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Heterologous expression of human Neuromedin U receptor 1 and its subsequent solubilization and purification

Hongyan Xia [a,*,1], Lihong Liu [b], Christoph Reinhart [a], Hartmut Michel [a]

[a] Max-Planck-Institute of Biophysics, 60438 Frankfurt am Main, Germany
[b] Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Box 7036, 75007 Uppsala, Sweden

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ABSTRACT

Human Neuromedin U receptor 1 (hNmU-R1) is a member of G protein-coupled receptor family. For structural determination of hNmU-R1, the production of hNmU-R1 in milligram amounts is a prerequisite. Here we reported two different eukaryotic expression systems, namely, Semliki Forest virus (SFV)/BHK-21 and baculovirus/*Spodoptera frugiperda* (Sf9) cell systems for overproduction of this receptor. In the SFV-based expression system, hNmU-R1 was produced at a level of 5 pmol receptor/mg membrane protein and the yield could be further increased to 22 pmol receptor/mg membrane protein by supplementation with 2% dimethyl sulfoxide (DMSO). Around 8 pmol receptor/mg membrane protein could be achieved in baculovirus-infected Sf9 cells. The recombinant hNmU-R1 from SFV- and baculovirus-based systems was functional, with a K_d value of [^{125}I] NmU-23 (rat) similar to that from transiently transfected COS-7 cells, where hNmU-R1 was first identified. With the aid of 1% *n*-dodecyl-β-D-maltoside (LM)/0.25% cholesteryl hemisuccinate (CHS), the yield of functional hNmU-R1 could reach 80%. The recombinant receptor from Sf9 cells was purified to homogeneity. The specific binding of the purified receptor to [^{125}I] NmU-23 (rat) indicated that the receptor is bioactive. This is the first report of successful solubilization and purification of hNmU-R1, and will enable functional and structural studies of the hNmU-R1.

1. Introduction

Neuromedin U (NmU), a smooth muscle contracting peptide, was first isolated from porcine spinal cord as two bioactive peptides NmU-25 and its cleavage product NmU-8 [1,2]. It has been subsequently isolated from various species [3,4]. Recently, two human orphan G protein-coupled receptors (GPCRs), hNmU-R1 and hNmU-R2, have been identified as the receptors for NmU [5–7]. Both receptors, which share approximately 50% amino acid identity, can couple to G protein of the Galpha (q/11) and Galpha (i) subfamily [8]. hNmU-R1 is primarily expressed in peripheral tissues, whereas hNmU-R2 is mainly found in specific regions of central nervous system such as spinal cord. The different distribution of NmU receptors suggests that they may have different physiological functions and pharmacological activities. However, the specific physiological role consequent to binding of the NmU peptide to these two receptors is still unknown.

Elucidation of these functions can be addressed from determination of the crystal structure of NmU receptors. For the structural determination by NMR or X-ray crystallography, substantial amounts (milligram) of protein are required. However, like other GPCRs, hNmU-R1 is present in minute amounts in natural cells and tissues. Thus, overexpression of the receptor in heterogeneous expression systems becomes a necessary strategy. *Escherichia coil* (*E. coli*) is a popular host organism for expression of foreign proteins because of its low lost, fast growth and simplicity of handling and scale-up. Despite these advantages, the *E. coli* expression system has limitations for production of mammalian membrane proteins because of its lack of machinery for post-translational modifications, different protein insertion and folding mechanisms from eukaryotic cells, as well as different membrane lipid composition which may be required for protein function [9,10]. Even if expression as inclusion bodies is achieved, refolding these proteins would be a challenge, given the current limited knowledge of GPCR folding [11]. In contrast to *E. coli*, as eukaryotic expression system, insect cells can perform post-translational modifications similar to mammalian cells and are hosts of choice to express GPCRs at high yield. High-level expression and purification of pituitary adenylate cyclase-activating polypeptide receptor, rhodopsin, P2Y12 receptor, beta-adrenergic receptor and histamine H1 receptor have been reported in baculovirus/Sf9 cells [12–16]. Semliki Forest virus (SFV)-

Abbreviations: BHK-21, Baby hamster kidney; BCIP, 5-Bromo-4-Chloro-3-Indolyl Phosphate *p*-Toluidinium; CHS, Cholesteryl hemisuccinate; HEGA-10, Decanoyl-*N*-hydoxyethylglucamide; DMSO, Dimethyl sulfoxide; DMEM, Dulbecco's Modified Eagle's Medium; *E. coli*, *Escherichia coli*; FCS, Fetal calf serum; FOS-12, FOS-choline-12; FOS-14, FOS-choline-14; FOS-16, FOS-choline-16; GPCR, G protein-coupled receptor; hNmU-R1, human Neuromedin U receptor 1; MOI, Multiplicity of Infection; LM, *n*-Dodecyl-β-D-maltoside; NTA, Ni-nitrilotriacetic acid; NBT, Nitro-Blue Tetrazolium Chloride; OG, Octyl-β-D-glucopyronoside; PMSF, Phenymethylsulfonylfluoride; SFV, Semliki Forest Virus; Sf9, *Spodoptera frugiperda*; TSA, Trichostatin A

* Corresponding author. Tel.: +46 18 4714587; fax: +46 18 4714673.
E-mail address: Hongyan.Xia@imbim.uu.se (H. Xia).

