Published in final edited form as:

Methods Mol Biol. 2015 ; 1298: 295–304. doi:10.1007/978-1-4939-2569-8_25.

Differential Effects of Overexpression of Rab5 and Rab22 on Autophagy in PC12 Cells with or without NGF

M. Caleb Marlin and Guangpu Li

Abstract

Macroautophagy selectively recycles damaged or unneeded proteins and organelles by degradation via targeting to the autophagosome. The following method seeks to identify candidate Rab GTPases that likely modulate autophagy in PC12 cells during nerve growth factor (NGF) starvation. This microscopy-based assay is a single cell-based quantification of the presence of autophagosomes by fluorescently labeled markers in response to the overexpression of Rabs and mutants in the presence or absence of NGF.

Keywords

Autophagy; LC3B; Rab; GTPase; GTP-binding protein; NGF; PC12 cells

1 Introduction

Macroautophagy is a bulk degradation/recycling stress response in eukaryotes. This complex system works to degrade intracellular components by targeting damaged, aggregated, or nonessential proteins and organelles to the autophagosome [1]. Though autophagy is continually active at basal levels, autophagy progression above basal level is highly regulated by growth factor stimulation or nutrient loss, particularly in neurons [2]. This work is aimed to provide useful tools for furthering our understanding of key regulators of autophagy.

The prototypical neurotrophin, nerve growth factor (NGF), binds to TrkA, its high affinity receptor, in mammalian neurons and stimulates survival and differentiation [3]. Phosphorylated TrkA dimers are internalized and trafficked from early, to late endosomes, and subsequently degraded at the lysosome. However, other pathways exist which divert TrkA from canonical endocytic trafficking and alter both the temporal and spatial regulation of TrkA. One such example is the signaling endosome, which is a particularly important subset of vesicles to TrkA signaling, neuron survival, and differentiation, though their biogenesis and function is not completely understood. As our understanding of the complex trafficking of TrkA increases, so does our appreciation for the endocytic and recycling machinery in the life of growth factor signaling.

TrkA enlists a plethora of prosurvival signaling cascades leading to a complex network of survival and differentiation signals that are necessary to the life of neurons. PI3K is one such

effector that promotes the activation of AKT and mTORC1 in response to NGF. mTORC1 inhibits autophagy by phosphorylation of the ATG1/ULK1 complex, an important component of the preautophagic complex and a critical step for initiation of autophagy [4]. Therefore, NGF/TrkA signaling leads to a decrease in autophagy and autophagosome production.

The endocytic trafficking of TrkA-positive vesicles is pivotal for the signaling and trafficking of NGF/TrkA in the neuron. Mechanisms governing the endocytosis, sorting, and trafficking of TrkA will have a profound impact on the life and health of the cell. The Rab family of small GTPases facilitate the fusion, sorting, and trafficking of all intracellular membrane compartments; endosomes and autophagosomes are no exceptions [5]. In particular, Rab5 and its subfamily member, Rab22, regulate the formation of NGF/TrkA-containing early and signaling endosomes [6]. Since endocytic trafficking is a major component of the TrkA signaling cascade, modulation of Rab5 and Rab22 will alter the physiologic outcomes of TrkA signaling, including prevention of autophagy. The current method aims to study the effects candidate Rabs may have upon the antiautophagic signaling of NGF in PC12 cells.

A reporter cassette based on the dsRed:LC3B:eGFP reporter [7] was modified by the replacement of dsRed with the more bright tandem dual tomato (tdTom) and inserted into pBI-Tet that contains a bidirectional promoter. The pBI-Tet vector allows for coexpression of both the new tdTom:LC3B:eGFP reporter and a candidate Rab (Fig. 1a). The tdTom:LC3B:eGFP reporter is expected to be processed by ATG4 at a glycine near the C-terminus in LC3B to cleave tdTom:LC3B from GFP to produce two separate cytosolic fractions under normal conditions. During autophagy LC3B-I is lipidated, forming LC3B-II, and causes a gel mobility shift downward during SDS-PAGE (Fig. 1b).

Transfection of PC12 cells with our modified constructs results in the overexpression of tdTom:LC3B:eGFP and the Rabs. Western blotting for LC3B, GFP, Myc-Rab5, Rab22, and actin confirms their expression in the cell (Fig. 2). Meanwhile, transfection of these cells under serum starvation allows for visualization of tdTom-LC3B-II-labeled autophagosome production via fluorescence microscopy (Fig. 3a).

Autophagy with this reporter is calculated by the percent cells that demonstrate a loss of GFP fluorescence and the production of ten or more tdTom-positive punctate autophagosomes, compared to total transfected cells. By using multiple conditions (e.g., serum free media with or without NGF) and overexpression of wild-type (WT) or a dominant-negative (SN) Rab mutant, one can quantify the effects of Rab overexpression on autophagy progression (Fig. 3b).

