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METHODS PAGE

Immunofluorescent Surface Labeling of Externalized Zymogen Granules or Endolysosomal Vesicles Following Exocytosis in Pancreatic Acini

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Version 1.0, April 11, 2014 [DOI: 10.3998/panc.2014.5]

Immunofluorescent surface labeling of intact cells with antibodies directed against intra-organelle luminal epitopes of trans-membrane proteins exposed on the extracellular surface following exocytosis is a common method we and others have utilized with many different cell types including CHOK1, HeLA, NRK and acini to measure exocytotic events (5, 13, 16, 18). External labeling can be evaluated by both direct and indirect methods such as microscopy and flow cytometry respectively, to measure the exocytosis of specific populations of vesicles at the plasma membrane (5, 9, 12, 13, 16, 18). Microscopy is the preferred method of evaluation over fluorescence activated cell sorting (FACS) when cells are transiently transfected at less than 100% efficiency or when evaluating secretory polarity (16, 18).

Secretion of stored, digestive zymogens from acinar cells of the pancreas and parotid glands occurs mainly via the classic ZG pathway. In addition there are two unique parallel pathways distinct from the classic ZG pathway that secrete newly synthesized zymogens known as the constitutive-like (CLP) and minor regulated pathways (MRP) (1-3, 10, 11). The CLP and MRP originate from immature secretory granules (ISGs) as small vesicles which traffic through endosomal intermediates prior to secretion. It is proposed that the CLP traffics through an apical endosomal recycling compartment while the MRP is stimulated by low-levels of secretagogues and traffics through sorting endosomes (2, 3). The fungal metabolite, brefeldin A (BFA) acutely inhibits secretion from the CLP and MRP but does not inhibit secretion from ZG's (4, 10). BFA functions by inhibition of guanine nucleotide exchange factors for the ADP-ribosylation factors ARF1 and ARF3 that mediate vesicle formation at *trans*-Golgi and endosomal compartments (15).

The CLP and MRP release relatively small amounts of zymogens in comparison to stimulated zymogen secretion via the classic ZG pathway. However they are essential for ZG formation/maturation and moreover, are proposed to indirectly regulate ZG exocytosis by delivering t-SNARE regulatory proteins to the apical membrane necessary for ZG

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docking and fusion (4). We have utilized cell surface labeling of the lysosome associated membrane protein (LAMP1) to analyze secretion from the CLP and MRP and Synaptotagmin 1 (Syt1) to detect exocytosis of ZG's **(Figure 1)** (16). Although this technique is done on fixed tissue, others have used spinning-disc microscopy in live acinar cells to image exocytotic events detected by expression of a pH-sensitive construct syncollin-pHluorin which localizes to ZGs (6). Upon exposure to the extracellular space, the rapid dissipation of the intragranular acidic environment induces pHluorin fluorescence. However, this method does not distinguish between ZG and CLP/MRP exocytotic events. Finally membrane-labeling with FM-143 dye has also been utilized in acinar cells to detect exocytosis but provides no information regarding the compartment undergoing exocytosis (8).

LAMP1 is a type 1 trans-membrane protein with a highly glycosylated intra-organelle domain that becomes exposed on the extracellular surface following exocytosis. Unlike Syt1 which is enriched in purified ZG fractions of acini, LAMP1 is not present on ZG's but is found in endolysosomal compartments (early and late endosomes and lysosomes) (16). Under normal conditions only a small fraction of total cellular LAMP1 is present on the plasma membrane. However, secretagogue stimulation induces an acute accumulation of LAMP1 at the plasma membrane via a lysosome-like secretory pathway **(Figure 2).** Of note, LAMP2 (which is also likely GRAMP92 seen in the literature (7, 14, 17)), is present in both ZG and endolysosomal compartments and therefore cannot be used to distinguish between these compartments.

1. Materials

1.1 Equipment and Reagents

1. Water-bath style incubator capable of holding temperature at 37 ^{o}C.
2. Tabletop mini-centrifuge
3. pH meter
4. Rotator or "Wheel"
5. 125 ml Polycarbonate erlenmyer flasks
6. Polycarbonate tubes (149566C – Fisher Scientific)
7. 1.7 ml microcentrifuge tubes
8. Rectangular coverslips, No.1, 24x50 mm (2975-245 – Corning)
9. There are 2 antibodies directed against the intra-organelle domain of proteins of interest that our lab has utilized: anti-LAMP1 (Enzo Life Sciences, cat#: ADIVAMEN001-F) and anti-Synaptotagmin1 (Sigma, cat#: S2177) both used at 1:20 in rat, mouse and human acini. LAMP1 antibody has also been successfully used for external labeling in CHO-K1, CHO-CAR, NRK and HeLA cells. These 2 antibodies can also be added simultaneously for dual external labeled samples.

