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Protein Trafficking into Autophagosomes

Andrew Young and Sharon Tooze

Summary

The methods described are designed to enable the assignment of an intracellular localization of secretory proteins, either soluble or membrane associated, to later secretory compartments, such as the trans-Golgi network (TGN) or endosome. These two subcellular compartments are closely linked through extensive protein trafficking, in both an anterograde and a retrograde direction. These compartments are likely to be important in the formation of autophagosomes during the process of autophagy. Our current knowledge of how autophagosomes form is scarce, and further investigation into the role that other subcellular compartments have in this process is needed.

Key Words: Autophagy; protein trafficking; subcellular fractionation; Atg; mAtg9;Golgi;endosome

1. Introduction

This chapter addresses methods to determine to which intracellular organelle a protein localizes. Determining where in a cell a protein localizes is often the first stage in characterizing a novel protein. The localization of a novel protein may provide clues as to its function or, if the protein is already of known function, then it is important to know where within the cell it performs that function. Here we will focus on techniques concerned with determining whether a protein localizes to pathways leading to the formation of autophagosomes. Protocols to determine whether a protein localizes to Golgi and endosomal membranes are described as these potentially have inputs into the induction of autophagy and formation of autophagosomes. Recent investigations into a transmembrane protein called mammalian Atg9 (mAtg9), originally identified

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in yeast as a protein required for autophagy, have exploited these techniques to start to gain an understanding of this question ***(1)***.

The analysis of the glycosylation of secretory proteins or transmembrane proteins (only proteins that have passed through the secretory pathway are glycosylated) can be informative in determining a protein's localization. However, not all proteins synthesized in the ER are N-glycosylated; some may be O-glycosylated. N-linked glycosylation is the most common form of glycosylation, which is particularly useful for pinpointing precisely where a protein is in the early secretory pathway. The presence of a NXS/T motif within the primary amino acid sequence of the protein is a good indication that the protein is glycosylated. However, not all these motifs will be glycosylated; for example, for reasons of steric hindrance, or limits in accessibility of the site to the glycosylation enzymes may inhibit glycosylation. Proteins that traffic to and though the Golgi acquire a complex form of N-linked glycosylation, which is detectable using a combination of endoglycosidase H (Endo H) and PNGase F glycosidases. Endo H cleaves only high-mannose forms of glycans present on proteins in the ER and early Golgi. The presence of Endo H–resistant glycosylation can therefore be used to determine whether the protein of interest has passed through the later compartments of the Golgi. PNGase F is able to also cleave off the complex forms of glycosylation that are added in later Golgi compartments and can therefore be used to determine if a protein is N-glycosylated.

Immunofluorescence analysis can be used to determine where a protein of interest is localized to at steady state by assessing its overlap with a panel of proteins that have a previously determined localization within the cell. Centrifugation methods can be used to the same end. Differential, velocity-controlled, or equilibrium centrifugation of cellular material after mechanical disruption of the plasma membrane can be used to separate subcellular organelles, and the distribution of marker proteins of previously determined localization can be compared to that of the protein of interest and its localization ascertained.

Examples of the marker proteins mentioned above are: protein disulfide isomerase (PDI), a protein resident in the lumen of the ER; GM130, a protein resident in the *cis*/medial cisternae of the Golgi apparatus ***(2)***; mannosidase II, a medial Golgi glycosidase; TGN38, at steady state displaying a predominantly trans-Golgi localization ***(3)***; EEA1, an early endosomally associated tether protein ***(4)***; and Rab proteins, specific Rabs that have been shown to localize to specific organelles ***(5)***. Antibodies to a majority of these proteins are commercially available. The best characterized and most frequently used marker for autophagosomes is microtubule associated protein (MAP) light chain 3, or LC3 ***(6,7)***. Several commercial antibodies are available, but use of these

should be carefully controlled with co-localization with GFP-LC3, or increased accumulation of LC3-II under conditions expected to induce autophagy, for example, vinblastine ***(8)***. GFP-LC3 expressed by transfection of plasmid is frequently used, but caution should be used in the interpretation of the results if GFP-LC3 is overexpressed as this may create artefacts.

