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A cleavable ligand column for the rapid isolation of large quantities of homogeneous and functional neurotensin receptor 1 variants from *E. coli*

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ABSTRACT

G protein-coupled receptors (GPCRs) are key players of cell signaling, thus representing important drug targets for the treatment of human diseases. Since inherent difficulties in receptor production and handling have precluded the application of many *in vitro* experiments, major questions about GPCR mechanisms and dynamics remain elusive to date. We recently used directed evolution in *Escherichia coli* on neurotensin receptor 1 (NTR1) for the generation of GPCR variants with greatly elevated functional expression levels and with excellent stability in detergent micelles. In this work we outline a highly efficient purification method for our evolved receptor variants, which is based on the application of an inexpensive, disposable high-affinity ligand column as the initial purification step. The ligand resin allows isolation of correctly folded GPCR variants directly from whole *E. coli* cell lysates at the scale of 10 mg and it permits preparations of agonist- and antagonist-bound receptor samples. The purification principle presented here was key to the first structures of signaling-active NTR1 variants (Egloff et al., 2014). Since *E. coli* is uniquely suitable for the production of fully deuterated proteins, our method provides the basis for an array of NMR experiments that were not feasible for GPCRs to date, but which will shed light on novel aspects of receptor function and dynamics.

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Introduction

The neurotensin receptor 1 (NTR1)[1] is a GPCR that is expressed in the human intestine and in the nervous system [1]. It binds to the 13-amino-acid peptide neurotensin (NT), which plays important roles in hypothermia, antinociception, the pathogenesis of Parkinson's disease, schizophrenia and in lung cancer progression [2–5]. Upon NT binding, the receptor triggers GDP/GTP exchange within heterotrimeric G proteins, which leads to downstream stimulation of phospholipase C and adenylyl cyclase that produce second messengers in the cytosol [6,7]. Due to a lack of experiments tackling NTR1 dynamics, little is known about the signal-transduction mechanism across the plasma membrane, but insights of this type would improve our understanding of the receptor in its signaling network and facilitate drug development.

Like many other GPCRs, NTR1 has been studied extensively in the contexts of native membranes and *in vivo* [8–10]. Investigations *in vitro*, on the other hand, were largely precluded due to receptor instability in detergent solution. Recently, this problem was approached by alanine-scanning mutagenesis, which generated a thermostable, but signaling-deficient NTR1 variant that was fused to T4 lysozyme for crystallization in a mild lipidic cubic phase environment [11]. In parallel, we have applied directed evolution technologies that generated NTR1 variants with significantly higher stabilities [12–15]. Several of the evolved NTR1 variants could be crystallized in harsh detergent environments by standard vapor diffusion experiments, and high-resolution structures were determined in the NT-bound state, thus confirming the structural integrity of the evolved variants [16]. One of the crystallized constructs, termed TM86V-ΔIC3A, was functionally characterized. It was signaling-active, it bound agonist and antagonist with high affinities and it exhibited residual desensitizing internalization behavior typical for a GPCR.

The evolved NTR1 variants may serve as useful model GPCRs for future biophysical studies. They exhibit up to 60-fold improved functional expression levels in *Escherichia coli* and thus benefit from several advantages of the prokaryotic expression host, such

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[1] *Abbreviations used:* NTR1, neurotensin receptor 1; IPTG, isopropyl-β-D-thiogalactopyranoside; CHS, cholesteryl hemisuccinate tris salt; HRV, human rhinovirus; IMAC, immobilized metal-ion affinity chromatography.

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as quick genetic modification strategies, growth to high cell densities, fast doubling times, inexpensive media, absence of glycosylation and robust handling. Furthermore, prokaryotic production now allows to fully deuterate large quantities of functionally expressed NTR1 variants, which will improve signal-to-noise ratios in many NMR experiments [17] that were previously not feasible with natively produced GPCRs.

