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1D4 – A Versatile Epitope Tag for the Purification and Characterization of Expressed Membrane and Soluble Proteins

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Summary

Incorporation of short epitope tags into proteins for recognition by commercially availabel monoclonal or polyclonal antibodies has greatly facilitated the detection, characterization, localization, and purification of heterologously expressed proteins for structure-function studies. A number of tags have been developed, but many epitope-antibody combinations do not work effectively for all immunochemical techniques due to the nature of the tag and the specificity of the antibodies. A highly versatile, multipurpose epitope tag is the 9 amino acid C-terminal 1D4 peptide. This peptide tag together with the Rho1D4 monoclonal antibody can be used to detect proteins in complex mixtures by western blotting and ELISA assays, localize proteins in cells by immunofluorescence and immunoelectron microscopic labeling techniques, identify subunits and interacting proteins by co-immunoprecipitation, and purify functionally active proteins including membrane proteins by immunoaffinity chromatography. In this chapter we describe various immunochemical procedures which can be used for the detection, purification and localization of 1D4-tagged proteins for structure-function studies.

Keywords

1D4 epitope tag; Rho1D4 antibody; 1D4-tagged proteins; Immunoaffinity chromatography; Immunocytochemical localization; Co-immunoprecipitation; Membrane protein purification; Protein expression

1. Introduction

The expression of proteins in heterologous cell systems including mammalian, yeast, insect and bacterial cells is widely used to identify, characterize, localize, and purify proteins for structure-function analysis. This has been greatly facilitated by engineering a short epitope tag within the protein for recognition by highly specific, commercially availabel monoclonal or polyclonal antibodies [1]. Epitope tags can be of any length and in principle inserted anywhere within the protein sequence. However, in most cases the preferred length is between 6–12 amino acids with the tag placed at the N or C terminus of the protein where it is less likely to affect protein structure and function and can be readily cleaved if required by

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inserting an additional protease–specific sequence between the protein and tag. In some applications epitope tags are generated as multiple copies to enhance binding or used with other protein affinity tags such as His-tags to facilitate protein purification and characterization of binding partners [2].

Some of the commonly used epitope tags include Flag (DYKDDDDK), HA (YPYDVPDYA), Myc (EQKLISEEDL) and 1D4 (TETSQVAPA). In principle these epitope tags can be used with a wide variety of immunochemical techniques to detect, localize, purify and characterize proteins and their interacting partners. In practice, however, the epitope-antibody combinations may only work well with specific techniques due to the affinity and specificity of the anti-epitope antibody and amino acid characteristics of the epitopes. For example, HA and Myc tags work well for the detection of tagged proteins by Western blotting and ELISA assays, localization of proteins in transfected cells by immunocytochemical techniques, and analysis of protein-protein interactions by co-immunoprecipitation, but in general these tags are inefficient for the purification of native proteins for structure-function studies. The Flag tag has been widely used with Western blotting, ELISA assays, co-immunoprecipitation and protein purification, but is limited in some applications due to relatively high nonspecific background labeling by many anti-Flag antibodies. Furthermore, the lysine residues in the Flag sequence can react with chemical fixatives (aldehydes) commonly used in sample preparations for immunocytochemistry and the tyrosine residue is susceptible to post-translational modification reducing the immunoreactivity of the Flag tag [3, 4].

One of the most versatile tags is the 9 amino acid 1D4 epitope derived from the C-terminus of bovine rhodopsin [5, 6]. This tag together with the monoclonal antibody Rho1D4, an IgG1, has been used with essentially all immunochemical techniques. These include 1) the detection of expressed proteins in cell extracts by Western blotting (Figure 1) [7]; 2) analysis of protein-protein and subunit-subunit complexes by co-immunoprecipitation (Figure 2) [8, 9]; 3) localization of proteins in cells by immunocytochemical techniques (Figure 3) [7, 10]; 4) ELISA-based assays [6, 11, 12] and; 5) purification of heterologously expressed proteins for structure-function analysis [13–17].

The 1D4 epitope has a number of advantages. The amino acid sequence is only present in rhodopsin and related photoreceptor proteins and is essentially devoid of charged residues which can result in nonspecific ionic interactions. The Rho1D4 immunoreactivity is insensitive to chemical fixatives due to the absence of reactive lysine residues and hence has been used with immunocytochemical labeling techniques for fluorescent and electron microscopy [7, 10, 18]. Since the Rho1D4 monoclonal antibody binds with high affinity to the 1D4 epitope, it is not necessary to insert multiple copies of the tag as commonly required for Flag tags. Furthermore, the Rho1D4 antibody is highly specific showing little if any nonspecific binding by immunofluorescence or Western blotting techniques (Figures 1 and 3). The binding properties of the Rho1D4 monoclonal antibody to its epitope have been systematically studied [5, 6]. The contribution of each amino acid residue within the epitope to Rho1D4 antibody binding has been evaluated by substituting each amino acid in the sequence with alanine for analysis by competitive ELISA assays.