2. Materials

2.1. N-Glycosylation

1. Endoglycosidase H (Endo H) and PNGase F (New England Biolabs).
2. Ball-bearing-based cell cracker (EMBL Workshop, Heidelberg, Germany).
3. Phosphate-buffered saline (PBS): 0.137 *M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 18 m*M* KH_2PO_4 (Sigma). Adjust to pH 7.4 with HCl if necessary and autoclave before storage at room temperature.
4. Cell scraper can be made from a semi-circular piece of rubber stopper (bung) mounted on the end of a plastic 10 mL pipet or purchased (e.g., from Fischer Scientific).
5. Trypan blue (Sigma).
6. Ultracentrifuge, and ultracentrifuge rotor, capable of achieving >100,000*g*, for example, Beckman TLA45.

2.2. Immunofluorescence

1. Glass cover slips, 22 mm^2 square, 1.5 mm thick, and glass microscopy slides, 76 × 26 mm (e.g. from Fisher Scientific).
2. Paraformaldehyde, 16% (v/v) (Agar Scientific), dilute to 3% using PBS, and add $MgCl_2$ and $CaCl_2$ to give 84 μ*M* and 96 μ*M* final, respectively.
3. PBS: 0.137 *M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 18 m*M* KH_2PO_4. Adjust to pH 7.4 with HCl if necessary and autoclave before storage at room temperature.
4. NH_4Cl, make up as a 1 *M* stock in water and dilute using PBS to the working concentration, 50 m*M* solution. Store both at room temperature.
5. Triton X-100 (Sigma). Made up as a 20% (w/v) stock and store at 4ºC.
6. Porcine skin gelatin (Sigma), make up as a 4% (w/v) stock and store in ~15 mL aliquots at −20ºC.
7. 3MM paper (Whatmann).
8. Parafilm (Pechiney Plastic Packaging).
9. Secondary antibodies conjugated to Alexa 488, 555, and 647 (Molecular Probes). These Alexa dyes have a strong signal and are quite fade resistant.
10. Mowiol 4-88 (Calbiochem). Add 2.4 g Mowiol to 6 g glycerol and 6 mL distilled water and incubate at room temperature for 2 h. Then add 12 mL 0.2 *M* Tris-HCl pH 8.5 and incubate at 53ºC, stirring occasionally, until the Mowiol dissolves. Make 1 mL aliquots and freeze at –20ºC for storage.

2.3. Subcellular Fractionation

2.3.1. Golgi Preparation

1. Six female Sprague-Dawley rats, weight 180–200 g.
2. Potassium phosphate buffer (PPB): mix sufficient 0.1 *M* K_2HPO_4 solution and 0.1 *M* KH_2PO_4 solution to achieve pH 6.7. Measure with pH electrode. Add $MgCl_2$ to 5 m*M* , pepstatin A to 5 μ*M* final and 1 complete EDTA free protease inhibitor tablet (Roche) per 50 mL.
3. Scissors, sharp/surgical quality.
4. Sucrose, ultra-pure.
5. Ultracentrifuge, and ultracentrifuge rotor, capable of achieving >100,000*g*, for example, Beckman SW28 rotor and SW28 tubes (or equivalent).
6. 150 μm mesh steel laboratory sieve (Endecotts Ltd, UK).