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based expression system is also useful, and several GPCRs, i.e. neurokinin-1 receptor, histamin H2 receptor, CB2 cannabinoid receptor and α2B-adrenergic receptor, have been efficiently expressed in SFV-based expression system [17–20].

Here, we report production of hNmU-R1 in two eukaryotic expression systems including SFV/BHK-21 cells and baculovirus/Sf9 cells. In the SFV/BHK-21 cell expression system, the expression level of recombinant protein could be increased to a range of around 22 pmol receptor/mg membrane protein by adding cell differentiation inducer DMSO. The recombinant receptor was then for the first time successfully solubilized from membranes of BHK-21 and Sf9 cells. Furthermore, the epitope-tagged receptor, produced from baculovirus-infected Sf9 cells, was purified to homogeneity by a two-step procedure utilizing Ni-NTA and monomeric avidin affinity chromatographies.

2. Materials and methods

2.1. Materials

Restriction enzymes were obtained from New England Biolabs GmbH (Frankfurt am Main, Germany). SP6 RNA polymerase, the cap analog m^7G (5′) ppp (5′) G, RNasin, and rNTPs were from Fermentas (Leon-Rot, Germany). NmU-8 (porcine) and NmU-25 (human) were obtained from Bachem (Weil am Rhein, Germany). [^{125}I] NmU-23 (rat) was provided by Phoenix Pharmaceuticals, Inc. (Karlsruhe, Germany). LM, FOS-choline-12 (FOS-12), FOS-choline-14 (FOS-14), and FOS-choline-16 (FOS-16) were from Anatrace Inc. (Maumee, OH, USA). Octyl-β-D-glucopyronoside (OG) was supplied by Glycon Biochemicals (Luckenwalde, Germany). Decanoyl-*N*-hydoxyethylglucamide (HEGA-10) was from BIOMOL GmbH (Hamburg, Germany). Digitonin, CHS, Monoclonal anti-polyhistidine antibody and Alkaline Phosphatase-coupled anti-mouse IgG were from Sigma (Munich, Germany).

2.2. Expression of hNmU-R1 in BHK-21 cells using SFV

To produce hNmU-R1 in SFV-based expression system, hNmU-R1 cDNA was amplified from plasmid Peak8 (Aventis, Frankfurt am Main, Germany), using forward primer (5′CGGGATCCGCTTGCAATGGCAGTGCG3′) and reverse primer (5′GGACTAGT GGATGGATCGGTCTCTTG3′), which introduced a BamHI site at the 5′ end and a SpeI site at the 3′ end to facilitate cloning. The BamHI- and SpeI-digested PCR product was inserted into pSFV2CAPhis$_{10}$ vector digested with the same enzymes, resulting in pSFV2hNmU-R1his$_{10}$ (Fig. 1). Expression of the recombinant receptor is driven from a subgenomic 26S promoter (PSG) contained in the vector. The N-terminus of hNmU-R1 was in frame with a viral capsid sequence as a translation enhancer and the C-terminus of the receptor was fused with a His$_{10}$ tag. In vitro transcription and in vivo packaging of recombinant SFV particles were done as described [17].

BHK-21 cells were used for virus infection and recombinant recepor production. BHK-21 cells were grown in 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Iscove's medium containing 10% fetal calf serum (FCS) and 4 mM glutamine. Cells were maintained at 37 °C with 5% CO_2 in a humidified incubator. BHK-21 cells of 80% confluence were infected with the activated virus at a multiplicity of infection (MOI) of 30, and harvested at 19 h post infection by centrifugation at 500 ×*g* for 10 min at 4 °C. The pellet was washed three times with PBS to remove the remaining infectious virus particles, and resuspended in breaking buffer (25 mM Hepes, pH 7.4, 100 mM NaCl, l5 μg/ml aprotinin, 50 μg/ml bestatin, 10 μg/ml soybean trypsin inhibitor, 1 mM phenymethylsulfonylfluoride (PMSF)) to a density of 5×10^6 cells/ml. The cells were lysed by nitrogen cavitation (Parr Instrument Co., Moline, IL, USA) at 500 psi (3.4 MPa) for 45 min and centrifuged at 500 ×*g* for 10 min at 4 °C to remove intact cells. The membrane pellet from ultracentrifugation (100,000 ×*g* for 1 h) was resuspended in the breaking buffer supplemented with 6% glycerol.

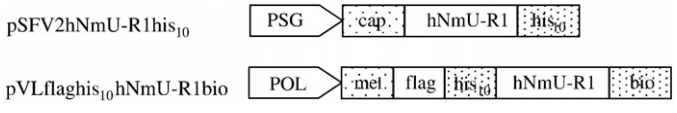

Fig. 1. Schematic representation of the expression constructs for production of hNmU-R1. PSG, subgenomic promoter from SFV; cap, coding region for the capsid protein from SFV as enhancer; his, coding region for 10 histidine residues; POL, baculovirus polyhedrin promoter; mel, encoding region for signal sequence of honeybee melittin; flag, coding region for the flag tag; bio, coding region for the biotinylation domain of the transcarboxylase from *Propionibacterium shermanii*.

