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Rab8-optineurin-myosin VI: Analysis of interactions and functions in the secretory pathway

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Abstract

The small GTPase Rab8 has been shown to regulate polarised membrane trafficking pathways from the TGN to the cell surface. Optineurin is an effector protein of Rab8 and a binding partner of the actin based motor protein myosin VI. In our lab we used various approaches to study the interactions between myosin VI and its binding partners and to analyse their role(s) in intracellular membrane trafficking pathways. In this chapter we describe the use of the mammalian two-hybrid assay to demonstrate protein-protein interactions and to identify binding sites. We describe a secretion assay, which was used in combination with RNA interference technology to analyse the function of myosin VI, optineurin and Rab8 in exocytic membrane trafficking pathways.

Introduction

The Rab proteins are a large family of small GTPases that participate in and regulate intracellular membrane trafficking pathways (Zerial and McBride, 2001). For example, Rab8 plays an important role in exocytic membrane traffic from the Golgi complex to the plasma membrane in polarised epithelial cells (Huber et al., 1993b), in photoreceptor cells (Moritz et al., 2001) and in polarised neurons (Huber et al., 1993a). It is localised in vesicles at/around the trans Golgi network, in recycling endosomes and at the plasma membrane in membrane ruffles (Peranen et al., 1996) (Ang et al., 2003). Rab8 binds to optineurin (Hattula and Peranen, 2000), an adaptor protein associated with the Golgi complex, which is known to bind to the actin based motor protein myosin VI (Sahlender et al., 2005) and to huntingtin, the protein mutated in Huntington's disease (Faber et al., 1998). The characterisation of Rab8 effector proteins and complexes is required to establish the precise role of Rab8 in exocytic membrane trafficking pathways. Furthermore, this approach will help us to understand the role of Rab8 and its effector proteins in a number of diseases.

Rab8 is linked by optineurin to myosin VI, which has been shown to be involved in endocytic and exocytic membrane trafficking pathways. Mutations in the myosin VI gene in mouse and humans are linked to deafness (Avraham et al., 1995) (Ahmed et al., 2003) and to hypertrophic cardiomyopathy (Mohiddin et al., 2004), and the myosin VI knock out mouse also has neurological abnormalities (Osterweil et al., 2005). The specific intracellular functions of myosin VI are mediated by a range of different interacting proteins (Buss et al., 2004). Optineurin was identified as a myosin VI binding partner in a yeast two-hybrid

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screen and was shown to link myosin VI to the Golgi complex and to mediate its function in the secretory pathway (Sahlender et al., 2005) (Au, 2007). This interaction between myosin VI and optineurin may be of medical interest, since mutations in the human optineurin gene cause primary open angle glaucoma (Rezaie et al., 2002). Optineurin is a 67 kDa protein that contains two leucine zippers and one zinc finger separated by large stretches of coiled coil. This myosin VI binding partner is the cellular target of the adenoviral protein Ad E3-14.7 K, which inhibits tumor necrosis factor alpha (TNF- α) induced cytolysis, an important defence mechanism to protect cells against viral infection (Li et al., 1998). Optineurin also links Rab8 to huntingtin, the protein mutated in the progressive neurodegenerative disorder Huntington's disease (Hattula and Peranen, 2000). Although a wealth of data suggests that polyglutamine-mediated aggregation of huntingtin is a major cause of the disease, new evidence suggests that loss of normal huntingtin function may also play a role in Huntington's disease pathogenesis. Wildtype huntingtin is present at/around the TGN and on vesicles and is suggested to play a role in endocytosis and exocytosis (Velier et al., 1998) (DiFiglia et al., 1995).

Therefore, to understand the function of Rab8, optineurin, myosin VI and huntingtin in exocytic membrane trafficking pathways, it is of vital importance to establish how they interact in defined protein complexes.

This article describes the experimental approaches we have used to identify and verify myosin VI binding partners and methods to test their function in the secretory pathway.