2.3.2. Endosome Preparation

1. Predialyzed Ficoll (Sigma).
2. Delta Refractometer (Bellingham and Stanley Ltd., UK).
3. 0.4 *M* TES (2-[2-hydrox-1,1-bis(hydroxymethyl)ethyl)amino]ethanesul-fonic acid) pH 7.4, sucrose, 0.5 *M* EDTA pH 7.4.
4. STE buffer: 250 m*M* sucrose, 10 m*M* TES pH 7.4 and 1 m*M* EDTA pH 7.4.
5. STM buffer: 250 m*M* sucrose, 10 m*M* TES pH 7.4, 1 m*M* $MgCl_2$.
6. Gradient maker, with reservoirs capable of holding at least 15 mL each.
7. Nycodenz (Axis-Shield, Norway).
8. Ultracentrifuge and ultracentrifuge rotor VTi50 (Beckman).
9. 39 mL Quick-seal centrifuge tubes (Beckman, polyallomer, 25 × 89 mm) and heat sealer for Vti50 rotor.
10. Peristaltic pump.
11. Fraction collector, capable of holding 30–40 tubes.
12. Potter Elvejhem homogeniser (Thomas Scientific).
13. 10-mL syringe and long (~10 cm) blunt-ended needle.
14. 2 mm diameter stainless steel tube ~10cm in length.

3. Methods

3.1. N-Glycosylation Analysis

1. Seed one 35-mm dish for each experimental data point to achieve 80–90% confluency (typically 1.6×10^6cells) on the day of the experiment.
2. Using a cell scraper, scrape the cells in PBS.
3. Homogenize the cells using the ball-bearing-based cell cracker (passing the cells through a narrow gauge needle can be just as effective). Check the cells for breakage using trypan blue (*see* **Note 1**). Keep the homogenate on ice.
4. Centrifuge the homogenate at 2200*g* for 10 min at 4ºC.

5. Centrifuge the resulting post nuclear supernatant (PNS) at 45,000 g in a Beckman TLA45 ultracentrifuge rotor (or equivalent) for 1 h at 4ºC to pellet the membranes. This allows resuspension of the proteins in a sufficiently small volume to allow glycosidase enzyme treatment.
6. Resuspend the membrane pellet in PBS containing protease inhibitors (*see* **Notes 2** and **3**).
7. Treat the resulting solution with the glycosidase enzymes Endo H and PNGase F (New England Biolabs [NEB]) as per the manufacturer's instructions.
8. Analyze by SDS-PAGE and Western blotting, and look for an increase in the protein's electrophoretic mobility upon removal of its N-glycosylation. Shown in **Fig. 1** is an example of a glycosidase digest performed on mAtg9.

3.2. Immunofluorescence

1. Seed the cells at least the day before at a sufficient density that they will be reasonably sparse upon fixation, approximately 50–60% confluency (typically 2.6×10^6 cells), in order that individual cells will be clearly visible in the microscope. Seed the cells in a 6-well dish, containing the cover slips. Depending upon the adherence properties of the cell type, pretreatment of the cover slips can help avoid cells washing off the cover slip during the procedure (*see* **Note 4**).
2. Fix the cells using 3% (v/v) formaldehyde (Agar Scientific) in PBS containing 84 μ*M* $MgCl_2$ and 96 μ*M* $CaCl_2$ (for subsequent staining with Rab antibodies, *see* **Note 5**). Alternatively, cells can be fixed using pure methanol at −20ºC (*see* **Note 6**) (if using methanol, leave out the quenching and permeabilization steps, **steps 3** and **4**).
3. Incubated cells in 50 m*M* NH_4Cl for 10 min to quench residual formaldehyde, wash three times with PBS.
4. Permeabilize the cells by incubating them in 0.2% (w/v) Triton X-100 in PBS for 5 min. Then wash three times in PBS.

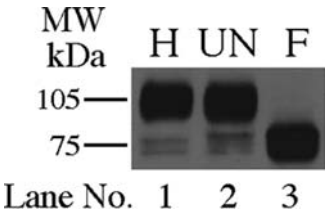

Fig. 1. Mammalian Atg9 is present in a late or post-Golgi fraction. mAtg9 with an HA-tag was transfected into HEK293 cells. After preparation of a lysate, aliquots were subjected to digestion with Lane 1, Endo H (H), Lane 2, untreated (UN), or Lane 3, with PNGase F (F). The samples were subjected to SDS-PAGE, followed by Western blotting with anti-HA antibodies. HA-mAtg9 has Endo H–resistant, N-linked glycans.