The 1D4 tag has been particularly valuable for the purification and characterization of membrane proteins since the binding of the Rho 1D4 antibody to its epitope is insensitive to mild detergents such as Triton X-100, CHAPS, octylglucoside and dodecylmaltoside widely used to solubilize membrane proteins. Importantly, the 1D4 peptide can be used to efficiently elute the 1D4-tagged protein from the immunoaffinity matrix under nondenaturing conditions. Examples of membrane proteins purified by the Rho1D4 immunoaffinity technique for structure-function analysis include members of the ABC transporters, P4-ATPases, tetraspanins, G-protein coupled receptors, and various channels [13, 16, 17, 19–21]. Importantly, this affinity tag has also been used to purify membrane proteins in sufficient quantities from native and expressed systems for high resolution X-ray crystallography [14, 16, 22]. Finally, the Rho 1D4 antibody can be efficiently produced and purified in large quantities and as a result the purified antibody is availabel to investigators at a reasonable cost (Flintbox http://www.rho1d4.com/).

A limitation of the 1D4 tag, however, is that it has to be placed at the C-terminus of a protein. This is because the Rho1D4 monoclonal antibody requires a free carboxylate group for high affinity binding [6]. Amidation of the carboxyl group lowers its immunoreactivity to the Rho1D4 antibody by over 100 fold.

Here, we describe in detail the methods used to purify, characterize and localize 1D4-tagged membrane proteins expressed in HEK293T cells using a Rho1D4-Sepharose immunoaffinity matrix. The procedures are for small batch preparations, but can be readily adapted for the purification of large quantities of 1D4-tagged soluble or membrane proteins from any of a variety of cells including yeast, bacteria or insect cells.

2. Materials

All solutions were prepared with distilled and deionized water using analytical grade reagents unless specified otherwise. 1D4-tagged protein is generated by PCR and established cloning procedures using a typical reverse primer containing appropriate restriction sites for cloning as follows: [restriction site / TTA/GGC AGG CGC CAC TTG GCT GGT CTC TGT/- - - - - -], where the underlined is the stop codon and the dash line represents the nucleotide sequence for the protein of interest.

2.1. Transfection of HEK293T Cells

1. Mammalian expression plasmid containing 1D4-tagged gene purified in $^{dd}H_20$
2. 5% CO_2, 37°C incubator
3. HEK293T cells (or another highly transfection efficient mammalian cell line)
4. DMEM (Dulbecco’s Modified Eagles’s Medium) with 4.5g/l D-glucose, L-glutamine and 110mg/l sodium pyruvate
5. Hyclone Bovine Growth Serum (BGS) or Fetal Calf Serum (FCS) (Thermo Scientific)
6. Cellgro Antibiotic-Antimycotic solution (Manassas, Virginia)

7 0.5% Trypsin-EDTA (10×) (Gibco Life Technologies)

8 Phosphate buffer saline (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.75mM KH_2PO_4, pH 7.4)

9 100mm diameter × 20mm sterile tissue culture plates

10 Sterile BBS (BES-buffered saline pH 6.96-HCl (50mM BES Free Acid, ULTRO Grade (Calbiochem, San Diego, Ca.), 280mM sodium chloride and 1.5mM sodium phosphate monobasic)

9 Sterile 1.0M calcium chloride

10 Vortex (Genie 2, Fisher Scientific)

2.2. Preparation of Membrane Fractions

1. Tris buffer saline (TBS) (20mM Tris pH 7.4, 0.1M sodium chloride)
2. cOmplete Protease Inhibitor (Roche) Dilute as suggested by manufacturer.
3. Low speed centrifuge
4. Sucrose solution 60% w/v in TBS
5. Beckman Optima TLX ultracentrifuge with TLS 55 swinging bucket and TLA 100 rotors
6. Beckman Ultra-Clear 11×34mm tubes and 13×56mm tubes (Beckman, Palo Alto, Ca)

2.3. Solubilization of Membrane Proteins

1 2% Triton X-100, 18 mM CHAPS or other mild detergent in TBS with cOmplete protease inhibitor

2 10mm magnetic flea stir bar

2 Beckman TLX ultracentrifuge with TLA100 rotor

3 Beckman 13×56mm centrifuge tube

2.4. Preparation of a Rho1D4-Sepharose Matrix

1. Purified Rho1D4 monoclonal antibody (see Note 1)
2. Sepharose 2B (not CL) or CNBr-activated Sepharose 4B (GE Healthcare BioSciences)
3. 0.15g cyanogen bromide (CNBr)