1.2 Buffers

1. HEPES
2. 1x PBS (Phosphate Buffered Saline) kept at 4 ^{o}C
3. Blocking Buffer: 1x PBS pH 7.4, 3% bovine serum albumin, 2% goat serum, and 0.7% cold water fish skin gelatin.

2. Methods

2.1 Isolation of Acinar Cells

1. We have been successful with this technique using isolated acini from rat, mouse and human.

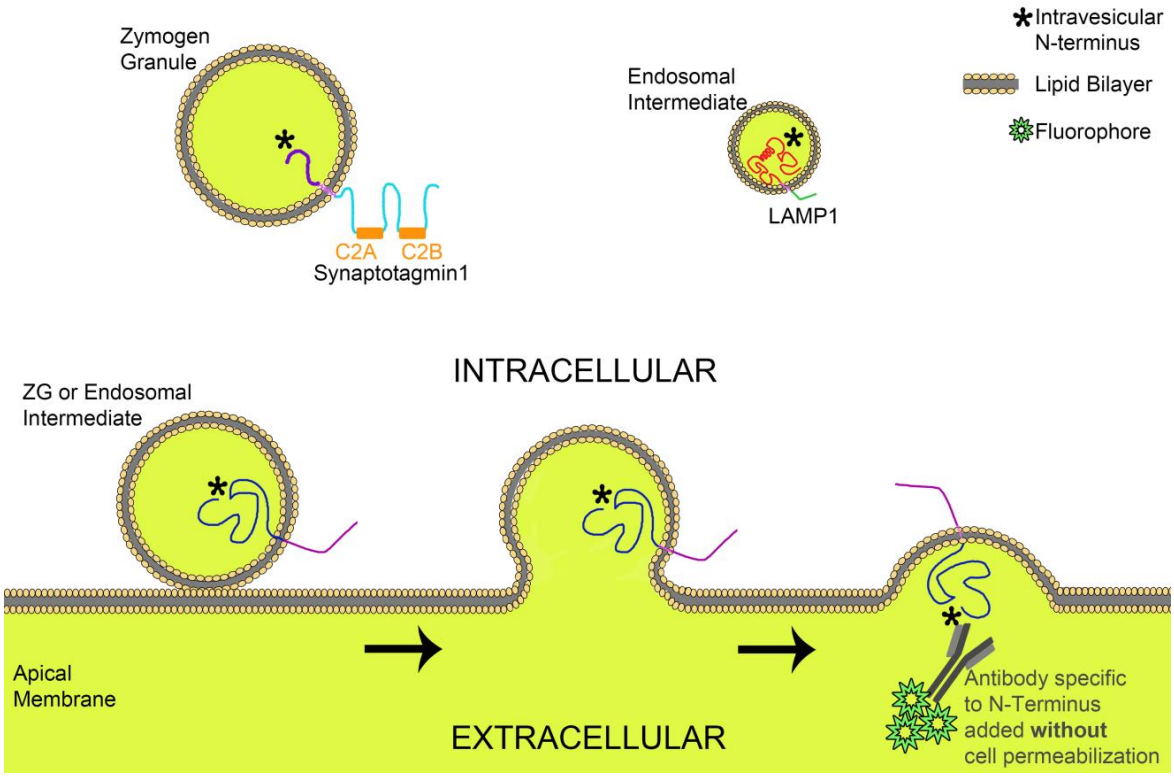

Figure 1. Antibodies are directed against the intra-organelle portion of a transmembrane protein (in this case the N-terminus) specific to a particular compartment (Syt1 – ZG, LAMP1 – endolysomal compartments). Under unstimulated conditions the N-terminus of the protein of interest is hidden inside the organelle. Upon stimulation, the organelle fuses with the apical membrane exposing the N-terminus of the protein of interest to the outside surface of the cell which can then be identified by the specific antibody and corresponding fluorophore.