In this work, we describe a highly efficient method for the isolation of evolved NTR1 variants directly from whole *E. coli* cell extracts, which is based on ligand binding and thus allows the enrichment of correctly folded receptors only. In contrast to previously documented NTR1 preparations from *E. coli* [18–20], the strategy presented here enabled us to purify within significantly shorter time larger quantities of receptor samples with improved homogeneity, even in very harsh short-chain glucosidic detergents. The method was key to the crystallization of several agonist-bound NTR1 variants in signaling-competent states [16], and we show that the purification concept is also applicable for the isolation of large quantities of functional receptors bound to antagonist.

Materials and methods

Materials

Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Biosolve. All detergents were obtained from Affymetrix. Cholesteryl hemisuccinate tris salt (CHS) and lysozyme were purchased from Sigma. Empty PD10 columns, NHS-activated Sepharose, SP Sepharose, Superdex-200 10/300 and HighLoad 16/600 Superdex-200 were obtained from GE Healthcare. Complete EDTA-free protease inhibitor tablets and DNase I were purchased from Roche. The neurotensin receptor 1 (NTR1) antagonist SR142948 was obtained from Axon Medchem. Amicon Ultra concentrators were purchased from Millipore. Ampicillin was obtained from AppliChem, Ni-NTA was purchased from Qiagen. Micro Bio-spin columns were obtained from Biorad.

Construct design

The NT ligand constructs for NHS-activated Sepharose coupling were expressed using a pAT223-derived vector (GenBank accession number **AY327138**) for IPTG-inducible expression. The open reading frame encoded an N-terminally Avi-tagged protein D (pD), which is at its C-terminus connected to an internal hexa-histidine tag, followed by the linker GS$(GGGS)_4$, a human rhinovirus (HRV) 3C protease site (LEVLFQGP), two glycines and amino acids 8–13 (RRPYIL) of human/rat NT (QLYENKPRRPYIL). This construct (Fig. 1) is referred to as pD-NT [16]. The mutant pD-NT constructs encoded alanine substitutions in the C-terminal NT8-13 moiety (R8A, R9A, P10A, Y11A, I12A, L13A or I12A + L13A) and the HRV 3C protease site was replaced by the non-cleavable linker GGGGSGG.

All NTR1 variants were subcloned into a pBR322-derived vector, which was originally obtained as a kind gift from R. Grisshammer (National Institute of Neurological Disorders and Stroke, National Institutes of Health, Rockville USA) [12,21]. The open reading frame of the modified vector encoded an N-terminal maltose-binding protein (MBP, including its periplasmic signal sequence) linked via a GSNN linker, hexa-histidine and an HRV 3C protease site (LEVLFQGP) to residue G50 of the receptor (sequential NTR1 numbering). The NTR1 variants were C-terminally truncated at G390 and linked via a HRV 3C protease site, a penta-asparagine linker, and a di-glycineserine linker to thioredoxin A (TrxA), which is followed by a deca-histidine tag. Amino acids V280-I295 of the intracellular loop 3 were deleted in all illustrated purification procedures involving NTR1 variants OGG7 and HTGH4 (Fig. 4C, front and middle) and in the TM86V-agonist complex purification in n-octyl-β-D-glucopyranoside (OG) (Fig. 4B). For all described TM86V purifications in n-nonyl-β-D-glucopyranoside (NG), amino acids E273-T290 of the intracellular loop 3 were deleted (Fig. 4C, back and Fig. 6).

Expression and purification of pD-NT constructs

Five milliliter 2 × YT medium containing 1% (w/v) glucose and 50 μg/ml ampicillin was inoculated with a single colony of *E. coli* BL21 harboring the pD-NT expression plasmid. The culture was incubated for 8 h at 37 °C. Subsequently, 1 ml of this culture was used to inoculate 1 L pre-culture (2 × YT, 1% (w/v) glucose, 50 μg/ml ampicillin), which was grown overnight at 37 °C to saturation. A 50 L fermenter (Bioengineering), containing 2 × YT medium, 0.6% (w/v) glucose, and 50 μg/ml ampicillin, was inoculated to OD_{600} 0.05 and grown to an OD_{600} of 1.5 at 37 °C, followed by induction with 1 mM IPTG, and growth was continued for 3 – 4 h. The pH was kept constant at 6.5. Cells were harvested using a continuous-flow centrifuge. Approximately 16 g (wet weight) of cells were typically obtained per liter of culture.

