Video Article

Detection of Detergent-sensitive Interactions Between Membrane Proteins

Nava Zaarur[1], Xiang Pan[1], Konstantin V. Kandror[1]

[1]Department of Biochemistry, Boston University School of Medicine

Correspondence to: Nava Zaarur at nzaarur@bu.edu

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Abstract

Our ability to explore protein-protein interactions is the key to understanding regulatory connections in the cell. However, detection of protein-protein interactions in many cases is associated with significant experimental challenges. In particular, sorting receptors interact with their protein cargo in the lumen of the membrane compartments often in a detergent-sensitive fashion, making co-immunoprecipitation of these proteins unusable. Binding of the sorting receptor sortilin to glucose transporter GLUT4 may serve as an example of weak luminal interactions between membrane proteins. Here, we describe a fast, simple, and inexpensive assay to validate the interaction between sortilin and GLUT4. For that, we have designed and chemically synthesized the myc-tagged peptide corresponding to the potential sortilin-binding epitope in the luminal part of GLUT4. Sortilin tagged with six histidines was expressed in mammalian cells, and isolated from cell lysates using Cobalt beads. Sortilin immobilized on the beads was incubated with the peptide solution at different pH values, and the eluted material was analyzed by Western blotting. This assay can be easily adapted to study other detergent-sensitive protein-protein interactions.

Video Link

The video component of this article can be found at https://www.jove.com/video/57179/

Introduction

GLUT4 is a glucose transporter protein which is expressed predominantly in fat and skeletal muscle cells where it mediates the effect of insulin on post-prandial blood glucose clearance[1]. Being a very stable protein, GLUT4 is regulated at a post-translational level. In the absence of insulin, GLUT4 is largely excluded from the plasma membrane (hence low basal permeability for glucose) and is localized mainly inside the cell in small insulin-responsive vesicles (IRVs) and trans-Golgi network (TGN) that is likely to represent the IRV donor compartment. Upon insulin administration, the IRVs fuse with the plasma membrane and deliver GLUT4 to the site of its functioning. This increases the permeability of the plasma membrane for glucose, so that glucose uptake from blood into adipocytes and skeletal myocytes rises 10 to 40-fold. After insulin withdrawal, GLUT4 is internalized into early/sorting endosomes and then retrieved to TGN where the IRVs are re-formed. Both sorting steps in the GLUT4 pathway, *i.e.* retrieval from the peripheral early endosomes to the perinuclear TGN, and the formation of the IRVs on the TGN donor membranes are enabled by the Vps10p family member, sortilin, which represents a type I transmembrane protein and a sorting receptor. According to one model, sortilin works as a transmembrane scaffold protein: it binds GLUT4 in the lumen of endosomes and TGN, and recruits retromer or clathrin adaptors to the cytoplasmic side of the donor membrane *via* its C-terminus[2,3]. This facilitates the distribution of GLUT4 into vesicular carriers that translocate GLUT4 between intracellular compartments.

The interaction of the cytoplasmic tail of sortilin with retromer and various adaptor proteins has been well documented. However, the binding of sortilin to GLUT4 (and to several of its other protein ligands) has been challenging to prove. In particular, attempts to co-immunoprecipitate sortilin and GLUT4 have not been successful probably due to the detergent-sensitive nature of the interaction between these two proteins. In addition, as a typical transporter protein, GLUT4 has 12 transmembrane domains and 6 luminal loops any combination of which may potentially represent a sortilin-binding site. At the same time, a large body of indirect evidence, such as substantial co-localization in the cell, cross-linking with membrane-permeable DSP, and the interaction in yeast two hybrid system suggest that sortilin can bind to GLUT4. Furthermore, using the latter approach in a combination with the alanine scanning mutagenesis, we have previously determined that the Vps10p domain of sortilin binds primarily to the first luminal loop of GLUT4. However, the proof of such an interaction in mammalian cells has been missing. Here, we have isolated His-tagged sortilin from transfected 3T3-L1 cells using cobalt resin and demonstrated that it can interact with chemically synthesized peptide corresponding to the first luminal loop of GLUT4 at pH 6 and pH 8 that resemble acidic milieu in the endosomal lumen and neutral environment in the lumen of the TGN membranes. No peptide binding was detected in control experiments where extract prepared from non-transfected cells was loaded on the same beads.