[1]There are several commercial suppliers of the authentic Rho1D4 antibody. For purification of 1D4-tagged proteins, the Rho1D4 antibody can be purchased at a reasonable price through UBC-UILO Flintbox (http://www.rho1d4.com/). For Western blotting and immunocytochemistry where smaller quantities are needed, the preferred suppliers are Millipore (MAB5356); ThermoFisher Scientific (MA1-722); Santa Cruz (sc-57432); Stress Marq (SMC-177C or D); PhosphoSolutions (1840 RHO). Some companies including Abcam, Sigma, Novus and others list different 1D4 monoclonal and polyclonal antibodies in their catalogue. These appear to differ in their specificity, affinity, and application.

4. 2.0 liters 20mM sodium borate pH 8.0
5. TBS (20mM Tris pH 8.0, 0.15M NaCl, 0.01% $NaN_{3)}$; and TBS with 50mM glycine
6. pH meter

2.5. Purification of the 1D4-tagged Protein

1. Ultra-Free-MC Centrifugal Filters (Millipore)
2. Table-top microcentrifuge such as a Labnet Spectrafuge Mini
3. Rho1D4-Sepharose matrix (50μl per two100mm × 20mm plates of transfected HEK293T cells)
4. Membranes prepared from two100mm × 20mm plates of transfected HEK293 cells
5. TBS with 0.2% Triton X-100 or 10 mM CHAPS or other mild detergent
6. 1D4 peptide (Ac-TETSQVAPA) (0.2mg/ml) in TBS/0.2% Triton X-100

2.6. Analysis of the Purified 1D4-tagged Proteins by SDS-gels and Western Blot

1. 6.5–12% Laemmli SDS page gel or a purchased pre-made SDS-PAGE
2. Laemmli gel buffers:
    a. Lower gel buffer (1.5M Tris, 0.4% SDS pH 8.8)
    b. Stack gel buffer (0.5M Tris, 0.4% SDS pH 6.8)
    c. Acrylamide (30%) in H_20
    d. 4× Loading buffer (0.7gms SDS, 4ml glycerol, 5ml stack gel buffer and 1.0ml prepared 1% bromophenol blue)
    e. Gel running buffer (dissolve 25mM Tris, 192mM glycine, 0.1% SDS in 1L H_20; pH should be 8.3 without adjustment)
3. Transfer buffer for Western blot (25mM Tris, 192mM glycine; pH 8.3 without adjustment)
4. Wet or semi-dry transfer apparatus
5. Nitrocellulose or polyvinylidene fluoride (PVDF) membrane for protein transfer
6. Methanol 10–20%
7. PBS with 0.5% Tween 20 (PBS-T)
8. Stock milk solution (5% powdered milk in ddH_20)
9. Rho1D4 antibody (Stock solution 1mg/ml (dilute 1:5000 for Western blots))
10. Chemiluminescence (ECL) detection solutions or a LiCor Odyssey infrared imager
11. Anti-mouse secondary antibody either conjugated to IR Dye 680 or 800 for imaging on Odyssey (LiCor) or conjugated to horseradish peroxidase for ECL imaging (Sigma A4416).

2.7. Detection and Localization of 1D4-tagged Proteins by Immunofluorescence Microscopy

1. Glass coverslips
2. Sterile poly-L-lysine-hydrogen bromide (0.02 mg in H_2O)
3. Sterile Sorensen's Phosphate Buffer, 0.1M, pH 7.4 (PB)
4. Paraformaldehyde 4% in PB
5. Normal goat serum (NGS)
6. Triton X-100
7. Blocking solution (10% NGS, 0.2% Triton X-100 in PB)
8. Labeling solution (2.5% NGS, 0.1% Triton X-100 in PB)
9. Rho1D4 monoclonal antibody (Stock solution 1 mg/ml (dilute 1:1000)
10. Secondary antibody (see Note)
11. Mowiol 4–88 mounting media (Polysciences Inc. Warrington PA)
12. Dihydrochloride DAPI nuclear stain (Invitrogen, Burlington, ON)
13. Microscope slides (25×75×1.0mm)

3. Methods

3.1. Preparation of Rho1D4-Sepharose Immunoaffinity Matrix

This recipe makes approximately 8 ml of activated beads. Use 2mg antibody per ml of activated beads. This prep of activated beads should preferably be used directly for antibody coupling. Due to inactivation, the activated beads should be used within 6 hours.