All pD-NT constructs were purified at 4 °C via the internal histidine-tag using immobilized metal-ion affinity chromatography (IMAC). A 100 g aliquot of the cell pellet (corresponding to less

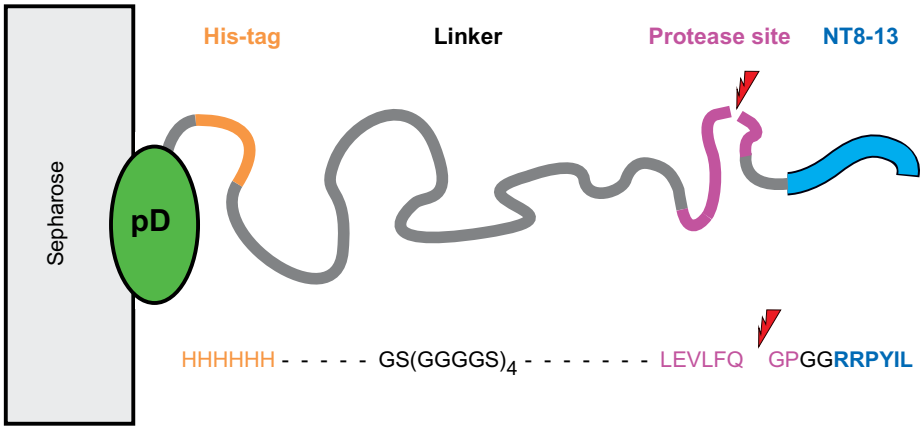

Fig. 1. Scheme of the NTR1 ligand-affinity Sepharose resin. pD-NT contains an internal His-tag allowing its own purification in large quantities via IMAC. The ligand construct can be coupled to NHS-activated Sepharose *specifically* via the pD domain, as this part contains all the primary amines encoded in the construct (all the lysines and the N-terminus). The long and flexible linker between pD and the C-terminal NT8-13 (the minimal receptor-binding epitope of NT) allows maximal accessibility of the GPCR ligand. A 3C protease site in close proximity to NT8-13 permits quantitative and mild elution of functional NTR1 variants in complex with the agonist by proteolytic cleavage.

than a fifth of the fermenter pellet) was resuspended by a Yellow Line DI 25 basic homogenizer (IKA) in 300 ml lysis buffer containing 50 mM HEPES, pH 8, 500 mM NaCl, 25 mM imidazole, pH 8, thirty complete EDTA-free protease inhibitor tablets, a spatula tip of DNaseI and 5 mM $MgCl_2$. The cells were lysed by one processing round of a T1.1 cell disrupter (Constant Systems) at 35,000 psi. Pelleting of cell debris was carried out in a Sorvall Evolution RC centrifuge at 30,000 × *g* using an SLA1500 rotor (tilted neck 250 ml tubes, SORVALL). The supernatant was loaded onto 12 × 4 ml pre-equilibrated Ni-NTA resin in empty PD10 columns (benchtop gravity flow). The resin was washed using a Cerex SPE Processor pressure-flow device (Varian) by 10 column volumes equilibration buffer (25 mM HEPES, pH 8, 500 mM NaCl, 25 mM imidazole, pH 8) and subsequently by 10 column volumes carbonate buffer (0.2 M $NaHCO_3$, pH 8.3, 500 mM NaCl, 5 mM imidazole, pH 8). The elution buffer contained 0.2 M $NaHCO_3$, pH 8.3, 500 mM NaCl and 300 mM imidazole, pH 8. The eluted protein was concentrated to approximately 20 mg/ml using four Amicon-15 Ultra concentrators and subsequently it was dialyzed (6000–8000 Da cutoff) against 3 × 1 L coupling buffer containing 0.2 M $NaHCO_3$, pH 8.3, and 500 mM NaCl (one dialysis step was performed overnight). The purified pD-NT constructs were diluted to 12 mg/ml with coupling buffer and they were frozen in liquid N_2 in 12.5 ml aliquots (typically 5–6 aliquots from one purification) and stored at −80 °C.