Protocol

1. Handling of the Peptide

1. Design and ordering of the peptide
    1. Choose the desired sequence of the peptide, and add a tag, such as myc epitope (EQKLISEED) at its either N- or C-terminus.
    2. Check the predicted solubility of the peptide in water, using peptide solubility calculator http://pepcalc.com/peptide-solubility-calculator.php. If the solubility is low, try to add another water-soluble tag to change the charge balance.
    NOTE: We used the peptide corresponding to the first luminal loop (fll) of GLUT4 tagged with the myc epitope (bold font) at the N terminus (Myc-fll-GLUT4): **EQKLISEED**LNAPQKVIEQSYNATWLGRQGPGGPSSIPPGTLTTLWA that has "good" predicted solubility in water.
    3. Order the custom peptide with at least 75% purity which is sufficient for the assay, and ask the provider for solubility test. Separate the order into at least two aliquots in case of unforeseen problems with dissolving the peptide.
    NOTE: Myc-fll-GLUT4 was ordered in two aliquots. Its reported solubility in ultrapure water is ≤10 mg/mL.

2. Dissolving the peptide
Note: Ultrapure water is the best solvent. If the peptide does not appear to be water soluble, refer to http://www.genscript.com/peptide_solubility_and_stablity.html for instructions.
    1. Prepare 100x working solution of the peptide in ultrapure water or in buffer of choice, with the concentration of 100 µg/mL.
    2. Separate the solution into 50 - 100 µL aliquots, and store them at -20 °C.

2. Handling of Cells

1. Cell culture
    1. Use Wild type (WT) cells as a negative control, and cells expressing the target protein tagged with six histidines (HisP).
    NOTE: We used 3T3 L1 pre-adipocytes stably transfected with Sortilin-myc/His[5].
    2. Grow the cells in Dulbecco's Modified Eagle Medium (DMEM) high Sugar, supplemented with 10% calf bovine serum, glutamine (2 mM) and penicillin/streptomycin (5 µg/mL) at 37 °C in 10% CO_2.
    3. Sit four 10 cm dishes of cells, transfect two of them with HisP and transfect the other two with control plasmid (WT). 48 h after transfection, cells should reach 80 - 90% confluency.

2. Preparation of the Cell Lysates
    1. Prepare lysis buffer (10 mM Hepes, 30 mM NaCl, 5% glycerol, 10 mM Imidazole, 0.5% Triton X-100 and protease inhibitor cocktail without EDTA for the isolation of His-tagged proteins, pH 7.4) and keep it at 4 °C:
    2. Wash cells three times with 10 mL of 1x phosphate-buffered saline (PBS) at 4 °C.
    3. Put the dishes on ice and add 500 µL of lysis buffer to each dish. Harvest the cell lysates using a cell scraper.
    4. Place the cell lysate from each dish into a different 1.5 mL tube. Label the tubes as WT-pH6, WT-pH8, HisP-pH6, HisP-pH8.
    5. Pass the cell lysates through a syringe with a 26G needle five times up and down, to complete lysis.
    6. Centrifuge the cell lysates at 16,000 x g for 10 min at 4 °C.
    7. Transfer the supernatants to new identically labeled tubes.
    8. Analyze protein concentration using the BCA Protein Assay Kit or other kit.
    9. Using lysis buffer, equalize protein concentration of all four lysates.
    10. Separate a small (*ca.* 20 µL) aliquot of each lysate and keep it at -20 °C for possible control experiments and/or trouble shooting.

3. Binding of His-tagged Proteins to the HisPur Cobalt Beads

1. Use commercially available wash buffer or prepare one (50 mM Na_2HPO_4/NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8) and keep it at 4 °C.
2. Prepare wash buffer with pH 6 by tittering wash buffer pH 8 with HCl. Keep it at 4 °C.
3. Gently swirl the bottle of the cobalt beads to make homogenous suspension.
4. Dispense 40 µL of the Cobalt beads suspension into four 1.5 mL tubes marked as above (step 2.2.4).
5. Add 1 mL of lysis buffer to each tube, and centrifuge the tubes at 1000 x g for 5 s. Aspirate the supernatant, and add 40 µL of lysis buffer to settled beads.
6. Add the cell lysates to corresponding tubes.
7. Incubate the tubes for 90 min at 4 °C on a tube rotator at 20 rpm.
8. Centrifuge the tubes at 1000 x g for 5 s and collect the supernatant. Keep the supernatant at 4 °C.
9. Add 500 µL of either pH 8 or pH 6 wash buffer to corresponding tubes and re-suspend the beads gently.
10. Centrifuge the tubes at 1000 x g for 5 s and discard the supernatants.
11. Repeat steps 3.9 and 3.10 for four times.