5. Wash three times with 0.2% (w/v) gelatin in PBS and leave in the last gelatin wash for at least 20 min to block.
6. Dilute the primary antibody in the gelatin solution and incubate with the cover slips for 20 min (*see* **Note 7**).
7. After washing three times with the gelatin solution, incubate with the secondary antibodies.
8. Wash the cover slips three times with the gelatin solution, three times with PBS, and then finally once with water.
9. Finally, drain cover slips by touching on 3 MM paper, and mount the cover slips on slides using Mowiol 4-88.

3.3. Subcellular Fractionation

3.3.1. Golgi Preparation

Rat liver Golgi are prepared as described by Slusarewicz et al. ***(9)***. The procedure uses a discontinuous sucrose gradient made up of sucrose solutions of different concentrations (*see* **Note 8**) in PPB. Organelles, and in particular Golgi membranes, are here subjected to differential centrifugation, which separates them by density.

1. Starve the rats for 24 h prior to the experiment (*see* **Note 9**).
2. Prepare six discontinuous sucrose gradients consisting of 13 mL of 0.86 *M* sucrose in PPB underlain with 7.5 mL of 1.3 *M* sucrose in PPB in Beckman Ultraclear SW28 tubes (*see* **Note 10**).
3. Sacrifice the rats by asphyxiation with CO_2 followed by cervical dislocation.
4. Excise livers using scissors and quickly immersed in 200 mL ice cold 0.5 *M* sucrose in PPB and swirl and squeeze the livers occasionally to expel any blood and to speed cooling.
5. Place 36 g of liver into fresh 0.5 *M* sucrose in PPB and cut into pieces with scissors to release as much blood as possible.
6. Decant excess liquid to leave a volume of less than 80 mL.
7. Mince the livers into small pieces, approximately 4–5 mm square, using scissors.
8. Homogenize the liver by gently pressing it through the 150 μm mesh steel laboratory sieve using the bottom of a 250 mL conical flask (*see* **Note 11**). Collect the homogenate in a plastic dish.
9. Pour the homogenate (H) into a 100 mL measuring cylinder and make up to a final volume of 80 mL with 0.5 *M* sucrose in PPB, then thoroughly mix.
10. Overlay 13 mL of the homogenate onto each of the six gradients and top up with 0.25 *M* sucrose in PPB.
11. Centrifuge in a Beckman ultracentrifuge using a SW28 rotor at 28,000 g for 1 h at 4ºC. Keep an aliquot of the homogenate and snap freeze in liquid nitrogen.
12. After centrifugation, remove the lipid layer on the surface of the gradient by aspiration.

13. Collect the intermediate (I) Golgi fraction from the interface between the 0.5 *M* and 0.86 *M* using a Pasteur pipet and bulb (approximately 2–3 mL from each gradient).
14. Pool the fractions from the six gradients and diluted to 8–9% (w/w) sucrose (0.25–0.28 *M*) using PPB. Check the concentration using the Delta refractometer.
15. Pour this intermediate fraction into two SW28 tubes (Beckman) and top up with 0.25 *M* sucrose in PPB. Keep an aliquot of the intermediate fraction and snap freeze in liquid nitrogen.
16. For each tube underlay 0.5 mL of 1.3 *M* sucrose in PPB (*see* **Note 12**).
17. Centrifuge at 7000 g in an SW28 rotor for 30 min at 4ºC.
18. Remove the supernatant by aspiration and collect the final membrane felt, the Golgi membranes (G) with a P200 Gilson pipet (or equivalent). This should yield 1–1.5 mL of Golgi membranes.
19. Divide into 100-µL aliquots and snap-freeze in liquid nitrogen, then store at −80ºC. These membranes can be thawed and refrozen at least twice without significant change of morphology or loss of enzymatic activity.
20. Analyze the fractions collected using organelle markers. **Figure 2** shows the result of immunoblotting for Mannosidase II (MannII), TGN38, and mAtg9. Note that Golgi cisternae are enriched by this preparation.

