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Immunochemical strategy for quantification of G-coupled olfactory receptor proteins on natural nanovesicles

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ABSTRACT

Cell membrane proteins are involved in a variety of biochemical pathways and therefore constitute important targets for therapy and development of new drugs. Bioanalytical platforms and binding assays using these membrane protein receptors for drug screening or diagnostic require the construction of well-characterized liposome and lipid bilayer arrays that act as support to prevent protein denaturation during biochip processing. Quantification of the protein receptors in the lipid membrane arrays is a key issue in order to produce reproducible and well-characterized chips. Herein, we report a novel immunochemical analytical approach for the quantification of membrane proteins (i.e., G-protein-coupled receptor, GPCR) in nanovesicles (NVs). The procedure allows direct determination of tagged receptors (i.e., c-myc tag) without any previous protein purification or extraction steps. The immunochemical method is based on a microplate ELISA format and quantifies this tag on proteins embedded in NVs with detectability in the picomolar range, using protein bioconjugates as reference standards. The applicability of the method is demonstrated through the quantification of the c-myc-olfactory receptor (OR, c-myc-OR1740) in the cell membrane NVs. The reported method opens the possibility to develop well-characterized drug-screening platforms based on G-coupled proteins embedded on membranes.

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1. Introduction

G-protein-coupled receptors (GPCRs) are a large family of seven-transmembrane domain proteins involved in cell-response pathways to a variety of external signals and important ligands, such as hormones, neurotransmitters, and inflammatory mediators [1,2,3,4]. In fact, GPCRs are targeted by approximately 60% of all therapeutic drugs [5]. Therefore, technology platforms (i.e., bioassay and biosensor microarrays) for drug screening based on the use of these receptors would improve the efficiency of the drug development pipeline at pre-clinical stages. Receptor binding assays developed for this purpose require building liposomes or bilayer arrays [6,7], that act as support for these transmembrane proteins to prevent denaturation during biochip processing and to ensure their biofunctionality [8–11]. Native liposomes, isolated directly from cell sources containing recombinant membrane proteins, retain the fluidity and lipid order of the original cell membrane, parameters that are relevant to maintain the functionality of the receptor [12,13]. Natural vesicles (NVs) produced from genetically engineered cells have been reported to be promising building blocks for sensing biodevices [14–19].

Deciphering the number of functional receptor molecules on a biochip or sensor array is a key issue in order to accomplish reliable and reproducible results. In addition to peptide tags combined with covalent labeling [20] or mutagenesis assays [21], GPCRs can be determined by methods such as single-molecule [22] or TIRF microscopy [23]. However, these approaches are not suitable for the development of well-characterized membrane-based protein

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biosensor arrays. For this reason, we have focused on developing a straightforward method for detecting and quantifying tagged-membrane proteins or receptors such as c-myc-GPCRs in the form of lipidic NVs. The tag peptide can be fused to either the N- or the C-terminus of the protein of interest without changing the tertiary structure [24] or biological activity of the protein [25]. Although other techniques such as fluorescence polarization [26] have been used to quantify c-myc fused proteins, such approaches allow the quantification of only c-myc-protein concentration and not the number of c-myc-protein molecules per NV. To the best of our knowledge, this is the first report that accurately quantifies c-myc-tagged-GPCRs directly in the form of lipidic NVs.

2. Material and methods

2.1. Chemicals and immunochemicals

Biochemical reagents such as c-myc peptide (*EQKLISEEDL*) were purchased from Sigma Chemical Co. (St. Louis, MO). The commercial monoclonal anti-c-myc IgG1 9E10 antibody was supplied by Roche Diagnostics (Mannheim, Germany). *N*-succinimidyl 3-maleimidopropionate ($M(CH_2)_2CO$) and *N*-succinimidyl iodoacetate (CH_2CO) cross-linkers were synthesized in our laboratory as previously described [27,28]. Two modified c-myc peptides (peptide *EQKLISEEDL-Cys*, named C_1, and peptide *Cys-EQKLISEEDL*, named C_2) were synthesized as C-terminal amides using standard Fmoc solid-phase protocols by the Platform of Peptide Synthesis by CIBER-BBN Biomedicine Scientific Services (http://www.ciber-bbn.es). The two peptides were characterized using mass spectrometry. Purities were >94%, as assessed by HPLC (data not shown). These peptides were used as haptens for the preparation of bioconjugates and as analytes for the competitive assays. The corresponding bioconjugates were characterized by MALDI-TOF-MS. Information on the suppliers of the other chemical and biochemical reagents used, is provided in the Electronic Supplementary Information (ESI).

2.2. Buffers and solutions

Phosphate buffer saline (PBS, 10 mM) solution was prepared as a 0.8% saline solution at pH 7.5. The PBST buffer contained PBS with 0.05% Tween 20 and the potassium dihydrogen phosphate/disodium hydrogen phosphate (PBT, 10 mM) buffer was prepared at pH 7.5 with 0.05% Tween 20. The substrate solution contained 0.01% TMB (3,3′,5,5′-tetramethylbenzidine) and 0.004% H_2O_2 in citrate buffer. TMB acts as a hydrogen donor for the reduction of hydrogen peroxide to water by horseradish peroxidase (HRP), producing a diimine with a blue color (λ = 650 nm). The addition of acid such as H_2SO_4 changes the colour to yellow (λ = 450 nm). More details about the general buffers/solutions are given in the ESI.