4. Binding of the Peptide to the His-tagged Protein Immobilized on the Beads

1. Using 100x working solution of the peptide (step 1.2.1), prepare 1x working solutions in either pH 6 or pH 8 wash buffer (two 100 µL aliquots for each, 1 µg/mL).

2. Add 100 µL of 1x peptide solution to the washed beads with the corresponding pH.
3. Incubate the beads for 30 min at 4 °C on a tube rotator at 20 rpm.
4. Centrifuge the tubes at 1,000 x g for 5 s, collect the supernatant and keep it at -20 °C.
5. Repeat steps 3.9 and 3.10 for four times.
   NOTE: In order to decrease possible background, the following steps could replace steps 4.4 & 4.5.
6. Take four micro columns with 30 µm pores, label them as in step 2.2.4, and place the columns in collection tubes.
7. After completion of step 4.3, transfer the incubation mixture into the columns, allow the solution to pass through by gravity. Keep the flow through at -20 °C.
8. Pass 500 µL of wash buffer with pH 6 or pH 8 through corresponding columns by gravity. Discard the flow through.
9. Repeat step 4.8 four times.
10. After the last wash, centrifuge the columns at 1,000 x g for 15 s.

5. Elution from Cobalt Beads

1. Use commercial Tricine sample buffer (200 mM Tris-HCl, 40% glycerol, 2% SDS, 0.04% Coomassie Blue, pH 6.8) and add β-mercaptoethanol to the final concentration of 2%.
2. Add 40 µL of Tricine sample buffer (with β-mercaptoethanol) to the washed beads and vortex the sample.
3. Heat the samples for 10 min at 100 °C, vortex the samples again, and centrifuge the tubes at 1,000 x g for 5 s.
   NOTE: In order to decrease possible nonspecific binding in the elution, the following steps could replace steps 5.1 - 5.3.
4. Add 40 µL of Elution Imidazole Buffer (50 mM Na_2HPO_4/NaH_2PO_4, 300 mM NaCl, 0.25 M imidazole) to the washed beads, vortex and incubate the tubes at room temperature for 20 min.
5. Centrifuge the tubes at 3,000 x g for 30 s, collect the supernatant (eluted samples) and transfer it to new labeled tubes.
6. Add 40 µL of Tricine sample buffer to each tube, heat the samples for 10 min at 100 °C, vortex and centrifuge the tubes at 1,000 x g for 5 s.
   NOTE: If micro columns are used, add the elution buffer to the washed beads, incubate for 20 min and collect the elution by centrifugation at 8,000 x g for 2 min.
   NOTE: Samples can be kept at -20 °C till electrophoresis is performed.

6. Electrophoresis and Western blotting

Note: The samples are ready for the separation by SDS-PAGE and subsequent Western blotting. Follow protocol of gel electrophoresis (https://www.jove.com/science-education/5065/the-western-blot) with the following modifications.

1. Use commercially available 10 - 20% Tricine gradient gels.
2. On the gel, load 20 µL of the eluted samples and 20 µL of lysates (step 2.2.10). For trouble shooting, it is recommended to load 20 µL of unbound material (step 3.8) and 10 ng of the peptide; before loading, add equal amount of Tricine sample buffer to these samples.
3. Carry out electrophoresis in commercial Tris/Tricine/SDS running buffer (100 mM Tris, 100 mM Tricine, and 0.1% SDS, pH 8.3).
4. Transfer the material from the gel onto a 0.45 µm nitrocellulose membrane using transfer buffer (25 mM Tris base, 192 mM glycine, pH 8.4) supplemented with 20% methanol.
5. Block the membrane with 3% Bovine Serum Albumin (BSA) for 1 h at room temperature. Using pre-stained molecular weight markers as guides, cut the membrane horizontally, blot the top part with an antibody against HisP, and blot the bottom part with an antibody against the myc epitope to identify the myc-tagged peptide.
   NOTE: In our particular case, cutting of the membrane is not required since both HisP and Myc-fll-GLUT4 can be detected with an anti-myc antibody.

