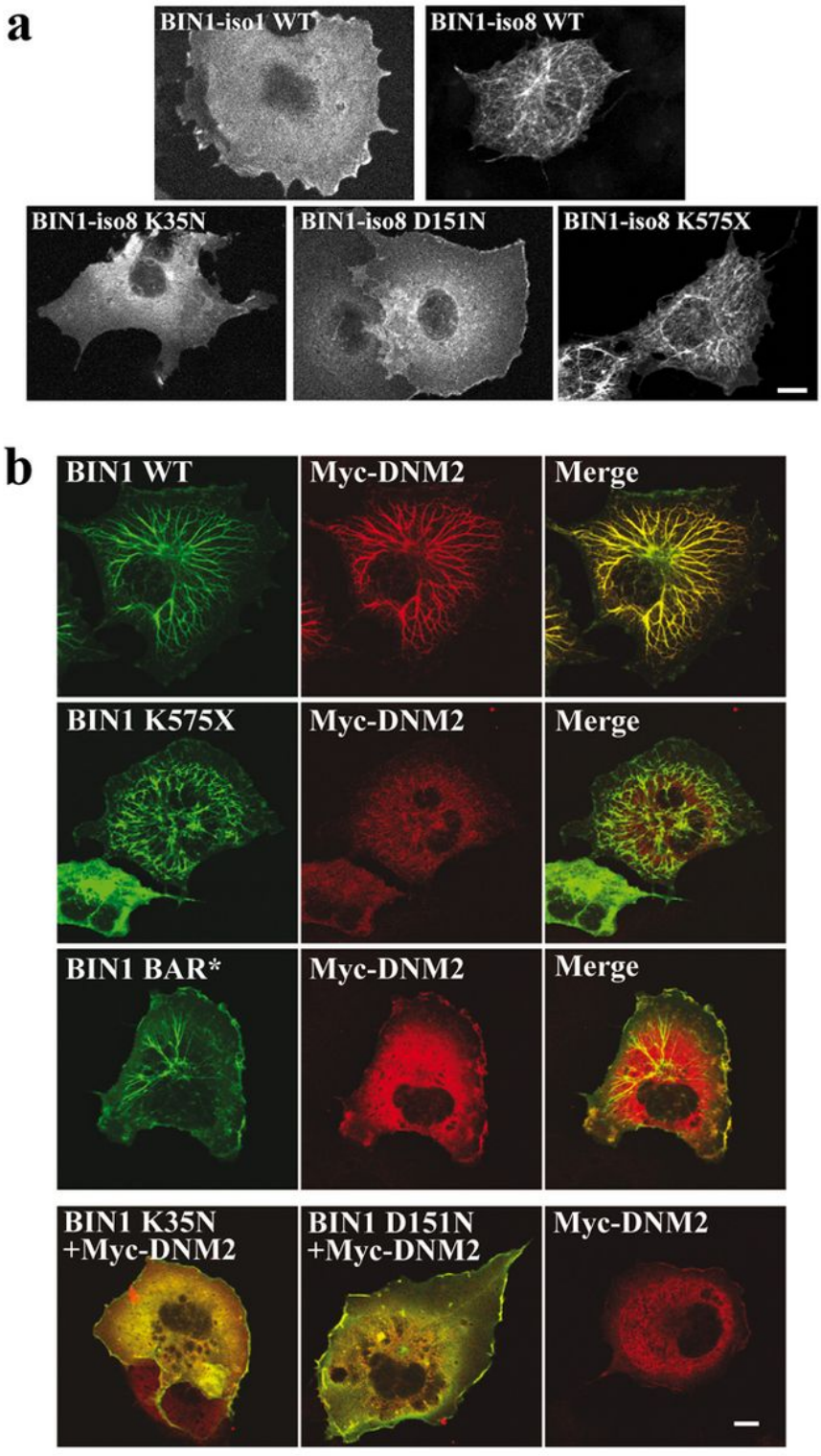

Figure 1

Ex vivo membrane tubulation induced by overexpression of BIN1, and recruitment of dynamin 2 to membrane tubules. (a) _Ex vivo_ membrane tubulation assays. COS-1 cells were transfected with BIN1-iso1 (neuronal isoform), and GFP-tagged BIN1-iso8 (skeletal isoform) wild-type and mutants. Mutants included mutations found in centronuclear myopathy patients in the BAR domain (K35N and D151N) and in the SH3 domain (K575X). Overexpressed BIN1-iso1 was detected with the anti-BIN1 C99D antibody and

BIN1-iso8 constructs were fused to GFP. BIN1-iso8 wild-type and K575X constructs induce membrane tubulation contrary to BIN1-iso1 and BAR domain mutants. (b) Dynamin 2 recruitment to BIN1-iso8 induced membrane tubules. COS-1 cells were transfected with Myc-tagged dynamin 2 and different GFP-tagged BIN1 constructs: wild-type and mutated full length BIN1-iso8 and wild-type BAR* (aa 1-282, lacking the SH3 domain at the C-terminus). Dynamin 2 localization was revealed with an anti-Myc antibody. Dynamin 2 recruitment to the membrane tubules induced by BIN1-iso8 was dependent on a wild-type SH3 domain, as both deletion of the SH3 domain or the last 19 aminoacids (K575X mutant) abrogated the recruitment. The bottom panel shows merge pictures of cells expressing DNM2 together with BAR domain mutants or DNM2 alone. Scale bar = 10 microm. Data first published in ref. 4 and reproduced with permission from Nature Publishing Group.