2 Materials

2.1 Cell Culture/Transfection

1. Tet-Off PC12 cells and BHK-21 cells.
2. Complete-DMEM: Dulbecco's Modified Eagle's Medium, 10 % horse serum, 5 % FBS, glutamine, and penicillin/streptomycin.

3. Lipofectamine-2000 (Invitrogen).
4. Poly-L-Lysine.
5. NGF.
6. 6-well and 24-well Plates.

2.2 Expression Plasmids

1. pBI-Tet/tdTOM:LC3:eGFP.
2. pBI-Tet/tdTOM:LC3:eGFP/Rab5(WT).
3. pBI-Tet/tdTOM:LC3:eGFP/Rab5(S34N).
4. pBI-Tet/tdTOM:LC3:eGFP/Rab22(WT).
5. pBI-Tet/tdTOM:LC3:eGFP/Rab22(S19N).

2.3 Antibodies

1. Rabbit anti-LC3B antibody.
2. Rabbit anti-GFP antibody.
3. Mouse anti-Myc monoclonal antibody.
4. Rabbit anti-Rab22 antibody.
5. Mouse anti-actin monoclonal antibody.
6. IRDye 800CW Goat anti-Mouse, LI-COR (#926-32210).
7. IRDye 680RD Goat anti-Rabbit, LI-COR (#926-68071).

2.4 Staining Material

1. Mount: ProLong Gold Antifade Reagent with DAPI (Invitrogen).
2. Glass slides: VWR Micro Slides, 25 × 75 mm 1.0 mm thick.
3. Coverslips: 12 Circular –1 (Fisher Scientific).
4. 16 % PFA: paraformaldehyde, 16 % Solution (Electron Microscope Sciences).
5. Fluorescence Microscope: Olympus, IX51 Inverted Microscope.

3 Methods

3.1 Plasmid Construction

1. MluI/NheI–LC3:eGFP and MluI/MluI–tdTOM cDNA fragments were inserted sequentially into Site I of the pBI vector and in the same reading frame.
2. NotI/SalI–Rab cDNAs were each inserted into Site II of a separate pBI vector.
3. A partial digestion product of tdTOM:LC3B:eGFP with MluI/NheI was transferred to the Site I of each pBI/Rab construct (*see* Note 1).

4. The resulting constructs have the tdTOM:LC3B:eGFP cassette in Site I and each candidate Rab cDNA in Site II of the pBI vector.

3.2 Cell Culture and Transfection

1. Maintain PC12 cell cultures in a 37 °C incubator with 10 % CO₂.
2. Place the desired number of coverslips in a 24-well dish. Add 500 µl of poly-L-lysine to coat the coverslips for >1 h at 37 °C (*see Note 2*).
3. Aspirate the poly-L-lysine solution and wash with sterile H₂O. Let dry for approximately 15 min (*see Note 3*).
4. Plate cells at approximately 70–80 % confluence in 400 µl of complete DMEM. Allow cells to adhere to the coverslips overnight.
5. In separate tubes, add 0.8 µg of DNA and 2 µl of Lipofectamine-2000 to 50 µl of room temperature serum-free DMEM, respectively, for every coverslip and incubate for 5 min (*see Note 4*).
6. Add the full 50 µl of lipofectamine-2000/DMEM mix drop-wise into the 50 µl of DNA/DMEM solution. Let stand for 20 min (*see Note 5*).
7. Add the full 100 µl DNA/Lipofectamine/DMEM solution dropwise into the 24-well dish with cells on coverslips. Gently swirl the media. Incubate for 24 h.
8. Check for expression under a fluorescent microscope. Replace the media with complete-DMEM for 24 h for recovery (*see Note 6*).
9. Aspirate the complete-DMEM recovery media and add serum-free DMEM supplemented with or without 100 ng/ml NGF for 24 h.

3.3 Fixing/Mounting Cells

1. In the 24-well plate, aspirate the media and wash coverslips with 1 ml of 37 °C PBS.

¹*Partial digest*: The tdTomato sequence is flanked by MluI sites while the LC3B:eGFP construct is flanked by MluI and NheI in the pBI vector. There is a SalI site within the linker region between LC3B and eGFP, therefore the NotI/SalI Rab fragment must be cloned into Site II first. In order to digest and purify the tdTOM:LC3B:eGFP cassette, it is partially digested by MluI and NheI. A short digest of 5–15 min at 37 °C should yield bands that include the fully digested tdTOM and LC3B:eGFP as well as the partially digested large full-length band consisting of tdTOM:LC3B:eGFP (~2 kb). Excise the full-length band and purify it.