7. Analysis of Results

1. Quantify the intensity of all Western blot signals using an Image Station or ImageJ program.
2. Normalize the signal from the myc-tagged peptide against the signal from HisP at both pH values.

Representative Results

Lysates were prepared from 3T3 L1 cells stably transfected with Sortilin-myc/His[5] and from WT 3T3 L1 cells, used as a negative control. Both lysates were incubated with cobalt beads at pH 6 or pH 8 and thoroughly washed. Beads with immobilized proteins were then incubated in the solution of Myc-fll-Glut4. After careful washes, proteins bound to the beads were eluted with 0.25 M Imidazole. Samples were subjected, along with the original lysates, to SDS-PAGE in a 10-20% Tricine gradient gel followed by Western blotting with anti-Myc antibody that allowed for the detection of both sortilin-myc/His (110 kD) and Myc-fll-GLUT4 (5 kD) (**Figure 1**).

Myc-fll-GLUT4 (10 ng) was loaded on the first lane of the gel as a reference. As the purity of the peptide is only 75%, contaminations are apparent (higher bands). Original cell lysates from both WT and Sortilin-myc/His expressing cells contain multiple bands recognized by the anti-myc antibody. Material isolated from Sortilin-myc/His expressing cells is much cleaner. In addition to some minor non-specific bands, it has only two myc-containing components: Sortilin-myc/His and Myc-fll-GLUT4. Contaminating peptides in the first lane do not appear to bind to Sortilin-myc/His. Negative control (WT eluate) has predominantly non-specific bands.

As GLUT4 passes through intracellular compartments, such as endosomes and TGN, that have different luminal pH, we wanted to assess interaction of GLUT4 with sortilin at pH 6 and pH 8 (**Figure 2**). Densitometry of results (not shown) has not revealed any significant differences in the ratio between Myc-fll-GLUT4 and Sortilin-myc/His retained on the beads at different pH values. We thus conclude that GLUT4 has the ability to interact with sortilin in both endosomes and TGN.

