Protein Expression and Purification 90 (2013) 160–169

Contents lists available at SciVerse ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Recombinant expression, detergent solubilisation and purification of insect odorant receptor subunits

Colm Carraher [a,b], Ali Reza Nazmi [c], Richard D. Newcomb [a,b], Andrew Kralicek [a,*]

[a] The New Zealand Institute for Plant & Food Research Limited, Private Bag 92169, Auckland 1142, New Zealand
[b] School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland 1142, New Zealand
[c] Biomolecular Interaction Centre, Department of Chemistry, University of Canterbury, Private Bag 4800, Christchurch 8140, New Zealand

ARTICLE INFO

Article history:
Received 14 March 2013
and in revised form 31 May 2013
Available online 13 June 2013

Keywords:
Insect odorant receptor
Orco
Zwittergent 3–16
Sf9 cell
Wheat germ
Cell-free
Protein expression
Purification
Liposome

ABSTRACT

Insect odorant receptors (ORs) are seven transmembrane domain proteins that comprise a novel family of ligand-gated non-selective cation channels. The functional channel is made up of an odour activated ligand-binding OR and the OR co-receptor, Orco. However, the structure, stoichiometry and mechanism of activation of the receptor complex are not well understood. Here we demonstrate that baculovirus-mediated Sf9 cell expression and wheat germ cell-free expression, but not *Escherichia coli* cell-based or cell-free expression, can be used successfully to over-express a selection of insect ORs. From a panel of 19 detergents, 1% w/v Zwittergent 3–16 was able to solubilise five *Drosophila melanogaster* ORs produced from both eukaryotic expression systems. A large-scale purification protocol was then developed for DmOrco and the ligand-binding receptor, DmOr22a. The proteins were nickel-affinity purified using a deca-histidine tag in a buffer containing 0.2 mM Zwittergent 3–16, followed by size exclusion chromatography. These purified ORs appear to form similarly sized protein-detergent complexes when isolated from both expression systems. Circular dichroism analysis of both purified proteins suggests they are folded correctly. We also provide evidence that when DmOrco is expressed in Sf9 cells it undergoes post translational modification, probably glycosylation. Finally we show that the recombinant ORs can be incorporated into pre-formed liposomes. The ability to recombinantly express and purify insect ORs to homogeneity on a preparative scale, as well as insert them into liposomes, is a major step forward in enabling future structural and functional studies, as well as their use in OR based biosensors.

Introduction

Insect odorant receptors (ORs)[1] are ligand-gated non-selective cation channels [1,2], with some evidence suggesting that their signaling is modulated metabotropically [2]. Together with ionotropic glutamate receptors (IRs) [3], ORs allow insects to detect volatile compounds associated with mating, predation and food localisation. ORs are localised in the dendritic membrane of olfactory sensory neurons housed in sensilla on the insects' antennae and maxillary palps [4]. Structurally these receptors contain seven transmembrane helices, but have an inverted orientation in the membrane with respect to G protein-coupled receptors, with an intracellular N-terminus and an extracellular C-terminus [5–7].

The insect odorant receptor complex comprises at least two subunits, one of which is the ubiquitous co-receptor, Orco [8,9], and a second that is a ligand-binding receptor subunit (OrX). Orco is essential for ion channel function in ORs [9] and is highly conserved at the sequence level across insect orders [10]. This is in stark contrast with the rapidly evolving ligand-binding ORs [11,12]. Although Orco does not generally respond to odours, a range of compounds that can activate this co-receptor have recently been identified (VUAA1–4 [13–16]). Functionally Orco is highly conserved, with *Drosophila melanogaster* null mutants of Orco capable of being rescued by Orco orthologues from other insect orders [17], and *in vivo* experiments demonstrating that Orco subunits from different species produce similar response profiles to those of odorants when paired with the same OrX [18,19]. Inhibitor experiments suggest that both Orco and OrX are involved in forming the pore of the ion channel [18]; however, this needs to be confirmed. Whilst there is evidence from resonance energy transfer and complementation studies that Orco and OrX form heteromers [6,20,21], the exact size and stoichiometry of the complex

* Corresponding author.
E-mail address: Andrew.Kralicek@plantandfood.co.nz (A. Kralicek).