It is necessary to finesse your organ digestion so the acinar cells are in small clumps (only 1-2 layers of cells in depth and groups of 10-15 cells). This allows for easier access to the apical lumen of the cell clusters. We generally have best results using pancreas from younger rats (50-75g) although we have also been successful with older larger animals and adult human pancreas. Refer to the Pancreapedia entry "Isolation of rodent pancreatic acinar cells and acini by collagenase digestion" for purification of acini (19).

2. After isolation gently centrifuge acini and suspend in DMEM or HEPES buffered salt solution containing glucose and amino acids at a concentration of 1 ml of "packed cells" per 15 ml buffer. Then take 3-5 ml of the cell suspension and further dilute to 20 ml in the same buffer. The buffer should be supplemented with 1.0 mg/mL BSA and 0.1 mg/ml SBTI. It is not recommended that cells be allowed to "rest" longer than approximately 15 min to minimize the loss of polarity which occurs with time.

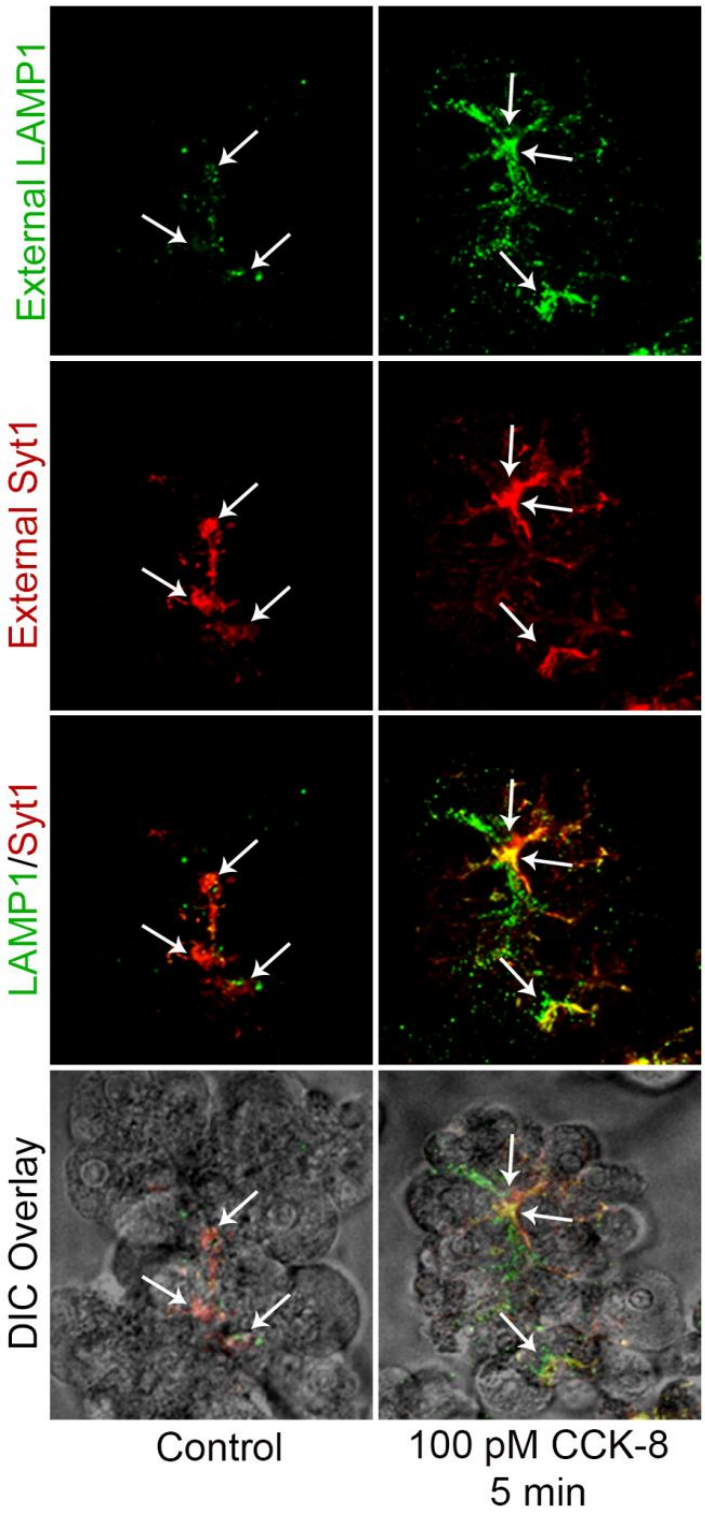

Figure 2. LAMP1 and Syt1 surface labeling was measured in intact rat pancreatic acini as control or using 100 pM CCK-8 for 5 min at 37°C. Acini were incubated at 4°C with anti-LAMP1 (1:100) and anti-Syt1 (1:50) simultaneously to label externalized antigens. Acini were then fixed in 2% formaldehyde. LAMP1 and Syt1 immunoreactivities were detected postfixation, by use of Alexa Fluor 488-conjugated anti-mouse IgG (1:100) and Alexa Fluor 546-conjugated anti-rabbit IgG (1:100), respectively. Each image is a reconstructed z-series obtained by confocal microscopy. White arrows indicate the apical regions of the acini. All images are representative of multiple determinations performed on at least 3 separate tissue preparations.

2.2 Cell Treatment and Surface Labeling

We have performed a comprehensive analysis of secretagogue stimulation (CCK, JMV-180, Secretin, Bombesin etc.) in rat acini using this technique. In general cells respond in a similar manner to that seen for amylase secretion. These results are being prepared for submission as a research paper.

1. Remove 5 ml of cell suspension, transfer to an empty 125 ml polycarbonate flask and add 10 ml of DMEM or HEPES buffer containing 1.0 mg/ml BSA and 0.1 mg/ml SBTI for each time point. (In a typical external label assay only approximately 50-100 µl of packed cells per treatment point are needed). Place each flask in a 37 ^{o}C water bath to treat as desired.
2. Transfer the cells to a prechilled 15 ml polycarbonate tube on ice and allow cells to pellet by gravity. Remove all but 1 ml of supernatant and transfer cells to a prechilled 1.7 ml microcentrifuge tube on ice. Allow cells to pellet by gravity and remove supernatant and extra cells leaving only 50-100 µl of packed cells.