Identification of myosin VI binding partners

The two-hybrid approach in yeast has been used extensively during the past decade as a tool to identify and characterise macromolecular interactions. The yeast two-hybrid system is an *in vivo* system that identifies interacting proteins by the reconstitution of active transcription factor complexes (Fields and Song, 1989) (Chien et al., 1991). A protein of interest is fused to a DNA-binding domain of a transcription factor, while a second protein is fused to an activating domain. If the proteins of interest interact, an active transcription factor is formed and expression of specific reporter gene occurs. The yeast two-hybrid system can be used in two main ways: to screen a library for novel interacting proteins (Stephens and Banting, 2000) or to further characterise binding of known interactors, for example by mapping interacting domains (Eugster et al., 2000). As a first step to identify myosin VI binding partners we performed a yeast two-hybrid screen. The myosin VI tail was used as a bait to screen a human umbilical vein epithelial cells cDNA library. To minimise self-activation of reporter gene expression by the myosin VI tail, we used the GAL4-based ProQuest Two-Hybrid System (Invitrogen), in which HIS3 reporter sensitivity was titrated with increasing concentrations of 3-amino-1,2,4-Triazole (3AT) as described in the ProQuest Instruction Manual (Invitrogen). Using this method one million yeast transformants were screened yielding 14 different positive interactors.

Verification of yeast two-hybrid screen results

Since one of the major problems with the yeast two-hybrid system is the isolation of false positive interactions, we used a range of different *in vitro* and *in vivo* assays to verify "true" myosin VI interacting proteins. First of all we used a mammalian two-hybrid system to demonstrate that the interactions occur in mammalian cells, where the expressed proteins are more likely to be in their native conformation. Furthermore, since myosin VI is not expressed in yeast, the mammalian cell is more likely to express, for example, regulatory kinases or phosphatases that might be involved in controlling the interaction between myosin VI and its binding partner. Next we identified and mutated the exact binding site and

showed for several binding partners such as optineurin, Dab2 and GIPC that highly specific binding to the C-terminal tail of myosin VI depends on a short amino acid sequence (Sahlender et al., 2005) (Spudich et al., 2007). In the case of Dab2 a single point mutation can abolish binding. To further test whether the interactions between the proteins were direct or part of a larger protein complex we performed GST pull down assays of bacterial expressed and purified myosin VI tail and *in vitro* translated optineurin or *in vitro* translated Rab8. In addition we immunoprecipitated myosin VI/optineurin containing protein complexes under native conditions from a variety of tissue culture cells to verify the interaction *in vivo*. We also performed localisation studies of myosin VI and its binding partners by indirect immunofluorescence to establish whether both proteins are present on the same intracellular compartment such as for example the Golgi complex or intracellular vesicles. Finally, we performed functional studies on siRNA knock down cells or on cells over expressing dominant negative mutants, to see whether myosin VI and its proposed binding partner were functioning in the same intracellular pathway and whether the ablation leads to similar phenotypes.

Since GST-pull down assays and co-immunoprecipitation are standard procedures described elsewhere in great detail (see for example (Monier and Goud, 2005) (Johansson and Olkkonen, 2005) we will focus first of all on the mammalian two-hybrid assay and its use to verify and further dissect protein-protein interaction. To identify and characterise proteins involved in constitutive secretion we have developed a SEAP assay, which is described in the second section.

Mammalian two-hybrid assay

The principles of the mammalian two-hybrid system are the same as for the yeast two-hybrid system, namely that the protein of interest is fused to a DNA binding domain and the potential interacting protein is fused to an activating domain of a transcription factor. If the proteins of interest interact, an active transcription factor is formed and expression of the luciferase reporter occurs. Figure 1 illustrates the components of the mammalian two-hybrid assay. Four plasmids carrying two proteins of interest fused to transcription factor domains, an inducible reporter gene and a constitutively expressed transfection control are co-transfected into Chinese hamster ovary (CHO) cells. pG5luc (Promega) codes for the inducible reporter – firefly luciferase under control of the GAL4 regulatory element. *Renilla* luciferase is coded by pRL-CMV (Promega), it is constitutively expressed and thus provides measurement of transfection efficiency. This allows variations in vector uptake to be taken into account when assaying induced firefly luciferase activity. The bait vector, pM (Clontech) codes for the GAL4 DNA binding domain fused to N-terminus of the myosin VI tail and the prey vector, pV16 (Clontech) expresses the myosin VI binding partner optineurin fused to the activating domain derived from the VP16 protein of herpes simplex virus. When two proteins interact, the reconstituted active transcription factor binds to the GAL4 regulatory DNA element in the pG5luc plasmid and the firefly luciferase is expressed. A *NotI* site was introduced into the vectors pM and pV16 downstream from the *SaII* site in the Multiple Cloning Sites (KJ Patel, MRC-LMB, Cambridge, UK). The modified vectors were referred to as pM* and pV16*. In this way, cDNA from the ProQuest yeast two-hybrid system was directly subcloned into the mammalian two-hybrid vectors.

