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IGF-I stimulates Rab7-RILP interaction during neuronal autophagy

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ABSTRACT

Restoration of autophagy represents a potential therapeutic target for neurodegenerative disorders, but factors that regulate autophagic flux are largely unknown. When deprived of trophic factors, cultured Purkinje neurons die by an autophagy associated cell death mechanism. The accumulation of autophagic vesicles and cell death of Purkinje neurons is inhibited by insulin-like growth factor, by a mechanism that enhances autophagic vesicle turnover. In this report, we identify Rab7 as an IGF-I regulated target during neuronal autophagy. Purkinje neurons transfected with EGFP-Rab7-WT and constitutively active EGFP-Rab7-Q67L contained few RFP-LC3 positive autophagosomes and little co-localization with GFP-Rab7 under control conditions. Upon induction of autophagy, RFP-LC3 positive autophagosomes increased and co-localized with GFP-Rab7. Conversely, expression of the dominant negative mutant EGFP-Rab7-T22N increased the accumulation of autophagosomes under control conditions, which accumulated even further during trophic factor withdrawal. There was no vesicular co-localization between Rab7-T22N and RFP-LC3 under control or trophic factor withdrawal conditions. During prolonged trophic factor withdrawal, a condition that leads to the accumulation of autophagic vesicles and cell death, Rab7 activity decreased significantly. IGF-I, added at the time of trophic factor withdrawal, prevented the deactivation of Rab7 and increased the interaction of Rab7 with its interacting protein (RILP), restoring autophagic flux. These results provide a novel mechanism by which IGF-I regulates autophagic flux during neuronal stress.

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Macroautophagy, herein referred to as autophagy, is a cellular housekeeping process that degrades the components of the cell through the lysosomal machinery [19,20]. Autophagy plays a vital role in cellular development, homeostasis, and survival under nutrient deprived or stressful conditions. Conversely, autophagy may contribute to cell death, particularly in the nervous system where conditions such as metabolic stress, aggregated mutant proteins and normal aging impair autophagy signaling leading to decreased autophagic vesicle turnover and subsequent autophagy-associated cell death [8,24]. This imbalance of autophagy signaling referred to as "autophagic stress" is thought to contribute to Parkinson's [1], Huntington's [18] and Alzheimer's disease [7], as well as, to stroke and other neuropathies [4,14]. Thus, there is a growing interest in identifying signaling proteins that control neuronal autophagy.

Rab7 is a small GTPase important in membrane trafficking through the endocytic pathway [22] particularly the fusion of late endosomes with lysosomes [5,21]. Recent studies have indicated a role of Rab7 in the late maturation of autophagosomes [11,13].

Studies from our laboratory demonstrate that the rate of autophagosome to lysosome fusion in cultured Purkinje neurons is regulated by insulin-like growth factor-I (IGF-I), an important neurotrophic factor for these neurons [2,3]. The ability of IGF-I to increase autophagic flux under conditions of trophic factor withdrawal (TFW) prevents autophagic vesicle accumulation and autophagy-associated cell death. To identify a potential mechanism by which IGF-I increases vesicle fusion and turnover, we examined whether IGF-I regulates Rab7 activity during neuronal autophagy.

GFP-Rab7, GFP-Rab7-Q67L and GFP-Rab7-T22N were provided by Bo van Deurs (University of Copenhagen). GST-RILP plasmids were provided by Aimee Edinger (University of California-Irvine). The adenoviral RFP-LC3 was from Aviva Tolkovsky (University of Cambridge, Cambridge, England). The polyclonal rabbit antibodies to LC3, GST, GFP, RILP, and Lamp1 were obtained from Abgent (San Diego, CA), Cell Signaling Technology (Beverly, MA), Clontech Laboratories (Mountainview, CA), Abcam Inc. (Cambridge, MA) and Sigma–Aldrich (Saint Louis, MO), respectively. Monoclonal Anti-Rab7 and goat polyclonal RILP were obtained from Sigma–Aldrich

Abbreviations: IGF-I, insulin-like growth factor I; RFP-LC3, MAP1-LC3 labeled with red fluorescent protein.