2.3. Expression of hNmU-R1 in Sf9 cells using baculovirus expression system

The coding region of hNmU-R1 was amplified by PCR using forward primer (5′CGGGATCCAGCTTGCAATGGCAGTGCG3′) and reverse primer (5′CCGGAATTCGGATGGATCGGTCTCTTG3′), which introduced a BamHI site at the 5′ end and an EcoRI site at the 3′ end. The PCR product was cloned into expression vector pVLflaghis$_{10}$bio, resulting in pVL-flaghis$_{10}$hNmU-R1bio (Fig. 1). In this construct, hNmU-R1 was fused with flag and His$_{10}$ tags at the N-terminus and biotinylation domain of *Propionibacterium shermanii* transcarboxylase at the C-terminus. The prepromelittin signal peptide from honeybee was used for proper targeting of the recombinant receptor, and the polyhedrin promoter was used to drive the expression of recombinant receptor. Recombinant baculoviruses were obtained by cotransfection of 0.1 μg of linearized BaculoGold DNA (BD Biosciences, Heidelberg, Germany) and 0.4 μg of recombinant transfer vector using Lipofectin Reagent (Invitrogen GmbH, Karlsruhe, Germany). The recombinant viruses were selected and amplified as described [21].

Sf9 cells were grown in TNM-FH medium with supplement of 5% FCS, 50 μg/ml gentamycin and 10 μg/ml vitamin B at 27 °C. Sf9 cells at a density of 2×10^6 cells/ml were infected with the recombinant viruses at an MOI of 3. The cells were harvested at 72 h post infection if not indicated otherwise, and used for membrane preparation as previously described for BHK-21 cells.

2.4. Radioligand binding assay

Ligand binding assay was performed as modified [6]. Membranes (1 μg of protein per assay) were incubated with different concentrations (0.01–3 nM) of [^{125}I] NmU-23 (rat) for 1 h at 4 °C in binding buffer (25 mM Hepes, 0.005% Triton X-100, 100 mM spermidine, 5 μg/ml aprotinin, 50 μg/ml bestatin, 10 μg/ml soybean trypsin inhibitor) in a final volume of 200 μl. Free and bound radioligands were separated by rapid filtration through 0.3% polyethylenimine-presoaked Whatman GF/F Filters. The filters were washed three times with ice-cold water. The bound radioactivity was quantitated using scintillation counter (1470 wizard, Wallac, Finland). Nonspecific binding was determined in the presence of non-labeled 1 μM human NmU-25. Specific binding was calculated by the difference between total binding and nonspecific binding. The results were analyzed using KaleidaGraph 3.5 (Synergy Software, Reading, PA, USA). Equilibrium dissociation constant (K_d) and receptor density (B_{max}) were calculated from the results of saturation binding experiments.

The binding assay for the solubilized receptor was performed as follows: 5 μl of solubilized aliquot was incubated with binding buffer containing 10 nM [^{125}I] NmU-23 (rat) in a final volume of 50 μl for 1 h at 4 °C. The final detergent concentrations were 0.1% LM, 0.3% OG, 0.3% HEGA-10, 0.1% digitonin, 0.1% FOS-12, 0.01% FOS-14, 0.01% FOS-16, and 0.1% LM/0.025% CHS, respectively. Nonspecific binding was determined in the presence of non-labeled 1 μM human NmU-25. The separation of bound ligand from free ligand and their quantification were as same as described above. For the purified receptor, 0.1% LM/0.025% CHS was included in the assay buffer with 0.5 μg receptor.

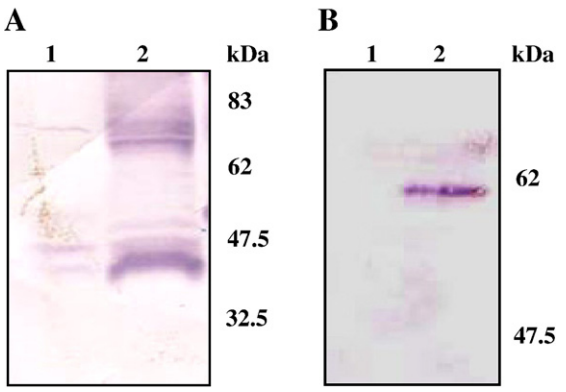

Fig. 2. Immunoblot of the recombinant hNmU-R1 produced in BHK-21 cells (A) and in Sf9 cells (B) with anti-His antibody. Each lane was loaded with 10 μg of membrane protein. Lane 1, membranes from uninfected cells. Lane 2, membranes from recombinant virus infected cells.

2.5. Solubilization of recombinant hNmU-R1

In order to find the detergent that yielded the highest amount of active receptor, we have tested both non-ionic and zwitterionic detergents. The non-ionic detergents were 1% LM, 1% digitonin, 3% OG, and 3% HEGA-10. The lipid-like zwitterionic detergents included: 1% FOS-12, 0.1% FOS-14, and 0.1% FOS-16. A combination of 1% LM and 0.25% CHS was also tested. For protein solubilization, crude membranes were resuspended in the ice-cold solubilization buffer (25 mM Hepes, pH 7.4, 500 mM NaCl, l5 μg/ml aprotinin, 50 μg/ml bestatin, 10 μg/ml soybean trypsin inhibitor, 1 mM PMSF) to a final concentration of 5 mg/ml protein, and incubated with different detergents at 4 °C in an end-over-end rotator for 1 h. After centrifugation at 100,000 ×*g* for 1 h, the supernatant was aliquoted and taken as solubilized fraction for Western blot and binding assays.