2.3. Equipment/Software

See the ESI.

2.4. Synthesis of peptide-bioconjugates

The two peptide haptens (C_1, C_2) were covalently linked to keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), conalbumin (CONA), ovalbumin (OVA), horseradish peroxidase (HRP), and aminodextrane (AD) by means of *N*-succinimidyl 3-maleimidopropionate ($M(CH_2)_2CO$) or *N*-succinimidyl iodoacetate (CH_2CO) cross-linkers at different peptide:Lys (protein) molar ratios (for additional information on the complete procedure, see ESI) using procedures previously reported [27,29] (Table 1). Tables 1-S and 2-S (ESI) provide additional information regarding the synthesized bioconjugates and the corresponding MALDI-TOF-MS recorded to obtain information on the number of attached peptide molecules. The bioconjugates were named $^{a}C_b$-Y-X, where *a* is the true hapten density (δ) calculated by MALDI-TOF-MS, C_b the peptide (C_1, C_2), Y the cross-linker ($M(CH_2)_2CO$ or CH_2CO), and X the macrobiomolecule used.

2.5. Monoclonal antibody production

The anti-c-myc monoclonal antibody (stock solution: 1.44 mg mL^{-1}, IgG mAb) was produced by Abyntek Biopharma (Bizkaia, Spain) in Balb/c mice, using the C_2 peptide conjugated to KLH with a maleimido derivative cross-linker as immunogen. The fused cells (spleen B cells from the selected mice with SP2\0 mouse myeloma cell line) were screened by competitive ELISA (see procedure below). For this purpose, we measured the binding of the supernatants to $^{x}C_2$-CH_2CO-CONA (where *x* corresponds to the bioconjugate synthesized at a ratio of 2:1:1, Tables Table 2 and 2-S) and $^{5}C_1$-$M(CH_2)_2CO$-CONA (0.2 μg mL^{-1} each) immobilized on the microtiter plates in the absence (zero concentration) or presence of the c-myc commercial peptide (ranging from 20 nM to 25 μM). The selected clones were subcloned again and subsequent screenings were performed in the same conditions. After these subsequent screenings, three cell clones were selected to produce the antibodies named $Ab894D12_{d9d10}$, $Ab894D12_{f7d12}$, and $Ab894D12_{f7f9}$ (Tables 3-S and 4-S).

2.6. Preparation of the protein-receptor nanovesicle solutions

The human olfactory receptor c-myc-OR1740 (ORL520 in OrDB) and pJH2-somatostatin receptor subtype 2 (SSTR2), used as negative control, were expressed heterologously in *Saccharomyces cerevisiae* yeast cultures, as previously described [9]. The yeast cells were mechanically disrupted [19] and the cell content separated. The membrane fractions were obtained, divided in aliquots, and frozen at −80 °C (~5 mg mL^{-1}, stock suspension). As a GPCR membrane receptor model, the fractions containing SSTR2 were used to evaluate the matrix effect of the immunochemical assay established for the quantification of c-myc-OR1740 integrated in the NVs. These NVs were produced and characterized as described in a recent publication by our group [30]. In brief, the characterization consisted in measuring: (i) total protein content (TPC) of NVs using the BCA Protein Assay; (ii) the average size of NVs through Dynamic Light Scattering and Cryo-EM; (iii) the concentration of NVs in the solutions (NV mL^{-1}) (Fig. 1-S) using Nanoparticle Tracking Analysis; and (iv) the zeta-potential of NVs in solution by using the Malvern Zetasizer instrument (Malvern Instruments, UK).

To perform the ELISA immunoassay, the stock suspensions of the membrane fractions were diluted to a TPC of 300 μg mL^{-1} in PBT and sonicated for 20 min in ice-cold water to homogenize the NV size. The solution was further diluted in PBT to a TPC of 60 μg mL^{-1} and passed through a sterile low protein-binding filter (Millipore, 13 mm diameter and 0.22 μm pore size). Finally, working solutions were obtained by adding PBT buffer until the desired concentration of NVs was reached. The SSTR2 blank matrix solution refers to PBT solutions of the NVs carrying the SSTR2 receptor without the c-myc peptide. The blank matrix was used at different concentrations (ranging from 10 to 50 μg mL^{-1} of TPC, or $1.47 \times 10^{10} - 7.33 \times 10^{10}$ NV mL^{-1}) [30] in the distinct experiments. The c-myc-OR1740 NV solutions were consistently analyzed at the same TPC concentrations as the reference SSTR2 blank matrix.

For the Western blot assays, 1 μL of the stock membrane suspension was loaded on each well of the gel (5 μg per well), and the assays were performed following the procedure described by Minic et al. [9] (see ESI for more details).

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