Coupling of pD-NT constructs to NHS-activated Sepharose

In one coupling reaction 25 ml of purified pD-NT construct at 12 mg/ml was coupled to 50 ml slurry of NHS-activated Sepharose (2 × 25 ml of a 1 to 1 slurry; #17-0906-01, GE-Healthcare). Briefly, the NHS-resin was divided equally into 10 empty PD10 columns, which were mounted to a Cerex SPE Processor pressure-flow device (Varian) at room temperature. The isopropanol-containing storage solution was drained and each column was washed with 60 ml of ice-cold 1 mM HCl. The columns were closed at the bottom prior to the addition of 2.5 ml of purified pD-NT per column. The resin was resuspended immediately in the pD-NT solution, followed by incubation for 2 h on a roller mixer (Stuart). The columns were subsequently drained and the coupling efficiency was analyzed by Bradford assays or by SDS–PAGE analysis of the unbound material in the drained solution (Fig. 2). The unreacted NHS-groups were quenched by washing each column with 10 ml of 100 mM Tris–HCl, pH 8.5 and further by resuspending the resin in 5 ml of 100 mM Tris–HCl, pH 8.5 (incubation on a roller mixer (Stuart) for 2 h at room temperature). Subsequently, 3 washing cycles using 12 ml of 100 mM sodium acetate, pH 4.5, 500 mM NaCl and 12 ml of 100 mM Tris–HCl, pH 8.5 per column and cycle were performed. To completely inactivate putatively co-purified proteases that may prevent long-term storage of the resin, each column was washed with 24 ml of 6 M GdmCl. Each column was then washed with 24 ml H_2O and with 16 ml of 20% ethanol. The resin from all columns was collected and stored in 20% ethanol as a 50-ml slurry containing 25 ml pD-NT Sepharose (bed volume). Note that the application of multiple small columns for the coupling reaction greatly facilitates the resuspension steps, compared to using one large column.

Large-scale expression and purification of agonist-bound NTR1 variants

Fermenter runs with *E. coli* BL21 Tuner cells harboring the NTR1 expression plasmids were performed as previously described [16]. In a typical purification, 50 g of cell pellet (corresponding to a 7% aliquot of a fermenter run) were resuspended by a Yellow Line DI 25 basic homogenizer (IKA) in 100 mL solubilization buffer containing 100 mM HEPES, pH 8, 20% (v/v) glycerol and 400 mM NaCl.