2.6. Purification of hNmU-R1 produced in Sf9 cells

The solubilized fraction was applied to a Ni-nitrilotriacetic acid (NTA) agrose column (Qiagen, Hilden, Germany) equilibrated with buffer A (25 mM Hepes, pH 7.4, 500 mM NaCl, 5 mM imidazol, 0.5% LM/0.125% CHS, l5 μg/ml aprotinin, 50 μg/ml bestatin, 10 μg/ml soybean trypsin inhibitor, 1 mM PMSF). The column was washed with 10 volumes of buffer B (same as buffer A except that buffer B contained 50 mM imidazol and 0.1% LM/0.025% CHS). Finally, the receptor was eluted with buffer C (25 mM Hepes, pH 7.4, 200 mM NaCl, 250 mM imidazol, 0.05% LM/0.0125% CHS, l5 μg/ml aprotinin, 50 μg/ml bestatin, 10 μg/ml soybean trypsin inhibitor, 1 mM PMSF). In order to chelate nickel ions released from the Ni-NTA affinity matrix, which could cause protein aggregation by binding to several His_{10} tags fused to receptor molecules, 20 mM EDTA was added to the elution fraction from the Ni-NTA column. The elution fraction was then passed through a monomeric avidin column (Pierce, Bonn, Germany) equilibrated with buffer D (same as buffer C without imidazol). After washing the column with 10 volumes of buffer D, protein was eluted with buffer D containing 2 mM biotin. For gel filtration analysis, the elution fraction from the monomeric avidin column was first concentrated with a 50-kDa molecular weight cut-off centricon (Ambion, Austin, TX, USA) and then loaded to the Superose-6 size exclusion column on a SMART system (GE Healthcare, Uppsala, Sweden).

2.7. SDS-PAGE and Western blotting

Protein concentration was determined using Bicinchoninic Acid Protein Assay Kit (Pierce, Bonn, Germany). The proteins were resolved by 10% SDS-PAGE and electro-transferred to PVDF membrane for immunoblotting. The PVDF membrane was blocked with 5% non-fat milk at room temperature for 1 h in PBST buffer (13.7 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 13 mM Na_2HPO_4, 1.5 mM NaN_3, 0.02% Tween-20), and followed by incubation with Monoclonal anti-polyhistidine antibody (1:5000 in PBST) at room temperature for 1 h. The membrane was washed three times and then incubated with Alkaline Phosphatase-coupled anti-mouse IgG (1:10,000 in PBST) at room temperature for 1 h. Finally, the PVDF membrane was developed in alkaline phosphate buffer (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing 0.33 mg/ml 5-Bromo-4-Chloro-3-Indolyl Phosphate *p*-Toluidinium (BCIP) and 0.165 mg/ml Nitro-Blue Tetrazolium Chloride (NBT).

3. Results

3.1. hNmU-R1 produced in SFV-infected BHK-21 cells

Following infection of BHK-21 cells with the recombinant viruses, expression of hNmU-R1 was analyzed by Western blot using anti-His

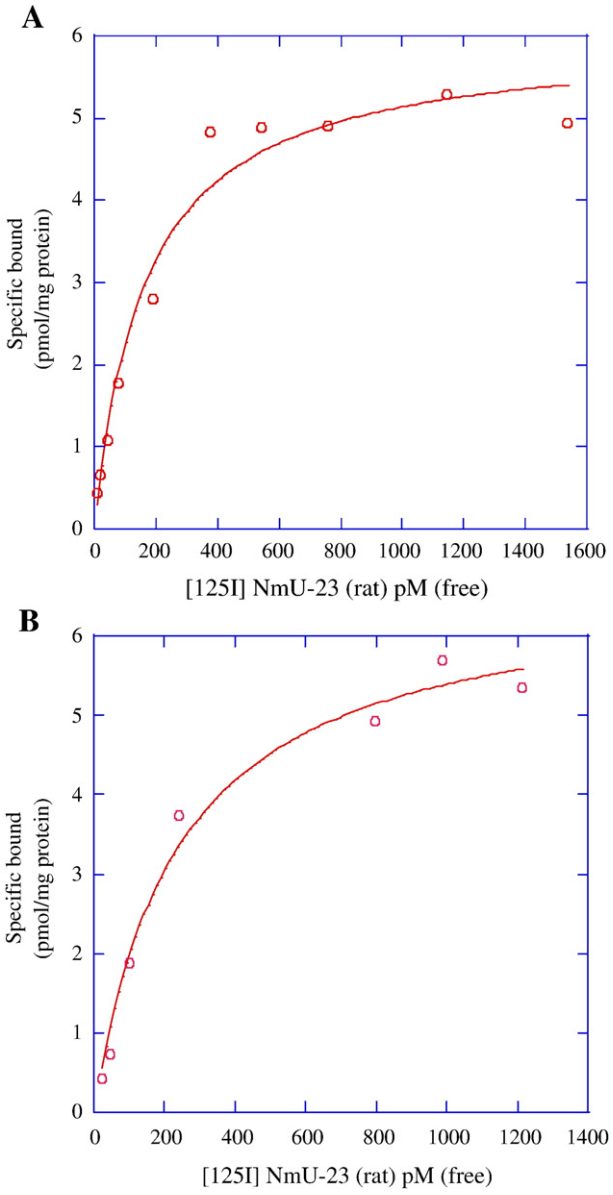

Fig. 3. Saturation curve of [^{125}I] NmU-23 (rat) binding to membranes from BHK-21 cells infected with SFV-hNmU-R1-his (A), and membranes from Sf9 cells infected by recombinant baculovirus for 96 h (B). BHK-21 cells were harvested at 19 h post infection and Sf9 cells were harvested at 96 h post infection. Data represent one of the two independent experiments performed in triplicate.