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(Saint Louis, MO) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

Animal procedures were approved by the Institutional Animal Care and Use Committee of The University of Colorado Denver and were conducted in accordance with policies for the ethical treatment of animals established by the National Institutes of Health. Primary rat cerebellar granule neurons were isolated from 7-day old Sprague–Dawley rat pups as described previously [26]. Cells were plated on laminin (Invitrogen; Carlsbad, CA) coated coverslips (Thermo Fisher Scientific Inc., Rock-ford, IL), or poly-L-lysine coated culture dishes (Becton Dickinson, Franklin Lakes, NJ) in basal modified Eagle's (BME) medium containing 10% fetal bovine serum, 25 mM KCl, 2 mM L-glutamine, and penicillin (100 units/ml)–streptomycin (100 μg/ml) (Invitrogen; Carlsbad, CA). Cytosine arabinoside (10 μM) was added to the culture medium 24 h after plating to limit the growth of non-neuronal cells. On day 2, neurons were infected with adenoviral vector expressing MAP1-LC3 labeled with red fluorescent protein (RFP-LC3) at a multiplicity of infection of 100 for 24 h. Autophagy was induced by removing the plating medium and replacing it with TFW medium (serum-free BME containing 5 mM KCl).

Rat cerebellar granule neurons were transfected using the Rat Neuron Nucleofector kit from Lonza Cologne AG (Cologne, Germany) at the time of plating using their optimized protocol. A nucleofection reaction containing 4×10^6 cells was plated onto three laminin-coated coverslips or a 35-mm culture dish and a nucleofection reaction containing 10×10^6 cells was plated onto a 60-mm culture dish. Immunofluorescence staining demonstrated that 60–80% of cells were transfected with Rab7 and the transfection efficiency was largely comparable for the different constructs (data not shown).

Immunofluorescent staining was performed as previously described [2]. Cells were incubated overnight at 4 °C with LC3 (1:50), RILP (1:250), dynein (1:250) or Rab7 (1:250) diluted in PBS containing 0.2% TX-100 and 2% BSA. Cells were then incubated with the appropriate Cy3 (1:500)- or FITC-conjugated (1:500) secondary antibodies and DAPI (1 μg/ml). Fluorescence imaging was performed on a Zeiss Axioplan 2 microscope equipped with a Cooke sensicam deep-cooled CCD camera and images were analyzed and subjected to digital deconvolution using Slidebook (Intelligent Imaging Innovations Inc.; Denver, CO).

After the appropriate treatment times, cells were stained at 37 °C with Hoechst (20 ng/ml) for 15 min to visualize cellular nuclei and live-cell imaging was performed as previously described [2]. Images of GFP-Rab7, RFP-LC3 and Hoechst fluorescence were captured on the FITC, Cy3 and DAPI channels, respectively, using a 63× water immersion objective.

Basic Western blotting methods were followed as previously described [2]. Primary antibodies were diluted as follows: LC3: 1:500, RILP: 1:1000 and Rab7: 1:1000 in blocking solution (phosphate-buffered saline containing 0.1% Tween 20 and 5% BSA) and incubated with the membranes overnight at 4 °C. Autoluminograms shown are representative of at least two independent experiments.

Pull-down assays were performed as previously described [23]. Briefly, GST alone or GST-RILP was transformed into *Escherichia coli* strain BL21 at 37 °C overnight. Luria broth was inoculated with the overnight culture and grown at 37 °C. Isopropyl β-D-thiogalactoside was added to induce protein production. The bacteria were washed with cold phosphate-buffered saline and resuspended in cold lysis buffer. Proteins were purified by adding pre-equilibrated Glutathione-Sepharose 4B beads (GE Healthcare; Piscataway, NJ) to the lysate and incubated for 30 min at room temperature. Beads were washed with lysis buffer, resuspended as 50% slurry and protein levels were quantified using the BCA assay. Each pull-down was performed in 500 μl with 150 μg of cell lysate and 15 μg of beads pre-equilibrated in pull-down buffer. Beads were rocked overnight at 4 °C and washed with cold pull-down buffer. Bound proteins were eluted with sample buffer containing DTT and separated by SDS-PAGE.