1. Dialyze purified Rho1D4 for a minimum of 8 h in 3 × 500ml 20mM sodium borate pH 8.0 at 4°C. Check concentration of antibody after dialysis (mg antibody/ml = Absorbance (at 280nm) ÷ 1.3).
2. Wash 8 ml Sepharose 2B with several changes of ddH_20 by low speed centrifugation. After the final wash resuspend the beads in 8 ml ddH_20 and pour the beads in a 50ml glass beaker containing a small stir bar.
3. Set up pH meter in fume hood. Place the beads on a magnetic stirrer and while gently stirring at room temperature add small amounts of 0.2N NaOH until solution is stable at pH 10.
4. In the fume hood weigh out 0.15g CNBr and add to beads with continuous gentle stirring while maintaining the pH between 10 and 11 with 0.2N NaOH for about 30 minutes at which time the pH should become relatively stable.
5. Add 40 ml of ice cold 20mM sodium borate buffer to the beads. Wash by low speed centrifugation with cold 40 ml borate 4 times to remove any excess CNBr.

6. Resuspend beads in 15ml sodium borate buffer and keep on ice for 5 min. Centrifuge down beads and determine the approximate amount of packed beads present (Since some of the beads get lost during the activation and centrifugation steps, the amount of beads present may be less than the starting amount). Add RhoD4 antibody (2mg/ml beads) to the beads with gentle stirring (see Note 2). It is best to perform this incubation in a tube that holds approximately the volume of beads and antibody combined to keep beads from drying out. Gently rock beads for 4 h at 4°C. Do not leave beads incubating overnight as this will cause the antibody to aggregate resulting in a loss in column efficiency.

7. To determine percentage of Rho1D4 bound to beads measure the absorbance of the supernatant at a wavelength of 280nm after low speed centrifugation of the beads. The absorbance should be reduced by 80–95% indicating efficient coupling of the antibody to the beads. (see Note 3)

8. The coupling reaction is stopped by the addition of TBS pH8.0 containing 0.05M glycine followed by low speed centrifugation. The immunoaffinity matrix is washed twice with the same buffer and once in TBS alone. The matrix is stored in TBS pH8.0, 0.01% NaN_3 at 4°C. The matrix should not be frozen as this can cause an inactivation of the antibody and disruption of the matrix structure.

3.2. Expression of 1D4-tagged protein HEK293T cells

1. Prepare complete growth medium by adding 8% BGS and 10ml/l antibiotic-antimycotic solution to DMEM.

2. At the end of day 1, trypsinize exponentially growing cells and add 5×10^5 cells to each of two 100mm dia. × 20mm plates in a final volume of 9 ml medium/plate.

3. On the morning of day 2, in a 1.5ml Eppendorf tube add plasmid DNA 2–20μg (see Note 4) to H_20 to a final volume of 0.372 ml for each plate of cells. Then add 0.123ml 1.0 M calcium chloride and mix well. Slowly add 0.495ml BBS while vortexing at low speed or by flicking tube with a finger. Incubate at room temperature for 10–20min. Calcium phosphate-DNA solution is then added dropwise to the cells while gently swirling medium in plate and subsequently the cells are placed in an incubator. After 8–16 hours replace the growth medium with fresh medium and incubate for an additional 16–35 h.

3.3. Preparation of the Membrane Extract from HEK293T Cells

1. The cells are removed from the two plates by pipetting or scraping and centrifuged in a 15 ml conical tube at 1500 rpm for 4 min. The medium is removed and the

[2]It is important to stir the Sepharose matrix gently so that disruption of the beads does not occur since this can result in an inefficient immunoaffinity matrix.
[3]Coupling should be complete within 2–4 hours at 4°C. Overnight coupling should be avoided as this can cause aggregation of the beads resulting in nonspecific binding of proteins to the matrix and inefficient protein purification. If the beads are disrupted, some of the absorbance at 280nm may be due to light scattering. For the absorbance at 280nm to reliably reflect protein concentration, then the absorbance at 260nm should be considerable less than at 280 nm. If the absorbance at 260nm is higher than 280m this likely reflects a significant amount of light scattering from fractured beads.
[4]For co-expression studies, plasmids for expression of each subunit or protein are added together in equal amounts or in amounts to be determined for optimal expression of both proteins.

cells are resuspended in 1.0 ml TBS containing cOmplete inhibitor. Incubate on ice for 20–60 min. During this time the cell homogenate is forced through a 22g needle 2–4 times to break open any remaining cells. At end of incubation the homogenate is passed through a 28g needle 12x and then layered on top of 60% sucrose/TBS in a 11×34mm tube and spun in a Beckman TLS55 rotor at 26,000 rpm (45,000g) for 30 min.