²*Reusing poly-L-lysine*: The Poly-L-Lysine solution used in coating the coverslips may be reused as long as care is taken to insure sterility. Maintain a working stock at 4 °C in the dark.

³*Keeping coverslips down*: The coverslips often float in the Poly-L-Lysine solution during the coating process. It has been an advantage to add the solution with the plate at a 45° angle to the bench, and slowly dispense the liquid at the bottommost point, where the coverslip and well wall meet. Once all wells have liquid added in them, then you can lay the plate flat and the solution will cover the coverslip and the coverslip should remain in the liquid.

⁴*Mixing lipofectamine-2000*: It is advantageous to calculate and prepare a master mix of the lipofectamine/DMEM mix to add to all of the DNA/DMEM solutions. For example, if you are making ten coverslips, add the DNA to DMEM into ten separate tubes. Multiply both the lipofectamine and the DMEM by ten and make a large mix. From this master mix, after the 5 min incubation, you can add 50 µl of the Lipofectamine-2000/DMEM mix to each DNA/DMEM mix in a dropwise manner.

⁵*Dropwise addition of DMEM-lipofectamine*: Per the manufacture's suggestion, add the lipofectamine/DMEM mix in a dropwise manner into the DNA/DMEM mixture (not vice versa). This facilitates the formation of the liposome/micelle complexes containing the DNA.

⁶*Recovering the cells*: Lipofectamine-2000 has cytotoxic effect on PC12 cells and increases basal autophagy in our system based upon preliminary experiments. Recovering the cells for 24 h in complete DMEM decreases the effect of Lipofectamine-2000 toxicity.

2. Fix cells on coverslips in fresh 4 % PFA at 37 °C for 15 min (400 µl per coverslip).
3. Aspirate and wash in room temperature with PBS three times (*see* Note 7).
4. Remove coverslips and lay *cell-up* on a kimwipe, dabbing excess liquid. Place 15 µl of Prolong-gold with DAPI onto the cells and place the coverslip cell-down onto the slide (*see* Note 8).
5. Let set overnight at room temperature in the dark. Image the next day.

3.4 Autophagy Assay

1. Transfect cells on coverslips in triplicate in a 24-well dish as outlined earlier (*see* Note 9).
2. Upon fixing and mounting, label slides in order to image cells in a blind manner (*see* Note 10).
3. Using a fluorescent microscope, count greater than 100 cells per condition (*see* Note 11).
4. The percent of autophagic cells per total fluorescent cells observed will be reported (*see* Note 12).

3.5 Western Blot for Protein Expression

1. Transfect PC12 cells at 80 % confluence in 6-well dishes in a similar manner found in Subheading 3.2 (*see* Note 13).
2. Lyse and collect 2 wells of each condition in 150 µl SDS-PAGE loading buffer.
3. Run samples on a 12 % acrylamide gel.
4. Stain for LC3B, GFP, Rab22, Myc, and Actin with specific antibodies.

⁷ *Washing off PFA*: PFA can quench fluorescence if left on the sample for too long. Our suggestion is three washes in PBS, which should adequately remove PFA. Also, samples can be left at 4 °C in PBS at this point and is a good stopping point if needed.

⁸ *Placing coverslips on slides*: Add the Prolong-Gold along one end of the coverslip. Lower a slide to meet the solution of the coverslip and allow the Prolong-Gold mounting solution to cover the entirety of the coverslip as the slide lowers. Using the forceps, push out any bubbles in between the coverslip and slide and remove excess mounting solution around the edge of the coverslip by vacuum.

⁹ *Triplicate experiments*: Triplicate samples increase the ability to get meaningful statistics. All three of the coverslips for one condition can fit onto the same slide. As in Note 8, lay out all three coverslips, add prolong-gold, and mount all three onto one slide.

¹⁰ *Labeling cells*: Making a random name for each sample is important for counting cells in an unbiased way. By using a lab mate, make a key that designates each sample with an unspecified name in a random order and relabel each slide accordingly. For example, the slide containing the controls is renamed *ID* and the slide containing Rab5:WT overexpression samples is labeled *3F*. After counting, the lab mate can disclose the key.

¹¹ *Counting cells*: Autophagic cells will exhibit a decrease in cytosolic GFP fluorescence and present ten or more red punctate, or autophagosomes labeled by tdTomato-LC3B-II. While counting, you will switch between the green and red channels in order to visualize the extent of autophagy. Autophagic cells must meet both conditions, loss of green and red punctate. If only one condition is met, it is not considered autophagic. Also, occasionally green fluorescence is observed within the red, LC3B positive, punctate autophagosomes. This is acceptable, as long as overall cytosolic green fluorescence is greatly reduced.