The relative luciferase activity was determined by calculating the ratio of inducible (firefly) to constitutively active (*Renilla*) luciferase activity for cells transfected with MyoVI-tail/pM* and optineurin/pV16*, divided by the ratio of inducible to constitutively active luciferase from cells transfected with MyoVI-tail/pM* and empty pV16*. A significant interaction was deemed to occur when the relative luciferase activity was greater than 25.

$$\text{Activity} = \frac{\text{Renilla/firefly from MyoVI-tail/pM}^* \text{ and optineurin/pV16}}{\text{Renilla/firefly from MyoVI-tail/pM}^* \text{ and pV16empty}}$$

Initially we used the mammalian two-hybrid assay to confirm the interaction between myosin VI and optineurin in mammalian cells *in vivo*. As a next step we used deletion mutants and site-directed mutagenesis to map the exact binding site for optineurin on myosin VI. Optineurin binds to the RRL motif (aa 1107-1109) in the globular tail domain of myosin VI. Mutation in the WWY, the Dab2 binding motif in the myosin VI tail, did not affect optineurin binding (Figure 2).

Protocol for the mammalian two-hybrid assay

The mammalian two-hybrid assay was performed in CHO (Chinese hamster ovary) cells obtained from the European Collection of Animal Cell Cultures. CHO cells were cultured in F-12 HAM medium supplemented with 10 % fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C and 5 % CO₂. For transfection the cells were seeded out and grown to about 50-60% confluency in 35 mm tissue culture dishes. The cells were transfected with highly purified plasmid DNA using FuGENE™ transfection reagent (Roche Diagnostics, UK) according to the manufacturer's instructions. Each dish was co-transfected with 0.8 µg pG5luc, 0.8 µg pRL-CMV, 1 µg MyoVI-tail/pM* and 1 µg optineurin/pV16* using 12 µl of FuGENE™. As a negative control background luciferase activity was measured in cells transfected with MyoVI-tail/pM* and empty pV16* together with both luciferase plasmids. As a positive control MyoVI-tail/pM* and Dab2/pV16* were used. Cells were grown for 48 to 72 h with a daily change of medium. To measure the luciferase activity a Dual-Luciferase Reporter Assay System (Promega) was used. After a single wash in PBS the cells were lysed in 500 µl Passive Lysis Buffer (Promega) for 30 min at room temperature on a shaker. The lysate including the cell debris was collected and either used immediately for quantitation of the luminescent signal or frozen at -70°C for later use. The Dual-Luciferase Reporter Assay was performed according to the instructions in the manual (Promega). The measurements were performed on an Orion Microplate Luminometer (Berthold Detection Systems). For the assay 10 µl samples of the cell lysates were pipetted onto a white 96-well plate. 50 µl of the luciferase assay reagent (LARII) was mixed with the samples and the firefly luciferase luminescence measured immediately. Into the same well 50 µl of the Stop & Glo Reagent was dispensed. This reagent quenches the firefly reaction and activates the *Renilla* luciferase, which gives the second measurement.

Notes:

1. A negative control must be done in each experiment as the transfection efficiency and luciferase activity may vary significantly from experiment to experiment.
2. As a standard control, an insert swap between pM and pVP16 is recommended.
3. We have used a luminometer which allows automatic substrate dispensing and processes the samples successively, one at a time. If only manual substrate addition and measurements are possible, it is not recommended to measure more than few samples at a time, because the luminescence fades very fast.
4. Although the Promega Manual recommends the use of relatively small quantities of a control reporter vector, i.e. ratios of up to 1:50 for co-reporter vector:experimental vector, we found that reproducible measurements can only be obtained when this ratio is at least 1:5.