2. Carefully remove the membrane fraction at the top of 60% sucrose, dilute with 2 volumes of TBS, and centrifuge in a 13×56mm tube at 30,000rpm (37,000g for 20 min in the TLA 100 rotor. Resuspend the pellet in TBS containing cOmplete inhibitor. The membranes can be directly used for protein purification as discussed below. Alternatively, the membranes can be frozen in 5% glycerol and stored at −30°C.

3.4. Solubilization of 1D4-tagged Membrane Protein from HEK293T Cells or Membrane Extracts

1. Prepare 0.6 ml 2% Triton X-100 or 36 mM CHAPS in TBS with cOmplete protease inhibitor in a 13×56mm centrifuge tube containing a flea magnetic stir bar. Many membrane proteins require phospholipids to maintain their activity. In such cases it is important to include phospholipids during solubilization and immunoaffinity chromatography. We typically use 0.2 mg/ml of brain polar lipid (Avanti Polar Lipids) or other suitable phospholipids.
2. While the detergent solution is stirring, add 0.6 ml of cells or membranes in TBS to the detergent solution at room temperature to result in a final detergent concentration of 1% Triton X-100 or 18 mM CHAPS. Continue stirring at 4°C for 10–20 min.
3. Remove the stir bar and centrifuge solubilized membranes in TLA 100 rotor at 40,000rpm (65,000g) for 10 min at 4°C to remove any aggregated material. Carefully remove the supernatant fraction containing the solubilized proteins and place the solution on ice. You may later want to analyze the pellet on SDS page to monitor for insolubilized proteins.

3.5. Purification of 1D4-tagged Membrane protein on a Rho1D4-Sepharose Matrix

1. Prepare 30ml TBS-T.
2. Add 50µl Rho1D4 Sepharose (packed volume) to an equal volume of TBS-T buffer and add to the Ultra-Free-MC filter. Wash the matrix 3 times with 0.5 ml TBS-T by a short centrifugation (~ 6 sec) in a microcentrifuge being careful not to dry out beads. (see Note 5)

[5]It is important that the immunoaffinity matrix is not centrifuged to complete dryness as this can irreversibly denature the protein resulting in low functional protein recovery. The amount of sulfhydryl reducing agents such as dithiothreitol (DTT) in the buffers should be minimized (less than 1 mM) since these reagents can cause some of the light and heavy chains to dissociate resulting in contamination of the purified protein. Immunoglobulin dissociation is particularly evident when SDS containing reducing agents is used to elute proteins from the matrix.

3. Retain a small aliquot (~ 50 μl) of the solubilized protein for analysis by SDS-PAGE and Western blotting. Add 0.55 ml of the solubilized proteins to the beads and incubate at 4°C for 20–30 min preferably rocking continuously or rocking by hand every 10 minutes. After first incubation, centrifuge in a microfuge for about 6 sec. Retain the flow through solution or unbound (UB) fraction and add second 0.6ml of solubilized protein to the matrix. Incubate as above and spin down again. Combining the two UB fractions and retain for analysis by SDS gel electrophoresis and Western blotting.

4. Wash the beads by centrifugation a minimum of 6 times each with 0.5ml TBS-T. During the washing procedure, be careful not to dry out beads.

5. Briefly spin the beads dry and immediately add 50 μl of 0.2mg/ml 1D4 peptide in TBS/0.2% Triton X-100 or TBS/10mM CHAPS. Incubate shaking for 20–30min at 18–20C. Spin down bound fraction. Add another 50ul peptide and incubate for 10min. It may take 2–4 elutions to remove all the protein bound to the antibody. To determine if significant amounts of protein is still bound, the matrix can be incubated in 100 μl of 4% SDS solution in the absence of reducing agent and eluted for analysis by SDS gel electrophoresis and western blotting. If this is carried out then the matrix should not be regenerated. Typically, 60 – 85% of the 1D4-tagged protein is recovered in the 1D4-peptide eluted fraction with minimal protein present in the subsequent SDS eluted fraction (see Note 6).

3.6. Analysis of Expression and Purification by SDS Gel Electrophoresis and Western Blotting

1 Prepare SDS page gel with appropriate acrylamide concentration for analysis of proteins of interest. Pre-made SDS gradient gels can also be used.

2 SDS sample preparation: Add one part fraction to two parts SDS gel loading buffer containing 5% 2-Mercaptoethanol. Heating the sample is not generally recommended for membrane proteins as it can generate multimeric proteins. In some instances, it is useful to compare the protein profiles for heated and unheated samples.

3 Sample analysis: Typically, 3 fractions from the immunoaffinity matrix are analyzed: input, unbound, and peptide eluted fractions (Figures 1 and 2). Accordingly, a SDS gel may have the following lanes: Molecular weight standards (colored), input (precolumn) fraction, unbound fraction, and bound (peptide-eluted) fraction. The set of lanes are repeated at least twice with a spacer lane separating the sets to facilitate cutting. One set is directly stained with Coomassie Blue or Silver Stain to analyze the protein components in the fractions and evaluate the purity of the 1D4-eluted fractions relative to the input.