antibody (Fig. 2A). A dominant band of 45 kDa was observed, which corresponds to the expected molecular weight of 44.9 kDa based on calculation of number of amino acids. The upper smearing band around 80 kDa detected by anti-His antibody probably represents the receptor in a homo-oligomerized form. In membrane preparations from mock-infected BHK-21 cells, only a nonspecific band was observed.

To analyze pharmacological properties of the recombinant hNmU-R1, saturation binding assays were performed using [^{125}I] NmU-23 (rat) as radio-ligand (Fig. 3A). In membranes of SFV2hNmU-R1his$_{10}$-infected BHK-21 cells, the saturation binding curve was best fitted with a single-site affinity curve. [^{125}I] NmU-23 (rat) bound the receptor with a high affinity (a K_d value of 0.14±0.028 nM) and a high density (a B_{max} value of 5.5±0.81 pmol receptor/mg membrane protein). No specific binding could be observed in membranes from mock-infected BHK-21 cells. The K_d value of the recombinant receptor was in good agreement with that of hNmU-R1 expressed in COS-7 cells (K_d=0.3, 0.61 and 0.72 nM) [5,6].

3.2. Effects of cell differentiation inducers on the production of hNmU-R1-his

After defining an MOI of 30 and incubation time of 19 h as optimal condition for expression, we tested effects of NmU-8 (porcine), DMSO, trichostatin A (TSA) and sodium butyrate on the production of the functional recombinant receptor. The results of one of two independent experiments that had a similar pattern are shown in Fig. 4. Porcine NmU-8 could increase protein production from 5 pmol receptor/mg membrane protein to 13 pmol receptor/mg membrane protein. Sodium butyrate, at a concentration of 2 mM, had a similar stimulation effect as porcine NmU-8, increasing hNmU-R1 production by approximately 2-fold. The level of hNmU-R1 was increased from 5 pmol/mg protein to 15 pmol/mg protein by adding 200 ng/ml of TSA. Addition of 2% DMSO had the most dramatic effect in enhancing expression, as it could increase hNmU-R1 production by four times, from 5 to around 22 pmol/mg protein. However, a combination of 2% DMSO and 100 nM porcine NmU-8 did not have synergic effect on receptor yield.

3.3. Functional solubilization of hNmU-R1 produced in BHK-21 cells infected with recombinant SFV

A critical step to obtain membrane proteins in an active, stable form for biophysical and structural studies is efficient solubilization. To maximize solubilization efficiency of functional protein, extensive efforts were made to screen a broad range of detergents including non-ionic and zwitterionic detergents. Most of the detergents did not show considerable effect on solubilization as revealed by Western blot analysis (data not shown), with the exception of few that were selected for further studies. In addition, other detergents that were proved to be gently mild to maintain membrane protein structure and function were included [22,23]. The list comprised the following detergents: 1% LM, 3% OG, 3% HEGA-10, 1% digitonin, 1% FOS-12, 0.1% FOS-14, and 0.1% FOS-16. FOS-cholines have been widely used for NMR conformational studies of membrane proteins because its phosphocholine headgroup mimics membrane environment [24]. Our results showed that FOS-cholines are efficient solubilizers in terms of total receptor, as demonstrated by a dominantly intensive band from Western blot analysis (Fig. 5A). However, low specific binding suggested that these detergents are not suitable for solubilization of the receptor in the functional form due to inactivation of part of the receptor. OG, one of the most successful detergents used in crystallization of membrane proteins, showed a

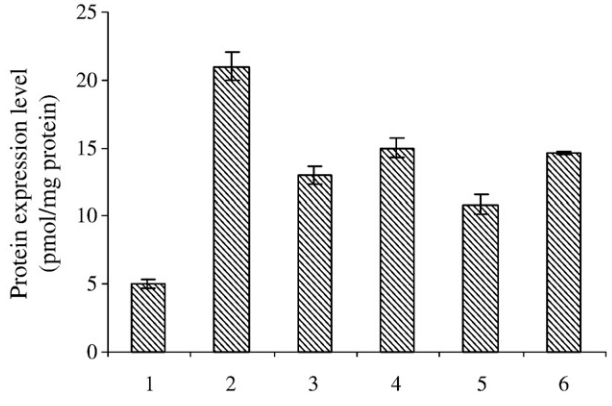

Fig. 4. Effect of different chemical reagents on the receptor production. Cells without any chemical reagents (1), cells with 2% DMSO (2), cells with 100 nM NmU-8 (porcine) (3), cells with combination of 2% DMSO and 100 nM NmU-8 (porcine) (4), cells with 2 mM sodium butyrate (5), and cells with 200 ng/ml TSA (6). BHK-21 cells were infected with SFV-hNmU-R1 and the additives were added at the same time as virus. Infected cells were harvest at 19 h post infection. The data shown here represent one of the two independent experiments (performed in triplicate) with a similar pattern.