[1] *Abbreviations used:* ORs, insect odorant receptors; IRs, ionotropic glutamate receptors; TEV, tobacco etch virus; IPTG, isopropyl-1-thio-β-D-galactopyranoside; CECF, continuous exchange cell-free; RNAP, RNA polymerase; MOI, multiplicity of infection; SEC, size exclusion chromatography; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; Ch, cholesterol; DGU, density gradient ultracentrifugation; MBP, maltose binding protein; PpiB, peptidyl-prolyl isomerase B; MW, molecular weight; CMC, critical micelle concentrations; SDS, sodium dodecyl sulphate.

1046-5928/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.pep.2013.06.002

or structure of any subunit are unknown. One approach to address these questions is to use recombinant gene technologies to express the OR subunits in either surrogate cells or *in vitro* to facilitate downstream structural and biochemical studies.

Most research on insect ORs has been conducted *in vivo* in *Drosophila* [6,22,23], whilst expression of these novel receptors in surrogate eukaryotic cell systems has been limited. Much of the *in vitro* research has focused on using the cell-based expression systems to study responses of OrXs to various ligands using calcium assays [2,3,5,24]. In addition, some OR subunits have been isolated from stably expressing surrogate cells and used for pull-down assays to investigate receptor subunit interactions [25]. *D. melanogaster* Orco has also been expressed in a rabbit reticulocyte cell-free system to study the topology of the protein using endogenous and engineered glycosylation sites [7].

Here we explore the use of prokaryotic and eukaryotic cell-based and cell-free expression systems for producing recombinant insect OR subunits. Baculovirus-mediated expression in insect Sf9 cells and cell-free expression using the wheat-germ system proved to be the most effective to produce these proteins for purification. We also test a diverse panel of detergents for their ability to solubilise OR subunits and develop protocols for the purification of his-tagged ORs from membranes. Finally, we show that the purified protein can be inserted into pre-formed liposomes either directly using cell-free synthesis, or post-purification using purified material from baculovirus expression.

Materials and methods

Cloning for Escherichia coli cell-based and cell-free expression studies

Four insect ORs (*Drosophila melanogaster* DmOr10a, DmOr22a and DmOr43b; *Epiphyas postvittana* EpOr1) were *de novo* synthesized by Genscript (Piscataway, NJ, USA) with a sequence encoding a 5' *Not*I site followed by the Tobacco Etch Virus (TEV) protease cleavage site and an *Nde*I site [26], then an *E. coli* codon-optimised version of the OR gene sequence followed by a 3′ *Bam*HI site. Restriction digests of these four constructs with *Not*I and *Bam*HI enabled cloning into the following derivatives of the tandem λ promoter vector pND707 [27], pND707-cMBP-his$_6$, pND707-ppiB-his$_6$, pND707-thioredoxin-his$_6$ to produce pND707 derivatives expressing different N-terminally peptide tagged versions of each OR with a C-terminal his$_6$ tag. Each resulting pND707-*Nde*I-cMBP-*Not* I-TEV-*Nde*I-OR-*Bam*HI-his$_6$ vector was then digested with *Nde*I to remove the MBP tag, and re-ligated to create pND707 derivatives encoding just the OR-his$_6$ sequence.

In order to create T7 promoter vectors expressing an OR with or without the N-terminal cMBP, PpiB or thioredoxin tags, cloning of the constructs was performed exactly as above except into the following pETMCSI [28] derivatives: pETMCSI-cMBP-his$_6$, pETMCSI-ppiB-his$_6$, pETMCSI-thioredoxin-his$_6$, respectively. To create tac promoter vectors expressing non-tagged versions of each OR, the pMALc2x derivative vector, pMALc2-*Nde* I-MBP-ProW-*Bam* HI-his$_6$, was cut with *Nde*I and *Bam*HI, and the ProW gene replaced with each *Nde*I-OR-*Bam*HI fragment, producing four pMAL2c-OR-*Bam*HI-his$_6$ derivative plasmids.