[6]In purification procedures employing small amounts of Rho1D4 affinity matrix (<100μl), it is, in general, not worth regenerating the matrix. In purification procedures employing larger amounts, the Rho1D4 immunoaffinity matrix can be regenerated by washing the matrix 3 times in 0.1M acetate buffer, pH 4 over a period of 5min., and then washing the matrix in TBS until the pH is 7.4. Although in principal, the Rho1D4 affinity matrix can be regenerated after each use, in practice the efficiency of the column will be reduce since some bound protein may not be removed with the peptide in the elution step. We have found that the regenerated matrix has lost about 25% of its immunoreactivity after this treatment.

The 1D4-eluted fraction can also be subjected to mass spectrometry for analysis of possible unknown interacting proteins. The other sets are transfer to polyvinylidene fluoride (PVDF) or nitrocellulose membranes for Western blotting with the Rho1D4 antibody and other antibodies of interest as required. In addition to the input, unbound, and bound fractions, it is often of interest to analyze the pellet fraction after detergent solubilization to determine the extent to which tagged proteins are solubilized and the SDS eluted fraction post peptide elution to determine the efficiency of elution of the tagged protein by the peptide.

4 Protein transfer for Western blotting. Before transferring low molecular weight proteins (10–40kDa) the gel should be soaked in transfer buffer containing 20% methanol for at least 15 minutes. High molecular weight proteins transfer better in 10% methanol and with only a brief wash in transfer buffer. As the transfer settings are dependent on the transfer apparatus used, the investigators need to consult the manual.

7 After transfer, the protein transfer blots are rinsed with ddH20 and left to dry at room temperature or used directly.

8 For PVDF, the blots must be briefly immersed in methanol and then rinsed in PBS before labeling. For nitrocellulose, the blots only have to be rinsed in PBS.

9 Blocking step. The blots are blocked with 1–2% milk in PBS for 1h at room temperature.

10 Labeling with Rho1D4. Remove the blot from blocking solution and apply Rho1D4 antibody diluted 1:5000 in PBS containing 0.2% milk; incubate on a rocker for 1h at room temperature.

11 Washing step. Remove the Rho1D4 antibody from the blot and wash every 15 min. 3 times by changing the PBS-T.

12 Labeling with secondary antibody. Apply secondary anti-mouse antibody diluted in PBS-T with 0.1% milk and incubate rocking for 20–40min at room temperature. (anti-mouse IgG IR dyes 680 and 800 can be diluted 1–10,000 and Sigma horseradish peroxidase anti mouse IgG can be diluted 1–5000.)

13 Washing step. Remove the blot from the secondary antibody solution and wash 3 times by immersing the blot in PBS-T for 15 min per wash.

14 Blots are ready for imaging by the LiCor Odyssey Imager or by ECL as per manufacturer's instructions.

3.7. Localization of expressed 1D4-tagged protein by immunofluorescence microscopy

1. Coat sterile glass coverslips for minimum of 20 min with sterile poly-L-lysine/ ddH_20 by floating the coverslips face down in the poly-L-lysine. Wash the coverslips with PB. These coverslips can be stored at 4°C for a few days.
2. Just before plating HEK293T cells, place coated coverslips face up in the plate containing medium. The transfection (see 3.2) can be done in a 100mm dia. ×

20mm TC plate with multiple coverslips or in a 6-well TC plate. (Divide the transfection reagent by three when transfecting in a 6-well plate).

3. After the transfection incubation time is completed remove the coverslips from TC plate and immerse in 4% paraformaldehyde/PB with cells facing up for 15 min.
4. Being careful not to disturb cells, gently wash 3 times in PB with 10 min incubation for each wash.
5. Block and permeablize cells with 0.2% Triton X-100, 10% goat serum in PB for 15–30 min.
6. Remove blocking buffer and add Rho1D4 antibody (dilute stock 1mg/ml solution 1:1000 in labeling solution (PB containing 0.1% Triton X-100 and 2.5% NGS) and incubate 1–2 hours room temperature.
7. Carefully wash 3 times in PB buffer with a 10 min incubation for each wash.
8. Add conjugated secondary anti-mouse antibody diluted in labeling buffer containing DAPI nuclear stain and incubate 30–60min at room temperature.
9. Carefully wash 3 times in PB buffer with a 10 min incubation for each wash
10. Mount by lightly covering the cells with Mowiol mounting media (or similar mounting medium) and turn coverslip face down on microscope slide. Seal the coverslip by outlining with nail polish.
11. Visualize by confocal scanning or conventional fluorescence microscopy.