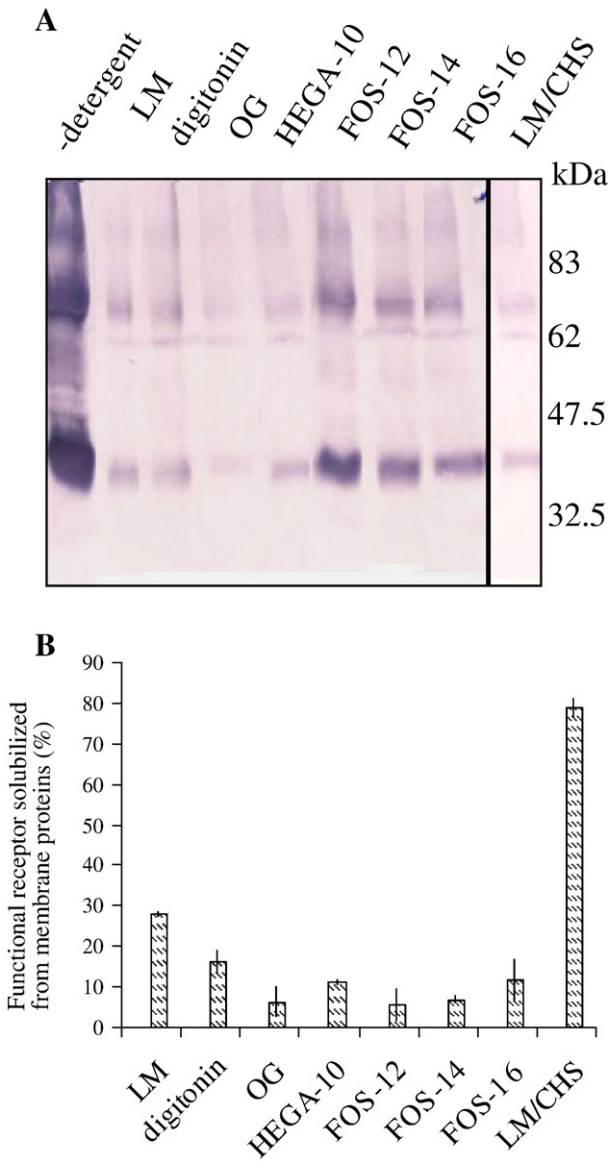

Fig. 5. Solubilization of the hNmU-R1 receptor with different detergents from membranes of BHK-21 cells infected by SFV-hNmU-R1-His. (A) Western blot analysis of solubilized proteins. (B) Specific binding of [^{125}I] NmU-23 (rat) to the solubilized hNmU-R1. Membrane proteins at a concentration of 5 mg/ml were incubated with different detergents at 4 °C for 1 h. After centrifugation at 100,000 ×*g* for 1 h, the same volume aliquots of the supernatants were used for Western blot and ligand binding assays.

lower solubilization efficiency for both total and functional receptors (Figs. 5A and B). Digitonin, which is another mild detergent widely used for solubilization of membrane proteins [25,26], had solubilization ability of total receptor similar to LM and HEGA-10 (Fig. 5A). However, in terms of active receptor, LM showed the highest yield (28%). Combination of 1% LM and 0.25% CHS could further improve the yield of functional protein to around 80%. Thus a combination of 1% LM and 0.25% CHS was used for solubilization in the following studies.

3.4. Expression of hNmU-R1 in Sf9 cells

An expression vector pVLflaghis$_{10}$hNmU-R1bio was constructed. The very late promoter polyhedrin of *Autographa californica* nuclear polyhedrosis virus (ACNPV) was used to drive the expression of recombinant receptor. The recombinant baculoviruses were used to infect insect Sf9 cells at an MOI of 3. Comparing to the membranes from mock-infected cells, the membranes from the recombinant viruses-infected cells showed a major band of 60 kDa (Fig. 2B), which corresponds to the size of recombinant hNmU-R1 fusion protein (flag tag 1.0 kDa + His$_{10}$ tag 1.4 kDa + hNmU-R1 44.9 kDa + bio tag 12.4 kDa). This also indicated the complete processing of the prepromelittin signal sequence in vivo. Maximal binding capacity of 8 ± 1.1 pmol receptor/mg membrane protein was observed at 72 h post infection. Saturation binding of [^{125}I] NmU-23 (rat) to cell membranes showed that the recombinant receptor has a single binding site, with a K_d value of 0.29 ± 0.016 nM (Fig. 3B), which was similar to that obtained for the receptor expressed in BHK-21 cells.