5. In our experience, CHO cells gave higher luciferase activity than HeLa cells possibly because of a combination of higher transfection rates and higher expression levels.
6. When luciferase activity is low, more concentrated lysate can be prepared by scraping cells into a smaller amount of lysis buffer followed by vigorous pipetting. In this way the volume of lysis buffer used for a 35 mm dish can be reduced to 200 μ l.

SEAP secretion assay

Myosin VI and optineurin are present at the Golgi complex and both proteins have been shown to play a role in maintenance of Golgi morphology and constitutive secretion (Sahlender et al., 2005) (Warner et al., 2003). To measure constitutive secretion, we expressed a secreted form of alkaline phosphatase (SEAP) in tissue culture cells and quantified the release of the enzyme into the tissue culture medium. The SEAP used is an engineered variant of the placental alkaline phosphatase (PLAP), which is normally anchored to the plasma membrane via a phosphatidylinositol-glycan (Berger et al., 1988b). The engineered version has a C-terminal truncation and is therefore efficiently secreted and no longer linked to the plasma membrane (Berger et al., 1988a). In contrast to other forms of alkaline phosphatases that are found in bones and intestine, SEAP is heat resistant and survives heat inactivation up to 65 °C, and so its activity can be measured in the presence of endogenous alkaline phosphatases. SEAP was originally described as a reporter enzyme to measure gene expression in eukaryotic cells (Cullen, 2000). The pSEAP2-control vector expresses SEAP under control of the SV40 early promoter and can be purchased from Clontech.

Measurement of SEAP secretion in fibroblasts from the myosin VI knock out mouse

To measure secretion in cells lacking myosin VI, fibroblasts were isolated from the myosin VI knock out mouse (Snell's waltzer mouse). A litter of new born mice was genotyped using a PCR based method as described in (Self et al., 1999). Skin and muscle tissue was cut into small pieces and incubated in 0.25 % trypsin for 15-30 min at 37 °C. After gentle pipetting large tissue lumps were allowed to fall to the bottom of the tube, and cell suspension was collected and the cells plated in growth medium (DMEM supplemented with 10 % fetal calf serum, 2 mM glutamine and 60 μ M 2-mercaptoethanol). The cells were split every 2-3 days and after several months a number of immortal cell lines from wild type and Snell's waltzer mice were generated. For transient transfections with the pSEAP2-control plasmid, the cells were grown on 35 mm dishes to 50-60 % confluency and transfected with 2 μ g of plasmid using FuGENE™ according to the manufacturer's instructions. The transfection efficiency of the pSEAP2-control plasmid was normalised by cotransfection of a second plasmid expressing GFP. Since the transfection efficiency of the mouse fibroblasts was generally very low, usually around 5%, for detection we used a chemiluminescent assay based on the substrate CSPD (disodium 3-(4-methoxyspiro(1,2-dioxethane-3,2'-(5'-chloro)tricyclo decan-4-yl)phenyl phosphate) (Clontech). Samples of the culture medium were taken every 24 hours for a total of 4 days and assayed in the chemiluminescent SEAP assay according to the manufacturer's handbook (Great EscAPE SEAP User Manual, Clontech). The assay was performed in 96-well flat bottom microtiter plates suitable for plate luminometers. In brief, 15 μ l aliquotes of the culture medium were diluted with 45 μ l of dilution buffer and heated up to 65 °C for 30 min. After cooling the samples to room temperature, 60 μ l of assay buffer and 60 μ l of diluted CSPD substrate were added, and 15-60 min later measurements were performed with a plate luminometer (Berthold Detection Systems, Orion Luminometer). Growth medium taken from untransfected cells was used for base line measurement.

Measurement of SEAP secretion in siRNA knock down cells

To study protein secretion in siRNA knock down cells, stable cell lines expressing SEAP were generated. The SEAP cDNA was subcloned from pSEAP2-control plasmid into the mammalian expression vector pIRES-neo2 using *NheI* and *HpaI* restriction enzymes. The resulting construct was transfected into two 35 mm culture dishes of HeLa cells using FuGENE. Two days after transfection the cells were trypsinised and transferred to a 10 cm culture dish. Selection commenced the next day with 500 µg/ml of the antibiotic G418. After 1-2 weeks single surviving clones were picked and transferred to a 24 well plate. 20-30 clones were tested for SEAP secretion and about 10 clones were selected with a range of levels of SEAP secretion. Selected clones were grown on and stocks were frozen in 10% DMSO, 25 % fetal calf serum in RPMI and kept in liquid nitrogen.