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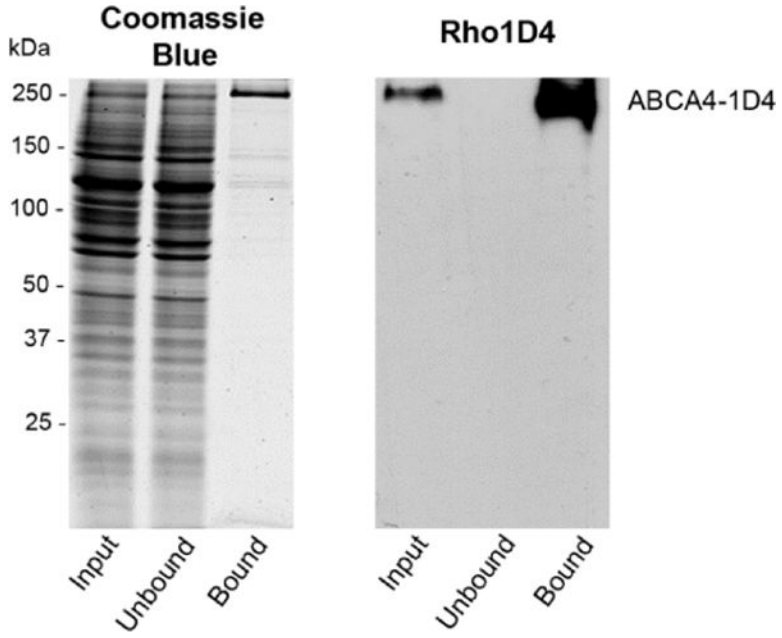

Figure 1.

Detection and purification of the 1D4-tagged ABC transporter ABCA4-1D4. HEK293T cells were transfected with pcDNA3 plasmid harboring the *ABCA4-1D4* cDNA. The cells were solubilized with 1% Triton X100 and incubated with Rho1D4 immunoaffinity matrix. After collecting the unbound fraction, the matrix was washed and the bound protein was eluted with the 1D4 peptide. The input, unbound and bound fractions were analyzed on SDS gels stained with Coomassie Blue and a Western blot labeled with the Rho1D4 antibody. This procedure produced a highly purified preparation of ABCA4-1D4.

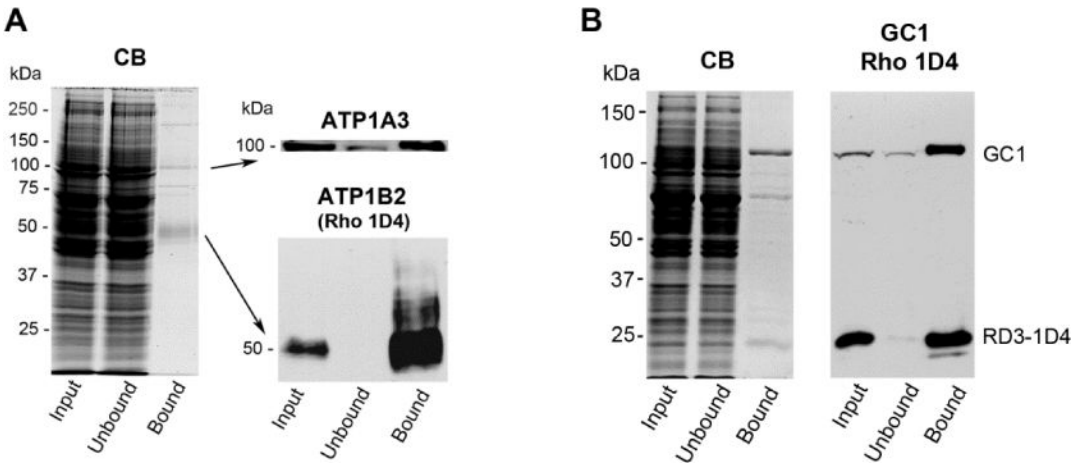

Figure 2.

Co-expression and co-immunoprecipitation of multisubunit proteins and interacting proteins from co-transfected HEK293T cells. A. HEK293T cells were co-transfected with pcDNA3 plasmids containing the cDNAs encoding the Na/K ATPase subunits ATP1A3 and the tagged ATP1B2-1D4. The cells were solubilized in Triton X-100 and applied to a Rho1D4 immunoaffinity matrix. The input, unbound, and bound (1D4 peptide-eluted) fractions were analyzed on SDS gels and Western blots labeled with an ATP1A3 polyclonal antibody and Rho 1D4 monoclonal antibody for the detection of ATP1B2-1D4. B. HEK293T cells co-expressing RD3-1D4 and guanylate cyclase 1 (GC1) were solubilized in Triton X-100 and applied to a Rho1D4 immunoaffinity matrix. The input, unbound, and bound fractions were analyzed on SDS gels and Western blots labeled with Rho1D4 antibody (RD3-1D4 detection) and anti-GC1 antibody (GC1 detection).