3. Resuspend cells in 200 □l of ice cold 1x PBS with primary antibody of interest and place on wheel kept in a cold room at 4 ^{o}C for 2 hr with constant slow rotation (acini have been in the presence of a balanced salt solution and 1.0 mg/mL BSA since initial isolation, this serves as the normally expected “blocking step” before adding primary antibody).
4. Transfer the microfuge tubes to ice, pellet the cells quickly in a tabletop mini-centrifuge, draw off the supernatant, resuspend the cells in 1 ml ice cold 1x PBS by gently flicking the bottom of the microcentrifuge tube and return to ice. Before fixation, during primary label incubation, the cells must be kept at 4 ^{o}C to prevent internalization of the antibody by endocytosis **(Notes 1, 2).**
5. Pellet the cells quickly in a tabletop mini-centrifuge and once again, draw off the supernatant and gently resuspend the cells in 1 ml of 2% formaldehyde in 1x PBS by flicking the bottom of the microcentrifuge tube. Incubate the samples for 10 min at RT with gentle agitation. (Acini tend to form clusters post fixation in formaldehyde however this is not a concern in regards to penetration of conjugated secondary antibody as it is added in blocking buffer containing BSA, goat serum and cold water fish skin gelatin, which coats the acini, preventing clustering).
6. Gently pellet the cells, remove supernatant, and rinse one time with 1x PBS (steps from this point on should be done at RT)
   a) At this point you can continue on to step 7 for external label only. If you want to co-label an internal antigen, add the primary antibody of interest in 200 µl of blocking buffer containing 0.2% TX-100 to permeabilize the cell. Incubate with gentle agitation for 1 hr at RT then rinse cells one time with 1x PBS and continue on with step 7.
7. Resuspend cells in 0.5 – 1 ml blocking buffer without TX-100, containing the secondary antibody(s) at desired concentration and incubate with gentle agitation for 1 hr. We normally use Alexa-conjugated antibodies from Invitrogen Life Sciences at 1:100 **(Note 3).**
8. Pellet the cells and rinse 2 times with 1x PBS.
9. Store cells in 0.5-1 ml 1x PBS at 4^{o}C until evaluation on a confocal microscope **(Note 4).**

2.3 Confocal Evaluation

1. Utilize an oil immersion 60x or 100x objective lens. Place the rectangular microscope coverslip directly onto the objective covered with oil then transfer approximately 200 µl of cell suspension onto the coverslip using a pipet tip with the tip cut off to avoid shearing the cells during pipetting.
2. Capture and save images in the z-plane using 0.5 µm steps.
3. The externalized label may be quantified using various software packages (typically available from the confocal manufacturer) and normalized to total cell volume. It would be difficult to list the many varied ways to use the many different microscopy software packages on the market for quantification. The best advice we can give is to contact the support persons available for the system each lab has access to.