Cloning for insect cell and wheat germ cell-free expression studies

Five odorant receptors from *D. melanogaster* were used in the eukaryotic expression systems, DmOrco, DmOr10a, DmOr22a, DmOr35a and DmOr43b. Each of the odorant receptor genes was tagged with a combination of N-terminal epitope tags to aid in protein purification, and visualisation on Western blots.

The tags His$_{10}$-Flag and His$_{10}$-Myc, were added to the N-terminus using PCR amplification. PCR primers were designed to add a TEV cleavage site to the N-terminus of each gene to aid PCR amplification and to enable the subsequent removal of epitope tags. These primers constituted the sequence 5′-GAA AAC CTG TAT TTT CAG GGA (10–15 bp of the target gene)-3′. The methionine-encoding ATG was removed to prevent translation of untagged protein and a new methionine codon added 5′ of the additional tag. These TEV modified constructs were inserted into the pCR8/GW/TOPO vector (Life Technologies).

Primers were then designed to add a Flag or Myc tag onto the TEV site, and a further round of PCR added the His$_{10}$ tag to the N-terminus of either the Flag or Myc epitope. This resulted in constructs of the general form Tag-TEV-Gene. All forward primers were designed with a CACC motif at their 5′ end. This allowed the PCR products to be inserted into the pENTR/D-TOPO directional cloning vector (Life Technologies, Carlsbad, CA, USA). All primers are listed in Supplementary Table 1. The genes were then gateway cloned into the destination vectors pDEST8 (Life Technologies) for baculovirus expression, and pEU-DEST for wheat germ cell-free expression, using the LR clonase II enzyme (Life Technologies). The vector pEU-DEST was made by digesting pEU-E01-MCS (CellFree Sciences) with *Eco*RV and *Kpn*I and inserting the PCR amplified *att*R1-Cm^R-*ccd*B-*att*R2-*KpnI* region of pDest8 (Life Technologies). All plasmids were transformed into DH5α chemically competent cells (Life Technologies). Plasmids were Sanger sequenced at Macrogen (Seoul, Korea) using plasmid-specific primers. DNA sequence analysis was performed using GENEIOUS Pro version 6.0.4 (Biomatters Ltd, New Zealand http://www.geneious.com).

Plasmids to be used for cell-free protein expression were transformed into DH5α chemically competent cells (Life Technologies) and DNA was extracted from 100 mL cultures using a Plasmid Midi-Kit (Qiagen). Those being used for wheat germ cell-free expression were further purified by a phenol/chloroform procedure according to the protocol from CellFree Sciences (Yokohama, Japan). The DNA was finally diluted to 1 μg/μL and frozen at −80 °C.

Escherichia coli cell-based expression studies

For expression trials under the control of tandem λ promoters, the pND707 vectors expressing an OR with or without the N-terminal cMBP, PpiB or thioredoxin tags were transformed into the *E. coli* strain, C43 BL21(DE3) [29]. Five hundred mL cultures were then grown at 30 °C with shaking at 200 rpm, to either A_{595} of 0.5 or 1.0 in LB containing 50 μg/mL ampicillin. Production of ORs was induced by rapid shift of each culture to 42 °C; specifically, the culture was immersed in a 70 °C water bath for approximately 2 min. Over-expression was then maintained by placing the cultures at 42 °C for a further 3 h with shaking at 200 rpm.

For expression trials under the control of the T7 promoter, the pETMCSI derivative vectors were also transformed into the C43 BL21(DE3) strain and 500 mL cultures were grown at 37 °C to A_{595} of 0.5 in LB containing 50 μg/mL ampicillin. Over-expression was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and the culture was incubated for three hours at 37 °C. In the case of the tac promoter expression trials, C43 BL21(DE3) strains transformed with each of the pMALc2x derivative plasmids were grown in 500 mL cultures at 37 °C to A_{595} of 0.5 in LB ampicillin. OR synthesis was induced by the addition of 0.4 mM IPTG and the cultures were left at shaking 20 °C for a further 40 h.

Escherichia coli cell-free protein expression

Continuous exchange cell-free (CECF) protein synthesis of tagged and untagged ORs was performed as described previously