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Isotopic labeling of mammalian G protein-coupled receptors heterologously expressed in *Caenorhabditis elegans*

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ABSTRACT

High-resolution structural determination and dynamic characterization of membrane proteins by nuclear magnetic resonance (NMR) require their isotopic labeling. Although a number of labeled eukaryotic membrane proteins have been successfully expressed in bacteria, they lack post-translational modifications and usually need to be refolded from inclusion bodies. This shortcoming of bacterial expression systems is particularly detrimental for the functional expression of G protein-coupled receptors (GPCRs), the largest family of drug targets, due to their inherent instability. In this work, we show that proteins expressed by a eukaryotic organism can be isotopically labeled and produced with a quality and quantity suitable for NMR characterization. Using our previously described expression system in *Caenorhabditis elegans*, we showed the feasibility of labeling proteins produced by these worms with ^{15}N,^{13}C by providing them with isotopically labeled bacteria. ^{2}H labeling also was achieved by growing *C. elegans* in the presence of 70% heavy water. Bovine rhodopsin, simultaneously expressed in muscular and neuronal worm tissues, was employed as the "test" GPCR to demonstrate the viability of this approach. Although the worms' cell cycle was slightly affected by the presence of heavy isotopes, the final protein yield and quality was appropriate for NMR structural characterization.

Drug design, lead generation, and optimization are greatly facilitated if the structure of the biological target is known. This is particularly true when complexes between a ligand and target can be obtained. Although X-ray crystallography remains the current "gold standard" for structural determination, recent advances in solution–state nuclear magnetic resonance (NMR)[1] techniques to overcome molecular weight limitations offer an alternative approach for structural determination [1]. An added advantage of NMR structure determination is that it is less sensitive to disordered regions of the protein [2], allowing the analysis of protein targets that could be refractory to crystallization. Moreover, NMR offers the possibility of quantitative dynamics and binding studies for membrane proteins (MPs) complexed with ligands and drugs in a solution closely resembling their native environment. Despite the increasing importance of structure-based methods in modern pharmacological research and the fact that approximately 60% of drug targets are MPs [3], only a small fraction of protein structures solved to date at atomic resolution correspond to MP structures with a native sequence. The G protein-coupled receptor (GPCR) family of MPs represents the largest class of drug targets because drugs designed to interact with GPCRs are marketed in virtually every therapeutic area [4–8]. Structure-based drug design for GPCRs is advancing at a steady pace due to several crystal structures solved during the past few years. However, bovine rhodopsin remains the only vertebrate GPCR with a native sequence whose crystal structure has been determined at atomic resolution. Thus, novel technologies to elucidate the structures and provide conformational dynamics of GPCRs in native-like environments remain both highly desirable and challenging.

The only GPCR structure solved to date by solid-state NMR is that of a ligand-free form of chemokine receptor CXCR1 [9], which was ^{15}N and ^{13}C labeled in *Escherichia coli,* solubilized with sodium dodecyl sulfate (SDS) from inclusion bodies, purified in hexadecyl- and dodecylphosphocholine (DPC), and refolded in phospholipic proteoliposomes by detergent dialysis. Another somewhat successful example of expression of a GPCR in bacteria is the serotonin receptor 5-HT4 [10], which also needed to be refolded from 6 M

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[1] *Abbreviations used:* NMR, nuclear magnetic resonance; MP, membrane protein; GPCR, G protein-coupled receptor; SDS, sodium dodecyl sulfate; DPC, dodecylphosphocholine; TG, transgenic; NGM, nematode growth medium; (b)opsin, bovine aporhodopsin; IHC, immunohistochemistry; PAGE, polyacrylamide gel electrophoresis; WT, wild-type; LC–MS/MS, liquid chromatography–tandem mass spectrometry; ANOVA, analysis of variance; SILAC, stable isotope labeling with amino acids in cell culture.

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urea. A major disadvantage of expressing mammalian GPCRs in bacteria is the uncertainty about the percentage of protein that is correctly folded in the final reconstituted purified sample.

Here we describe the feasibility of triple isotopic labeling (^{2}H, ^{15}N, and ^{13}C) of proteins expressed in a eukaryotic system (*Caenorhabditis elegans*). We chose worms heterologously expressing bovine rhodopsin, a GPCR critical for vision signaling, as our primary target for proof of concept for two reasons: (i) rhodopsin's signature absorbance allows a convenient quality control for protocol optimization and (ii) rhodopsin's well-characterized biochemical properties allow functional comparisons of isotopically labeled and nonlabeled samples. Some advantages of this particular expression system include the following: (i) mammalian GPCRs expressed in transgenic (TG) worms are post-translationally modified and properly folded, (ii) they exhibit the same pharmacological, photochemical, and G protein signaling properties as do their counterparts obtained from a native source, (iii) scalability, (iv) phenotypic diversity, and (v) relatively facile genetic manipulation [11,12]. Proteins expressed in the worms can be easily labeled simply by providing them with ^{15}N,^{13}C-labeled *E. coli* or adding $^{2}H_2O$ to the worm culture media.

Here we demonstrate the feasibility of isotopically labeling mammalian GPCRs in the *C. elegans* expression system to characterize their structure, stability, interactions, and dynamics in solution by NMR. This strategy leverages the power of the *C. elegans* protein expression system for producing experimental quantities of GPCRs (or other MPs) combined with isotopic labeling to produce samples suitable for structure determination with state-of-the-art NMR methods.