Results shown represent the mean ± SEM from three independent experiments. Statistical differences between the means of unpaired sets of data were evaluated using one-way analysis of variance followed by a post hoc Tukey's test. A p value of <0.05 was considered statistically significant.

Cerebellar cultures were transfected with GFP alone or Rab7-WT, Rab7-Q67L, or Rab7-T22N mutants tagged to GFP, infected with RFP-tagged LC3, a cellular marker for early-to-late autophagosomes, and then incubated overnight in the absence or presence of trophic factors as previously described [2]. In living Purkinje neurons transfected with EGFP-Rab7-WT (Fig. 1A; g and h) and EGFP-Rab7-Q67L (Fig. 1A; i and j), the EGFP signal was punctate throughout the cytoplasm and perinuclear region in control and TFW conditions. Conversely, expression of the dominant negative mutant EGFP-Rab7-T22N, which remains bound to GDP, resulted in a diffuse cytosolic signal under both control and TFW conditions (Fig. 1A; k and l). In the presence of trophic factors (0 h control), Rab7-WT and Rab7-Q67L expressing neurons contained few RFP-LC3 positive vacuoles indicative of basal autophagy (Fig. 1A; m and o, Fig. 1B). There was little co-localization of GFP-Rab7 with RFP-LC3 (Fig. 1A; s and u, Fig. 1C). At 16 h of TFW, there was a significant increase in RFP-LC3 positive autophagosomes (Fig. 1A; n and p, Fig. 1B, $^{**}p < 0.01$, $^{*}p < 0.05$ compared to Rab7-WT and Rab7-Q67L control, respectively) and increased co-localization with Rab7 (Fig. 1A; t, v, Fig. 1C, $^{**}p < 0.01$, $^{*}p < 0.05$ compared to Rab7-WT and Rab7-Q67L control, respectively). Rab7 colocalization with RFP-LC3 appeared higher in Rab7-Q67L cells compared to Rab7-WT (Fig. 1C). Since autophagosome turnover is rapid in these neurons, the observed increase in LC3-II is modest compared to the increase observed when vesicle degradation is inhibited [3]. Expression of Rab7-T22N resulted in an increased accumulation of autophagosomes under control conditions (Fig. 1A; q, Fig. 1B, $^{\#}p < 0.05$ compared to Rab7-WT and Rab7-Q67L control) and an even further accumulation of autophagosomes after TFW (Fig. 1A; r, Fig. 1B). There was no vesicular co-localization between Rab7-T22N and RFP-LC3 under control or TFW conditions (Fig. 1A; w and x, Fig. 1C). These data confirm previous findings in CHO and HeLa cells that active Rab7 co-localizes with autophagosomes [11,13]; whereas, an inactive Rab7 fails to co-localize with the vesicular compartment of autophagosomes [11]. Although Gutierrez et al. [11] reported the presence of Rab7-T22N on autophagic vacuole membranes, it is important to note that the EGFP Rab7-T22N signal observed was distinct from Rab7-WT and Rab7-Q67L and was not vesicular, but rather a diffuse cytosolic stain similar to our observations and others using Rab7-T22N [5,6,13].

During autophagy, cytosolic LC3-I is lipidated to form LC3-II, which binds to the autophagosomal membrane, thus making it a marker of autophagic activity [17]. We analyzed the effects of the Rab7 mutants on LC3-II expression and turnover in Purkinje neurons subjected to TFW. Rab7-WT, Rab7-Q67L or Rab7-T22N transfected cultures were subjected to TFW for 0, 2, 4, and 6 h in the absence or presence of bafilomycin A_1, a vacuolar ATPase inhibitor that inhibits vesicle degradation ensuring accurate measurements of total LC3-II [9]. TFW alone did not result in measurable levels of LC3-II in Rab7-WT and Rab7-Q67L transfected cells confirming previous data [2] that the turnover of autophagic vesicles was rapid in these cultures (Fig. 1D, top blot). However, expression of Rab7-T22N resulted in a dramatic time-dependent increase in LC3-II expression indicative of autophagosome accumulation. As expected, in the presence of bafilomycin A_1, TFW resulted in LC3-II induction and accumulation in Rab7-WT and Rab7-Q67L transfected cells, as well as, in Rab7-T22N transfected cells (Fig. 1D,

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