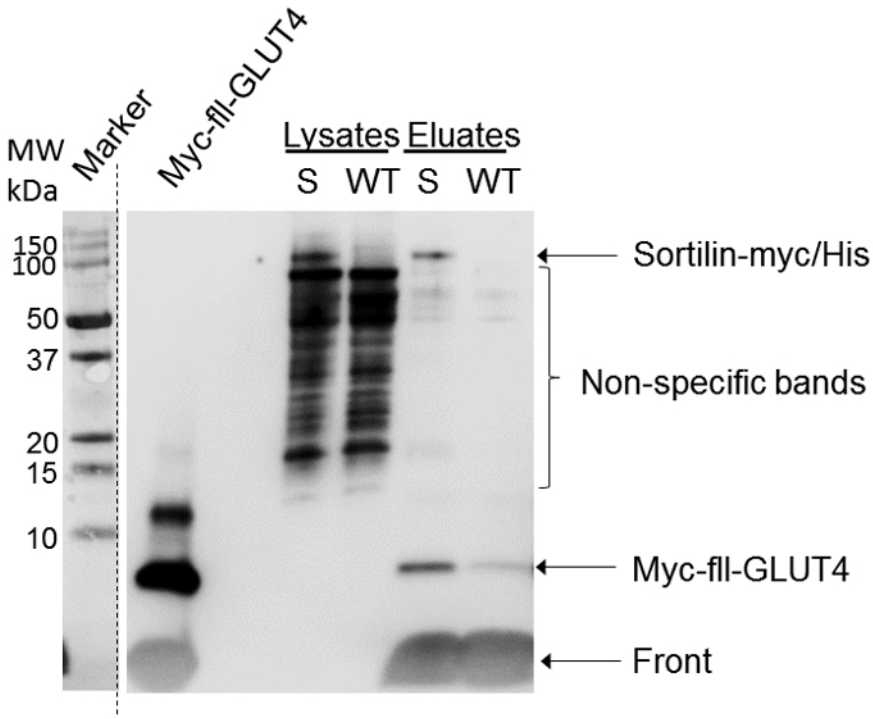

Figure 1. Interaction of Sortilin-myc/His with Myc-fll-GLUT4 on cobalt beads. Lysates prepared from WT 3T3-L1 cells and 3T3-L1 cells stably expressing Sortilin-myc/His (S) were passed over cobalt beads, washed, incubated with Myc-fll-Glut4 at pH 8, washed again, and eluted with Imidazole buffer. Myc-fll-Glut4 (10 ng) was loaded on the first lane as a reference.

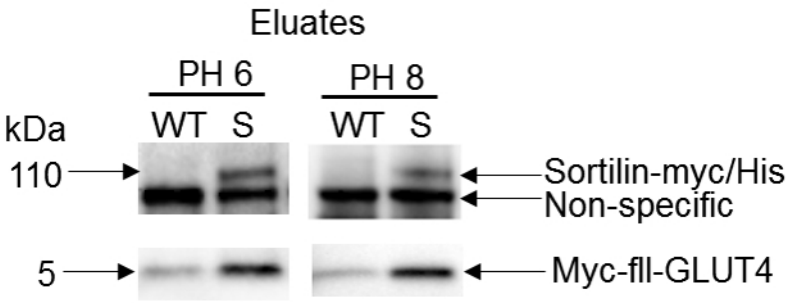

Figure 2. Sortilin-myc/His interacts with Myc-fll-GLUT4 at both pH 8 and pH 6. Myc-fll-Glut4 was incubated with material immobilized on the beads at pH 6 and pH 8, washed, and eluted with Glycine sample buffer. The figure has been adapted from Pan X., *et al*[3].

Discussion

Sortilin is an evolutionary conserved multi-ligand protein receptor that is involved in both signaling at the plasma membrane and in intracellular sorting events[6,7]. However, the search for the authentic sortilin's ligands (some of which are luminal or integral membrane proteins) is complicated as the interaction of sortilin with some of its binding partners appears to be sensitive to detergents. Therefore, an easy and a widespread approach for studying protein-protein interactions, co-immunoprecipitation, cannot be readily applied. Some research groups have used co-immunoprecipitation to demonstrate the interaction between sortilin and its potential ligands[8,9]. However, these experiments have been carried out either in 0.1% Triton or in 0.6% CHAPS which casts doubts in the completeness of membrane solubilization.

Other researchers studied the interaction between sortilin and its ligands by surface plasmon resonance[10,11]. Although surface plasmon resonance is arguably the best approach to the problem, it requires expensive equipment and significant amounts of pure recombinant proteins which is costly and time consuming.

Here, we describe a technique that, in combination with other methods, such as cross-linking, and the yeast two hybrid system, strongly suggest that sortilin can bind to GLUT4. The assay is fast and easy and can be readily adapted to other proteins and different sizes of peptides.

There are a few critical steps in this assay. The first one is the peptide's solubility in water. Another one is the selection of the proper control. Clearly, a significant amount of the biological material can interact with cobalt beads *via* endogenous His-reach sequences or non-specifically. Therefore, it is essential to immobilize on the beads lysates of WT cells and cells transfected with the protein of interest, in our case Sortilin-myc/His. In such an experimental design, material bound to the beads will differ in only one protein, Sortilin-myc/His, so that a certain degree of background binding of the peptide to control beads can be tolerated. Still, the background binding of the peptide to the beads can be reduced

by increasing the stringency of the final washing steps. The use of mini columns for washing the beads may decrease background binding even further.

Because of the nature of the "GLUT4 pathway", we wanted to address binding of Myc-fll-GLUT4 to sortilin at different pH values. We realize that pH in the lumen of early/sorting endosomes can fall below 6. However, pH lower than 6 disrupts binding of His-tagged proteins to cobalt beads which may be considered as a limitation of the method.

Isolation of the target protein on cobalt beads using the His epitope is preferable to other affinity purification steps, such as immunoprecipitation with specific antibodies, in terms of the yield and non-specific binding. Still, it is completely possible that isolation of the target protein on any other resin (preferably, magnetic beads) with proper controls will prove successful.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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