3.5. Purification of hNmU-R1 produced in Sf9 cells infected with the recombinant baculovirus

Solubilization of Sf9 membranes containing the recombinant receptor was done with 1% LM/0.25% CHS as described above. After two-column chromatography purification, the receptor was nearly pure as shown by silver stained SDS-PAGE gel (Fig. 6A). The major band of approximate 60 kDa detected by Western blot analysis (Fig. 6B), represented the purified receptor. The two minor bands of slightly higher molecular mass did not cross-react with the anti-His antibody, suggesting that they are contaminants from the affinity column. The final yield of purified receptor from 105 mg solubilized fraction was 96 μg as revealed by protein quantification of the purified receptor (Table 1). Using [^{125}I] NmU-23 (rat) as ligand, saturation binding assay was attempted. However, due to inability to obtain a high concentration of [^{125}I] NmU-23 (rat), the ligand could not saturate the receptor. This indicated that the purified receptor is in a low affinity state. If the K_d was taken as 0.3 nM, the specific activity of the purified hNmU-R1 in the presence of 10 nM [^{125}I] NmU-23 (rat) was 1520 pmol/mg protein. As the receptor is of low affinity, the data would underestimate the real activity of the receptor. To verify its homogeneity, the purified protein was concentrated and analyzed by analytical gel filtration column in a SMART system. The elution profile displayed a single peak (Fig. 7), indicating that the purified receptor is in a homogeneous form.

Table 1
Purification of the hNmU-R1

Fraction	Total protein (mg)	Specificity (pmol/mg)	Yield (%)	Purification fold
Membranes	410	8.5	100	1
Solubilized membranes	105	27	81	3
Elution from Ni-NTA	0.85	295	7.1	34
Elution from monomeric avidin	0.09	1520	3.9	178

The amount of receptor present in the different fractions was determined in the presence of 10 nM [^{125}I]NmU-23 (rat). Protein concentration was determined with bicinchoninic acid using bovine serum albumin as standard.

4. Discussion

GPCRs constitute the largest group of integral membrane protein superfamily in human [27]. With the exception of bovine rhodopsin, which is abundant in natural source (300 mg per 200 bovine retina's) and represents one of the most stable and detergent-tolerant GPCRs [28], most of the GPCRs are produced in a very limited amount in their natural tissues and cells. Thus, a heterogeneous expression system is required to achieve adequate amount of proteins for structural studies. Although there are examples of GPCRs overexpressed at a high level [12–19,22,29], it is difficult to outline general rules for a successful overproduction of a desired membrane protein [9]. The expression level for a given GPCRs is significantly different from one system to another, therefore, trial and error experiments are always necessary for production of a specific protein.

hNmU-R1 expressed in Sf9 cells using baculovirus expression system had a similar binding affinity to [^{125}I] NmU-23 (rat) as the receptor from SFV-infected BHK-21 cells. However, the detected binding sites of around 8 pmol/mg membrane protein were less than that of hNmU-R1 from BHK-21 cells, demonstrating the variable capacity of different expression systems for production of the same protein. With the aim of acquiring sufficient material for structural studies, scale-up of the cell culture for large amount of protein production is required. Despite less binding sites in Sf9 cells, baculovirus expression system is still our choice for production of this receptor. Firstly, comparing to BHK cells (at a density of 1×10^6 cells/ml), Sf9 cells can grow to a higher

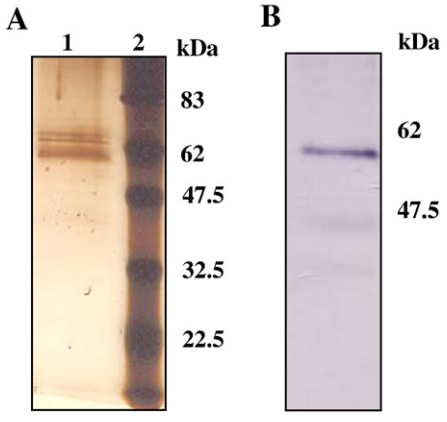

Fig. 6. Purified hNmU-R1 from Sf9 cells by Ni-NTA and monomeric avidin column. Proteins were separated by 10% SDS-PAGE and analyzed by silver staining (A) or Western blot against anti-His antibody (B). One microgram of purified hNmU-R1 was loaded to the gel. Lane 1, purified hNmUR1. Lane 2, protein marker.

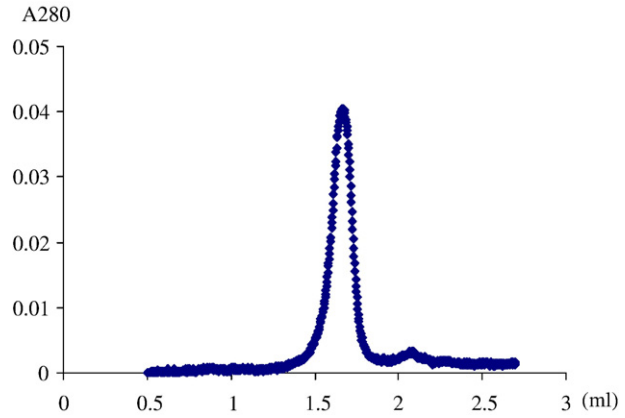

Fig. 7. Gel filtration analysis of purified hNmU-R1 produced from Sf9 cells. Twenty-five microgram of the elution fraction from monomeric avidin column was applied to Superose-6 column and elution of the protein was monitored at 280 nm.

cell density ($1–2 \times 10^7$ cells/ml). From 1 l of cell culture, around 230 μg of active receptor can be obtained in Sf9 cells instead of around 85 μg of active receptor in BHK-21 cells. Secondly, once the recombinant baculovirus is produced and verified, it can be easily multiplied from stock virus by infecting insect cells. In contrast, due to biosafety reasons, recombinant SFV has been modified to replication-deficient type, therefore, it cannot be propagated after infecting the cells. The recombinant SFV has to be prepared from in vitro transcribed mRNA for each infection. Thus SFV preparation is time-consuming and costly. Furthermore, a high MOI of 30 had to be used for high-level production of hNmU-R1 in BHK-21 cells, which made SFV-based system unsuitable for large-scale production of the receptor.