¹² *Graphing the autophagy assay*: For triplicate experiments, the average of each condition and the standard error of the mean are reported. For Fig. 3 graph, we used Prism 7 and included the average, standard error of the mean, and the number of samples per condition. With the data, Student's *t*-test calculated the statistical difference of each condition compared to control.

¹³ *Transfecting BHK*: PC12 cells have a low transfection efficiency, therefore it is advantageous to use a more easily transfected cell line to validate overexpression. To that end, we transected BHK cells in a similar manner to that performed in PC12 cells, with only minor modification. Only 1 well of a 6-well plate was used and only half the amount of lipofectamine-2000 was needed (1 µl instead of 2 µl). Also, because this cell line is not tetracycline regulatable, we cotransfected with the regulatory pTet-Off vector (0.4 ng of DNA for pTet-Off and 0.4 ng of DNA for the pBI constructs).

Acknowledgments

The authors' research program is supported by the NIH/NIGMS grant R01 GM074692 (to G.L.).

References

1. Mizushima N. Autophagy: process and function. *Genes Dev.* 2007; 21:2861–2873. [PubMed: 18006683]
2. Lee J-A. Neuronal autophagy: a housekeeper or a fighter in neuronal cell survival? *Exp Neurobiol.* 2012; 21:1–8. [PubMed: 22438673]
3. Huang EJ, Reichardt LF. Trk receptors: roles in neuronal signal transduction. *Annu Rev Biochem.* 2003; 72:609–642. [PubMed: 12676795]
4. Hosokawa N, Hara T, Kaizuka T, Kishi C, Takamura A, Miura Y, Iemura S, Natsume T, Takehana K, Yamada N, Guan JL, Oshiro N, Mizushima N. Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. *Mol Biol Cell.* 2009; 20:1981–1991. [PubMed: 19211835]
5. Chua C, Gan B, Tang B. Involvement of members of the Rab family and related small GTPases in autophagosome formation and maturation. *Cell Mol Life Sci.* 2011; 68:3349–3358. [PubMed: 21687989]
6. Wang L, Liang Z, Li G. Rab22 controls NGF signaling and neurite outgrowth in PC12 cells. *Mol Biol Cell.* 2011; 22:3853–3860. [PubMed: 21849477]
7. Sheen JH, Zoncu R, Kim D, Sabatini DM. Defective regulation of autophagy upon leucine deprivation reveals a targetable liability of human melanoma cells. *Cancer Cell.* 2011; 19:613–628. [PubMed: 21575862]

Fig. 1. pBI/LC3B/Rab constructs and protein expression. **(a)** Schematic illustration of the plasmid constructs. The tdTom:LC3B:eGFP cassette was inserted into the MCS I in the pBI-Tet plasmid. Each of the Rab cDNAs was cloned into the MCS II allowing for coexpression of the tdTom:LC3B:eGFP fusion protein and the Rab GTPase. **(b)** Western blot analysis of protein expression. The full-length tdTom:LC3B:eGFP is the pro-LC3B analog. Once cleaved by ATG4 at a Gly residue close to the C-terminus it becomes LC3B-I. Upon addition of Phosphatidylethanolamine (PE) by the ubiquitin-like ATG3-ATG14-ATG8 cascade, LC3B-I is converted to LC3B-II, which exhibits faster mobility during SDS-PAGE and is seen as a distinct lower band. eGFP is detected as the cleavage product and as a part of the pro-LC3B band

Fig. 2.

Overexpression in BHK and PC12 cells. All tdTOM:LC3B:eGFP constructs, as indicated, were transfected into BHK cells (**a**) and PC12 cells (**b**) for 24 h, and the cells were recovered in full growth media for an additional 24 h (PC12 cells only) before harvesting for SDS-PAGE and Western Blot. Samples were probed with antibodies for LC3B, eGFP, Myc, Rab22, and Actin as indicated. Note that the expression level of Rab22(SN) mutant is significantly lower than that of Rab22(WT)

Fig. 3.

Fluorescence microscopy and the autophagy assay in PC12 cells overexpressing Rab5 and Rab22 with or without NGF. PC12 cells on coverslips were transfected for 24 h in 24-well plates and recovered in complete DMEM for an additional 24 h. Cells were then treated with serum-free DMEM supplemented with or without NGF for 24 h, followed by fixation and visualization by fluorescence microscopy (a). The number of autophagic cells was counted and expressed as the percentage of total transfected cells. Autophagic cells are defined as cells containing greater than ten red fluorescent punctate autophagosomes along with the loss of green fluorescence. Greater than 100 cells were counted per condition (b)