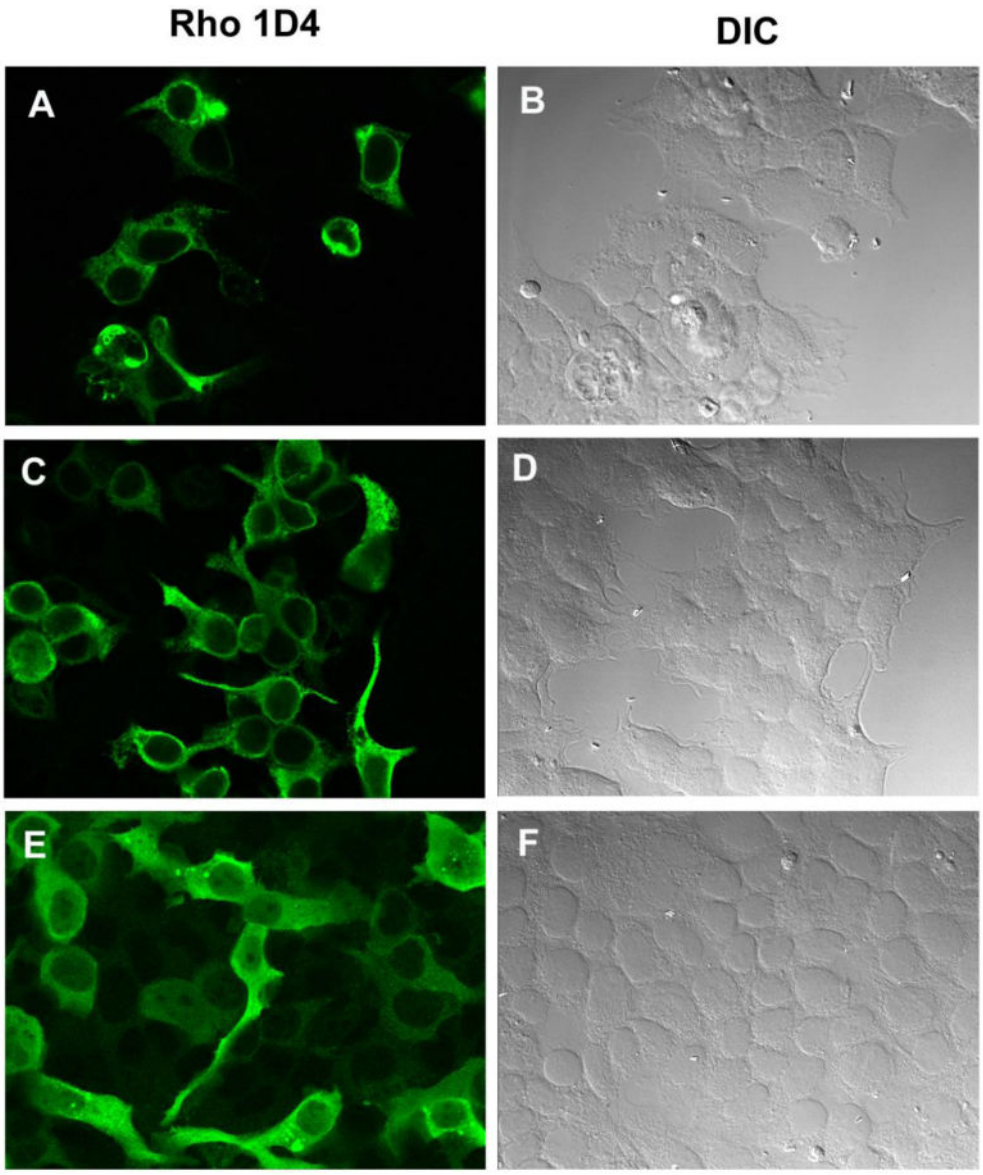

Figure 3.

Immunofluorescence microscopy of HEK293T cells expressing ABCA4-1D4 (A,B); co-expressing ATPase subunits ATP1A3 and ATP1B2-1D4 (C,D); and interaction proteins RD3-1D4 and guanylate cyclase GC1 (E,F). The cells were labeled with the Rho1D4 antibody for immunofluorescence imaging. The total number of cells was visualized using differential interference contrast (DIC) imaging. Not all cells expressed the ATPase subunits and hence serve as internal control for antibody specificity.