Interestingly, DMSO, TSA, and sodium butyrate could enhance hNmU-R1 production in SFV-infected BHK-21 cells. They have been reported to enhance gene expression in several systems. In mammalian expression system, sodium butyrate enhanced expression level of different tissue plasminogen activators from 2- to 9-fold in CHO cells, and D6 chemokine receptor by 10-fold in L1.2 murine pre-B cell line [30,31]. An increase of reporter expression was also observed in baculovirus-transduced mammalian cells by addition of sodium butyrate or TSA [32]. In dipteran *Drosophila melanogaster* S2 cells and lepidopteran *Trichoplusia ni* BTI Tn 5B1-4 cells, supplement of DMSO and sodium butyrate could enhance expression of human cyclooxygenase and tumstatin [33,34]. TSA and sodium butyrate even reactivated silenced, virally transduced genes in a manner that is neither promoter nor cell-type-specific [35]. As histone deacetylase inhibitors, TSA and sodium butyrate can cause hyperacetylation of histone, relaxation of chromatin structure. The change of the chromatin structure is supposed to correlate with activating gene transcription and increasing protein expression [33,36]. However, for SFV-based expression system, the mechanism is different. The recombinant virus directly starts synthesis of its own proteins as well as the recombinant receptor once it enters host cells. Thus TSA and sodium butyrate cannot be directly involved in the recombinant protein production at transcription level. They might indirectly increase recombinant protein production by improving production of other proteins, which are usually insufficient for translocation and modification of quickly synthetic recombinant protein. Recently, it has been reported that DMSO in the culture medium can significantly improve the functional expression level of recombinant GPCRs in *Pichia pastoris*. Out of 20 tested GPCRs, DMSO could improve functional expression of 16 GPCRs up to 6-fold [37]. DMSO can also increase the functional expression of human bradykinin receptor in BHK-21 cells, and hNmU-R2 in BHK-21 cells and in *P. pastoris* [38,39]. It has been reported that DMSO can alter the expression pattern of yeast [40], and upregulate genes involved in lipid synthesis [41]. The exact mechanism of DMSO in enhancing recombinant protein production is still unknown. However, DMSO may improve protein production by stabilizing proteins as suggested [33]. In contrast to the beneficial effect of DMSO, TSA and sodium butyrate on production of functional recombinant hNmU-R1 in BHK-21 cells, the same was not observed in Sf9 cells infected with recombinant baculoviruses, suggesting cell-specific dependence of these agents on the functional protein production.

Detergents play an important role in studies of membrane protein structure and function. They are indispensable as solubilizing agent to extract membrane proteins from their native lipid bilayer environment for subsequent purification and crystallization. The transfer of membrane proteins from a membrane environment to a detergent environment may result in a loss of function, which is strongly detergent-dependent [42]. The choice of detergents, buffer composition, and ionic strength has an important effect on the solubility efficiency and function of a specific protein. Because no literature was found for the solubilization of hNmU-R1, a series of detergents had to be tested. Among our selected detergents, LM was the most efficient in solubilizing hNmU-R1 from BHK-21 cell membranes in the functional form. Addition of 0.25% CHS to solubilization reaction in the presence of 1% LM could result in high recovery of functional receptor (around 80%). The benign effect of CHS on improvement of solubilization recovery has been reported [22,43–45]. It was suggested that its structural similarity to cholesterol contributes to protein integrity, function and stability [45,46].

The recombinant hNmU-R1 produced in Sf9 cells was purified by two-column chromatographies. A major band of around 60 kDa by silver staining and Western blot verified that the intact receptor had been obtained. The sharp peak from the analytic gel filtration showed the homogeneity of the receptor. The unresolved dissociation constant of the purified receptor, which was limited by reaching a high concentration of [^{125}I] NmU-23 (rat), indicated that the purified receptor is in a low affinity state, which could be caused by delipidation during the purification step. It is known that some cofactors and lipids bound to protein can affect the binding properties of recombinant GPCRs and the loss of such cofactors during purification step could lower the specific activity of otherwise fully active receptor [15,47,48]. However, the specific binding of [^{125}I] NmU-23 (rat) to 1520 pmol/mg protein (the K_d is taken as 0.3 nM) indicated that the purified hNmU-R1 is bioactive. The functional study of the purified receptor will be further investigated by adding different lipids to the radio-ligand binding assay or by [^{35}S] GTPγS assay using reconstituted receptor.

In conclusion, functional recombinant hNmU-R1 could be produced in both SFV/BHK-21 cell and baculovirus/Sf9 cell expression systems. Cell differentiation inducers were shown to enhance the level of the receptor produced in BHK-21 cells. Due to its lower cost, growth to a higher cell density and scale-up capacity, the baculovirus system is favored for large-scale production of the protein for structural studies in future. The receptor was successfully solubilized with LM/CHS, and purified to homogeneity from Sf9 cell membranes. To our knowledge, this is the first report on the solubilization and purification of hNmU-R1, and it opens possibilities for biophysical and structural studies.

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