3.3.2. Endosome Preparation

The endosome preparation is from rat liver according to the protocol of Ellis et al. ***(10)***, which essentially consists of a continuous Ficoll gradient on which organelles are separated by density.

1. Prepare the gradients the day before the experiment. Dissolve predialyzed Ficoll (Sigma) in water, 1 mL water per gram of Ficoll. After the Ficoll has dissolved, dilute it until the refractive index is 1.37, to give a 25% (w/v) solution.

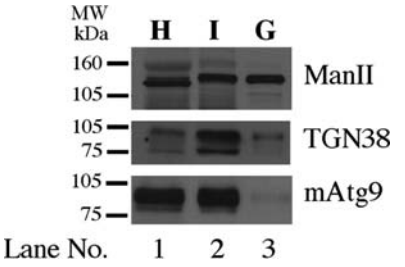

Fig. 2. Mammalian Atg9 fractionates in a similar fashion to TGN38, but not Mannosidase II. Fractions from the Golgi preparation, homogenate (H), intermediate fraction (I), and purified Golgi (G) were analyzed by SDS-PAGE, followed by Western blotting with antibodies to Mannosidase II (MannII), TGN38, and mAtg9.

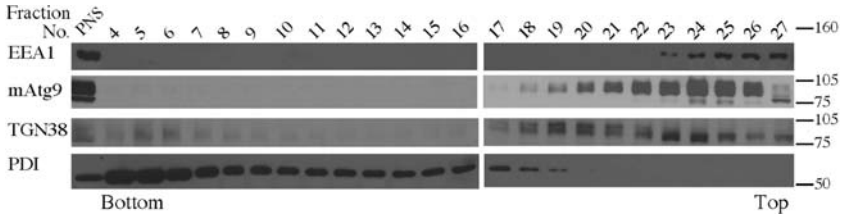

Fig. 3. Mammalian Atg9 is found in fractions partly overlapping with TGN38, but not EEA1 or PDI. Rat liver postnuclear supernatant (PNS) was subjected to the endosome gradient analysis as described. Fractions were collected and numbered as shown. The individual fractions were analyzed by SDS-PAGE and immunoblotted with the antibodies shown. mAtg9 sediments more closely to the Cation-independent mannose-6-phosphate receptor (CI-MPR, data not shown; see **ref. *1***) but can be found in membranes containing TGN38, as expected from **Fig. 2**. The overlap with EEA1 is only partial, and EEA1 exhibits a different profile, peaking at Fraction. No. 27, whereas mAtg9 has a peak at Fraction. No. 24.

2. Add 400 m*M* TES pH 7.4, solid sucrose and 0.5 *M* EDTA pH 7.4 to the 25% Ficoll solution to give 22% Ficoll, 250 m*M* sucrose, 10 m*M* TES and 1 m*M* EDTA.
3. Use buffer STE to dilute the 25% Ficoll to 1% Ficoll solution.
4. Add 15 mL of the 22% Ficoll to the chamber of the gradient maker closest to the exit tube and 15 mL of the 1% Ficoll to the other chamber. Stir the 22% Ficoll chamber by means of a magnetic stir bar.
5. Place 4 mL of a 45% (w/v) Nycodenz (Axis-Shield, Norway) solution into the bottom of a 39 mL Quick-Seal centrifuge tube (Beckman, polyallomer, 25 × 89 mm).
6. Overlay the Ficoll gradient onto the Nycodenz by connecting the gradient maker to a peristaltic pump and placing the tubing into the neck of the centrifuge tube such that the Ficoll solution drips down onto the Nycodenz.
7. To start pouring the gradient, turn on the pump and open the tap separating the chambers simultaneously. Once poured, leave the gradients to sit overnight at 4ºC in order to let them smooth by diffusion.
8. Sacrifice the rat by asphyxiation with CO_2 followed by cervical dislocation and excise the liver and transfer it to a beaker containing 3 mL/g liver of ice cold STM buffer.
9. Homogenize the liver in a 4ºC cold room using a Potter Elvejhem homogenizer (Thomas Scientific) fitted with a Teflon pestle rotating at 2400g, with three complete up-and-down strokes.
10. Centrifuge the homogenate at 1500*g* for 10 min at 4ºC.
11. Load the resulting post nuclear supernatant (PNS) on top of the Ficoll gradients, 5 mL per gradient, using a syringe coupled to a long blunt-ended needle.