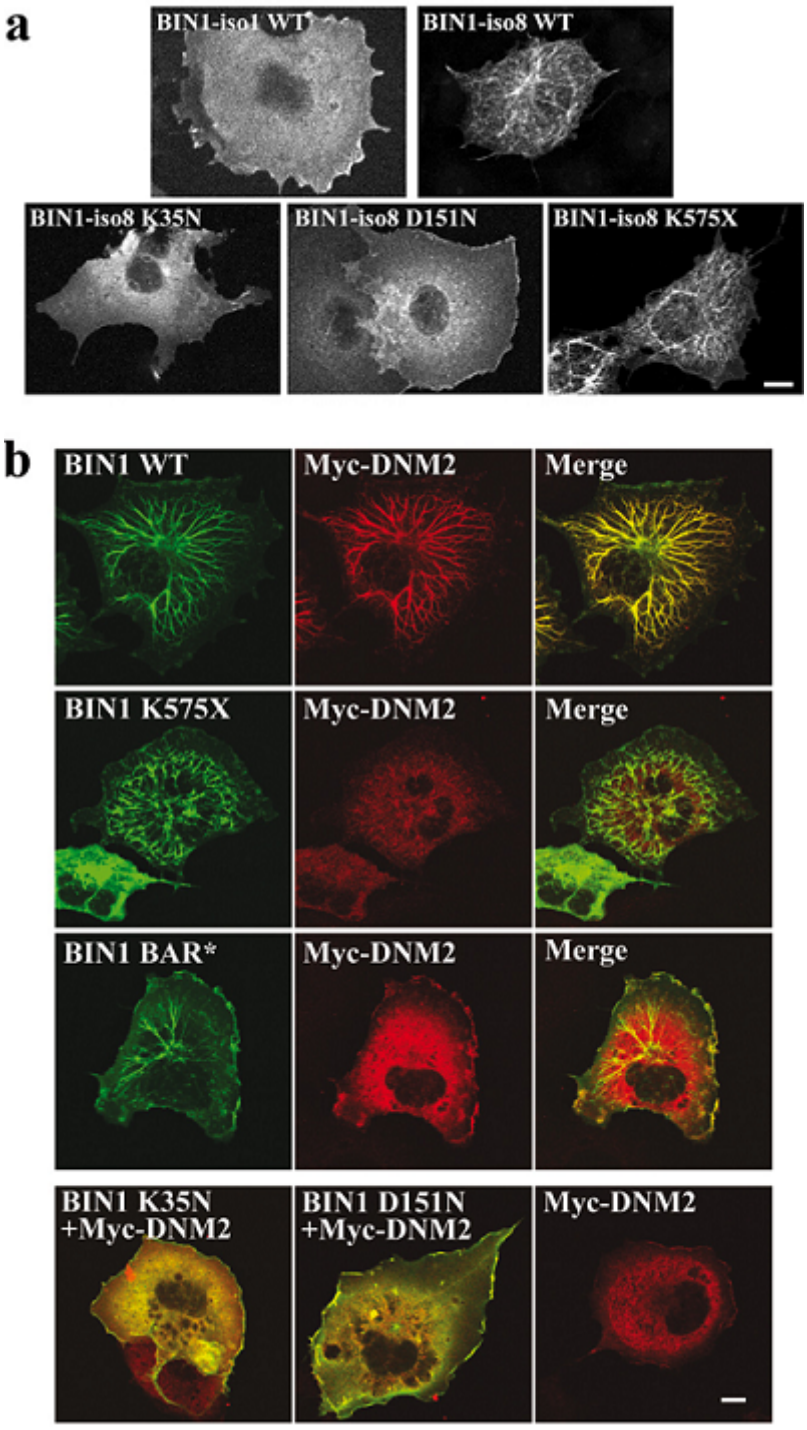
a
BIN1-iso1 WT
BIN1-iso8 WT
BIN1-iso8 K35N
BIN1-iso8 D151N
BIN1-iso8 K575X
b
BIN1 WT
Myc-DNM2
Merge
BIN1 K575X
Myc-DNM2
Merge
BIN1 BAR*
Myc-DNM2
Merge
BIN1 K35N
+Myc-DNM2
BIN1 D151N
+Myc-DNM2
Myc-DNM2

Figure 2