3. Anticipated Results

With freshly prepared high quality acinar cell preparations a distinct apical pattern of localization is typically achieved **(Figure 1).** The small number of antigens on the relatively small surface area of the apical membrane is one of the most challenging aspects of the procedure. Acini must be prepared in smaller clusters as large clusters of cells and/or lobules do not allow penetration of the antibodies to the apical lumen. The level of staining is greatly enhanced within 2 to 5 min of secretagogue stimulation. LAMP1 and syt1 show both distinct and overlapping staining patterns. High doses of CCK-8 or carbachol will result in pronounced basolateral labeling over time (15-30 min) as previously described using other techniques (6). As shown in Fig 3, the external labeling is typically distinct compared to intracellular label and provides an excellent control measure to ensure cells are not permeabilized during handling. The culturing of acinar suspensions overnight results in a loss of secretory polarity but not in secretagogue responsiveness.

4. Notes

1. Because this experiment is labeling EXTERNAL antigens, no detergent is used in any buffer as this would compromise the integrity of the plasma membrane. Likewise, great care must be taken when working with cells as rough handling can lead to cell breakage and undesired intracellular labeling. Intracellular labeling of broken cells is often quite obvious when contrasted to intact cells. Indeed control experiments can be conducted by comparing total intracellular label to externalized label as the labeling patterns are typically quite distinct **(see Figure 3).**

2. Acid washing may be used as a control experiment to ensure surface labeling. Following LAMP1 or Syt1 labeling, rinse coverslips 3 times with ice-cold PBS followed by washing an additional 5 times in acid wash buffer containing (in mM) 100 glycine, 20 acetate, 50 KCl, pH 2.2 at 4°C. Continue on with fixation of the cells. Note there should be a complete loss of surface label with acid washing (see Ref 18).

3. Addition of secondary antibody alone in conjunction with addition of a primary antibody that does not bind an external epitope (followed by the appropriate secondary antibody) are additional experiments that can be performed to

show the absence of non-specific external label (see Ref 16).

4. For antibody uptake/endocytosis experiments Alexa 488-conjugated anti-LAMP1 is available from BioLegend (cat. no. 121608). Syt1 conjugated to a fluorophore is not commercially available but it is possible to conjugate a fluorophore to the antibody directly using an antibody labeling kit from Invitrogen. In the assay add conjugated antibody to cells for 2 h at 37°C. Treat cells at desired time points with desired reagents and then fix as described above (10).

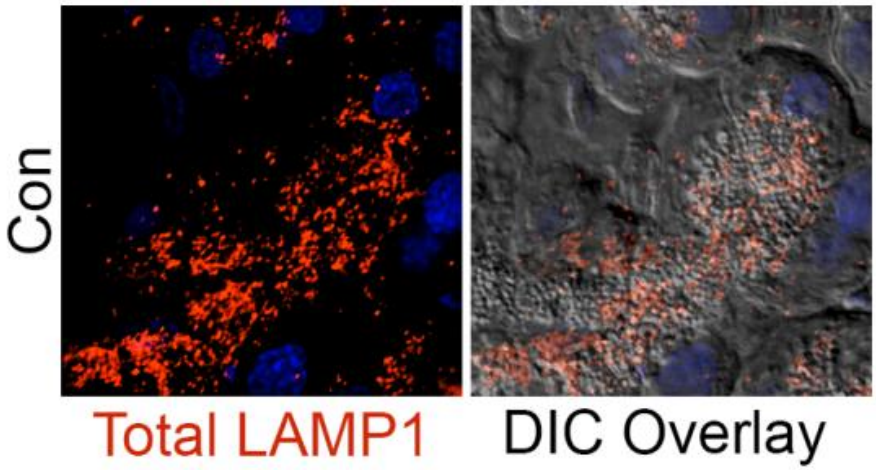

Figure 3. Total LAMP1 viewed by brightfield immunofluorescence of cryostat sections from rat pancreatic lobules is present in a punctate pattern throughout the cytoplasm of the acini. Each image is a reconstructed z-series representative of multiple determinations performed on at least 3 separate tissue preparations. LAMP1 immunoreactivity was detected by use of AlexaFluor 546 – conjugated anti-mouse (1:100). Nuclei are labeled with DAPI.

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