Materials and methods

Maintenance of worms and generation of TG worm lines

Worms used for this study were maintained by standard methods [13], including culture on nematode growth medium (NGM) plates (0.25% peptone, 51 mM NaCl, 25 mM K_3PO_4, 5 μg/ml cholesterol, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) with OP50 bacteria, cryostorage, and recovery from stocks. Compositions of media and solutions, as well as detailed protocols for their use, were published previously in Ref. [13]. TG worm lines expressing bovine aporhodopsin ((b)opsin) in either muscles or neurons also have been described previously [11,12]. Hermaphrodites expressing (b)opsin in muscles were crossed to males expressing (b)opsin in neurons. By screening for the fluorescent marker DsRed in F3 progeny, we obtained a homozygous worm line expressing (b)opsin in both muscles and neurons ([M,N](b)opsin).

Stable isotope labeling of HB101

Unlabeled *E. coli* HB101 were grown in an incubated shaker (I2500 series, New Brunswick Scientific, Edison, NY, USA) (37 °C, 180 rpm) with M9 minimal medium of the following aqueous composition: 42.25 mM Na_2HPO_4, 279.41 mM KH_2PO_4, 8.56 mM NaCl, 18.70 mM NH_4Cl, 113.51 μM $CaCl_2$, 8.92 μM EDTA–Na_2, 15.41 μM $FeCl_3$, 1.50 μM $CuSO_4$, 1.19 μM $MnSO_4$, 0.1673 μM $ZnSO_4$, 0.2080 μM $CoCl_2$, 40.93 nM biotin, 33.24 nM thiamine, 2 mM $MgSO_4$, and 22.20 mM glucose. The medium was adjusted to pH 7.4 with 10 M NaOH.

The same culture conditions were used to culture isotopically labeled HB101 except that approximately 99% $^{2}H_2O$ (for ^{2}H labeling), 18.35 mM $^{15}NH_4Cl$ (for ^{15}N labeling), and 10.74 mM labeled glucose ($^{13}C_6H_{12}O_6$) (for ^{13}C labeling) were substituted for either H_2O, NH_4Cl, or glucose, respectively, in M9 minimal medium. All media were sterilized by filtration.

Stable isotope labeling of nematodes

For solid-phase culturing, worms were grown on peptone-free NGM plates with 51 mM NaCl, 25 mM K_3PO_4, 5 μg/ml cholesterol, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ in either H_2O or 700 g/L $^{2}H_2O$. For liquid-phase culture, worms were grown in S-medium (100 mM NaCl, 39.79 mM KH_2PO_4, 10.22 mM K_2HPO_4, 12.93 μM cholesterol, 10 mM citric acid monohydrate, 20.66 mM KOH, 3 mM $CaCl_2$, 3 mM $MgSO_4$, 24.89 μM $FeSO_4$, 55.32 μM EDTA–Na_2, 15.58 μM $ZnSO_4$, and 11.69 μM $CuSO_4$) in either H_2O or 800 g/L $^{2}H_2O$. Isotope-labeled worms were provided with HB101 containing the same isotope (e.g., ^{13}C,^{15}N-labeled HB101 for ^{13}C,^{15}N-labeled worms) using previously described worm culture protocols [14].

Analysis of worm brood sizes

Worms were synchronized to L1 (first larval stage) by standard methods [14]. Six L1 animals were transferred onto peptone-free NGM plates specially made with isotopic media and then provided with HB101 labeled with the same isotope. Total F1 larvae were counted.

Analysis of growth rates

Approximately 200 synchronized L1 worms were transferred into H_2O or $^{2}H_2O$ S-medium and provided with unlabeled or isotopically labeled HB101. Lifetime cycles (from L1 to L1 progeny) were quantified. The ratio of the lifetime cycle of control worms (46 ± 2 h) raised under nonlabeling conditions over the experimental worm lifetime cycle was defined as the relative growth rate.

Analysis of egg hatching rate

Synchronized young adult worms were raised in 70% $^{2}H_2O$ containing S-medium and provided ^{2}H-labeled (98%) HB101. A total of 100 of their eggs were transferred to S-medium containing unlabeled, ^{13}C-labeled, or ^{15}N-labeled HB101. Hatched F1 L1 worms were then observed for 4 days.

Immunohistochemistry

Immunohistochemistry (IHC) was performed as published previously [11,12]. Briefly, age-synchronized day 1 animals were sandwiched between two cover glasses, buried in dry ice for 30 min, and then fixed with 100% methanol (10 min) followed by 100% acetone (10 min). Then worms were washed with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4{\cdot}2H_2O$, and 1.76 mM KH_2PO_4, pH 7.4) for 0.5 h and incubated with PBS containing Alexa-488-conjugated 1D4 antibody and 0.1% Triton X-100 overnight at 4 °C. Stained worms were subsequently washed three times with PBS and examined by confocal microscopy. All experiments were done with a Leica TCS SP2 confocal microscope (Leica Microsystems, Bannockburn, IL, USA). Either live worms immobilized with 10 mM NaN_3 on 2% agarose pads or methanol/acetone-fixed worms were used. Fluorescent probes employed were DsRed (λ_{ex} = 543 nm, λ_{em} = 580–630 nm) and Alexa-488 (λ_{ex} = 488 nm, λ_{em} = 510–530 nm).

Immunoblotting

Immunoblotting was carried out by a published protocol [11]. Briefly, worms were sonicated and centrifuged to remove debris. The resulting supernatant was mixed in electrophoresis loading buffer, vortexed, and centrifuged briefly, and then samples were analyzed by immunoblotting after SDS–PAGE (polyacrylamide gel electrophoresis) on 4–12% Bis–Tris polyacrylamide gels (Invitrogen,