HeLa cells stably expressing SEAP were routinely cultured in the presence of 500µg/ml G418, but medium with no antibiotic was used during knock down experiments. For siRNA knock down SEAP expressing cells were plated on 6 or 24-well plates, transfected with myosin VI or optineurin siRNA duplexes twice (on day 1 and 3), medium was changed on day 4 and SEAP was let to accumulate in the medium for further 1-2 days. On the day of the assay, small aliquots of culture medium were collected and used for assay immediately or were frozen at -70°C. The basic protocol for the SEAP activity assay was taken from www.genetherapysystems.com and slightly modified as described. Growth medium, which did not have contact with cells, was used for base line measurement.

Protocol for SEAP assay

Growth medium from control or siRNA treated SEAP expressing cells was collected and heated to 65°C for 30 min. In a 96 well plate 10µl of 0.05% Zwittergent (Calbiochem) (0.5% stock in water, dilute 1:10 before the assay) was mixed with 20µl of heated culture medium (or 20µl heated growth medium for base line measurements) followed by 200µl PNPP substrate. To prepare the substrate PNPP (para-nitrophenyl phosphate disodium salt, Sigma 104 phosphatase substrate) was dissolved in 1M Diethanolamine pH 9.8 and 1mM MgCl₂ to 1 mg/ml immediately before the assay. The reaction was incubated for 20-30 minutes at room temperature in the dark and the absorbion measured at 405 nm in a spectrometer such as the Anthos HTII plate reader or similar.

Notes:

1. SEAP secretion data have to be normalised to the cell number, as cell density may vary dramatically between cells treated with different siRNA oligos.
2. We have extensively tested the HeLa cell lines stably expressing SEAP and found that they behave as normal HeLa cells in all standard applications such as transfection, siRNA knock downs or immunofluorescence. They can be successfully used for assaying secretion after transfection of different constructs (for example, dominant negative tail of myosin VI or Rab8Q67L mutant), given that the transfection efficiency is high enough so that the decrease/increase in secretion in transfected cells is not masked by bulk SEAP secretion by cells, which do not express the construct of interest.
3. Since only small amounts of culture medium are needed for the assay and the cells themselves are left intact, the cells from the secretion assay can also be used, for example, for Western blotting to monitor levels of protein depletion/expression or for immunofluorescence if plated on coverslips.

4. The SEAP is constitutively secreted and thus always present in the culture medium. In order to see small changes in secretion after manipulating the cells some extra work may be required to find suitable experimental conditions for each protein/construct of interest. For example, HeLa clones with different levels of SEAP secretion can be tested and SEAP can be allowed to accumulate in freshly changed medium for different times – from 6 hours to 3 days.

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Figure 1. Schematic flow diagram of the mammalian two-hybrid assay

Both plasmids coding for the two proteins of interest fused to transcription factor domains (pM and pV16) are co-transfected with an inducible reporter gene (pG5-luc) and a constitutively expressed transfection control (pRL-CMV) into CHO cells. After 72 h the cells are lysed and the lysates assayed in 96 well plates using the Dual-Luciferase reporter Assay System, which supplies the LARII and Stop&Glo reagents as substrates to assay the firefly and *Renilla* luciferase, respectively.

Figure 2. Mammalian two-hybrid assay to characterise interaction of optineurin and myosin VI Optineurin in pVP16 vector was transfected into CHO cells together with the pM vector encoding the globular tail of myosin VI (aa 1034-1276) (GT-LI), the deletion mutant lacking the large insert (GT Δ LI), or the tails carrying point mutations RRL->AAA (aa 1107-1109) or WWY->WLY (aa 1183-1185). The luciferase activity was assayed as described in the text. The graph shows the result from a single representative experiment.

Figure 3. SEAP assay to measure constitutive secretion in myosin VI and optineurin knock down cells

A stable HeLa cell line expressing SEAP was transfected twice with siRNA specific for myosin VI (MyoVI) or optineurin (Opt). The activity of alkaline phosphatase secreted into the culture medium was measured 48 h after the last transfection. The results shown are mean $\pm$ SD from three independent experiments.