12. Heat seal the tubes closed and centrifuge in a VTi50 rotor (Beckman) at 50,000g (206,000*g*) for 1 h at 4ºC using slow initial acceleration and no braking for deceleration.
13. After centrifugation, remove the top of each tube using a scalpel. Working in a 4ºC cold room, pass a 2 mm diameter stainless steel tube down to the bottom of the centrifuge tube.
14. Connect the metal tube via the peristaltic pump to a fraction collector. Precalibrate the pump speed to run at ~1 mL/min with water and program the fraction collector to move to the next tube every 60 s (*see* **Note 13**).
15. Snap-freeze the fractions in liquid nitrogen and store at −80ºC.
16. Analyze the fractions by SDS-PAGE and Western blotting. Shown in **Fig. 3** is an analysis of EEA1, TGN38, and PDI, compared to mAtg9.

4. Notes

1. Trypan blue is a dye that crosses the plasma membrane but is actively pumped out of live cells. Dead cells or disrupted cells cannot pump out the dye and stain blue.
2. The membrane pellet can be difficult to spot after centrifugation; mark the top of the centrifuge tube on the side facing outwards in order to determine on which side of the tube the pellet should appear after removal from the rotor. Often the pellet can appear as a round opaque spot.
3. The membrane pellet from the high-speed spin (>100,000*g*) can be difficult to resuspend. Resuspension is achieved through a combination of disruption with a glass bulb made from melting a disposable glass pipet, repeated pipetting in a small volume of the PBS, and vortexing or shaking at 4°C.
4. One method of cover slip pretreatment is to overlay the cover slips with a 0.1 mg/ mL solution of poly-D-lysine and incubating them for ≥15min at room temperature before washing three times with water to remove any that has not stuck to the cover slip.
5. For subsequent staining with Rab antibodies, in order to remove the non–membrane associated cytoplasmic pool of the Rab, cells can be prepermeabilized with 0.05% saponin in 80 m*M* Pipes pH 6.8, 1 m*M* $MgCl_2$, 5 m*M* EGTA (a physiological buffer) for 5 min before fixation and then washed with PBS before fixation.
6. Different antibodies will work best with different fixation methods, which must be empirically determined.
7. For economy, use only 100 µL of diluted antibody solution. In order to do this, line the bottom of a plastic container with wetted 3MM paper (Whatmann) and place a sheet of Parafilm (Pechiney Plastic Packaging) on top of the paper. The wetted 3MM paper will maintain a humidified atmosphere and the Parafilm will cause the 100 µL of antibody solution to form a droplet. Drain the cover slip on dry 3MM paper by touching the edge against the paper, and invert the cover slips onto to the antibody droplets using tweezers.

8. To ensure the sucrose solutions are the exact concentrations, check them using a Delta refractometer (Bellingham and Stanley Ltd., UK). Often it is necessary to either dilute or add more sucrose to the solution, despite careful preparation.
9. The rats are starved so as to stop lipoprotein synthesis, which would otherwise dramatically alter the Golgi's physical characteristics.
10. Underlaying of the heavier sucrose solution is performed using a syringe connected to a long (~10 cm) metal needle by passing the needle down to the bottom of the tube.
11. This relatively gentle method of homogenization reduces the possibility of cisternal unstacking by mechanical shear ***(11)***.
12. The dense sucrose will act as a cushion to stop the membranes impacting on the bottom of the tube and potentially breaking.
13. The varying density of the Ficoll will mean that the volume of the fractions collected will vary. To allow comparison with published markers, it is recommended that the refractive index is measured. This will also allow the quality of the gradient formed after centrifugation to be checked. The gradient should be a linear gradient.

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