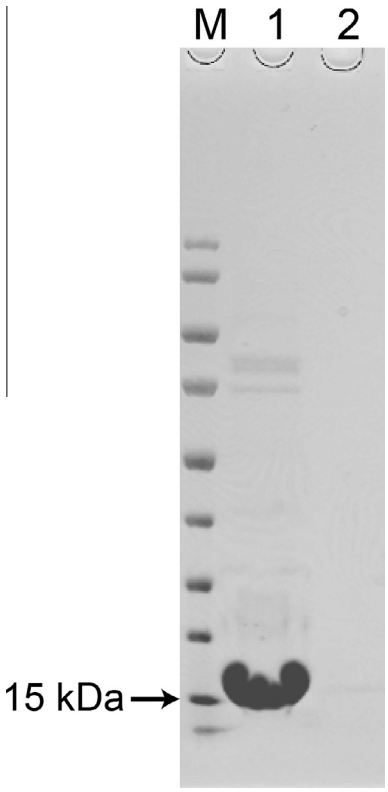

Fig. 2. SDS–PAGE analysis of the pD-NT purification outcome and of the coupling efficiency to NHS-activated Sepharose. pD-NT was expressed in *E. coli*. It was purified by IMAC, followed by dialysis for the removal of small-molecule contaminants containing primary amines, which appeared to act as competitors during NHS-coupling. Lane 1 shows the purity of the pD-NT construct (M_W = 16 kDa) after dialysis, but *prior* to the coupling to NHS-activated Sepharose. Lane 2 represents a fair loading of the same solution *after* exposure to NHS-activated Sepharose. The absence of pD-NT in lane 2 indicates a highly efficient coupling reaction.

All subsequent steps were carried out at 4 °C. 0.5 mL of 1 M $MgCl_2$, a spatula tip of DNase I, 200 mg lysozyme, 20 mL of a solution of 6% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS)/1.2% (w/v) CHS, and 34 mL of 10% (w/v) N-decyl-β-D-maltopyranoside (DM) were added to the resuspended cells while stirring. The mixture was incubated while stirring for 15 min. Sonication was performed for 30 min in an ice-water bath using a Sonifier 250 (Branson) at a duty cycle of 30%, output 5, with sonication tip extension from Heinemann (13 mm) and a stirring bar at 250 rpm within the extraction mixture. Subsequently, 4 mL of 0.5 M EDTA was added to the extraction mixture, followed by another 30 min incubation while stirring. The suspension was transferred to one 250-ml tilted neck centrifugation tube (SORVALL) and centrifuged for 30 min at 15,000 rpm (SLA 1500 rotor). The supernatant was transferred to a 250-ml glass bottle and mixed with 5 mL slurry pD-NT-ligand resin that was pre-equilibrated in NT wash buffer 1 (25 mM HEPES, pH 8, 10% (v/v) glycerol, 600 mM NaCl, and 0.5% (w/v) DM). The NT binding reaction was incubated on a roller mixer (Stuart) overnight. Subsequently, the mixture was centrifuged at 400 × *g* for 10 min, and 90% of the supernatant was decanted.

Using the remaining supernatant, the pelleted pD-NT resin was transferred to an empty PD10 column, and it was washed on a bench top in a cold room on a Cerex SPE Processor pressure-flow device (Varian) with 75 mL of wash buffer 1. The resin was subsequently washed by 40 mL of NT wash buffer 2 containing 25 mM HEPES, pH 7, 10% (v/v) glycerol, 150 mM NaCl, 4 mM DTT and 0.4% (w/v) NG. In the NT wash buffer 2 and in all subsequent

purification buffers, various detergents at concentrations depending on their particular critical micelle concentration were tested for individual *in vitro* applications. For simplicity, only the one detergent (NG) is mentioned in this description (see Fig. S1 for a summary of alternatively applied detergents). The resin was resuspended in a small volume of wash buffer 2 within the column, containing 0.7 mg of HRV 3C protease (produced in house), followed by incubation for 2 h. The eluted protein (10 mL) was diluted threefold with SP binding buffer, containing 10 mM HEPES, pH 7, 10% (v/v) glycerol, 4 mM DTT, and 0.3% (w/v) NG, and it was subjected to another PD10 column (gravity flow) containing 5 mL SP Sepharose (bed volume), which had been pre-equilibrated with SP binding buffer. The SP resin was washed with 10 mL SP binding buffer, followed by 25 mL SP wash buffer containing 10 mM HEPES, pH 7.7, 10% (v/v) glycerol, 35 mM NaCl, 4 mM DTT, and 0.3% (w/v) NG, followed by another 3 mL SP binding buffer. Elution was carried out by ~15 mL SP elution buffer containing 10 mM HEPES, pH 7, 10% (v/v) glycerol, 350 mM NaCl, 4 mM DTT, 0.3% (w/v) NG and 500 nM NT1 (GPGGRRPYIL). NT1 corresponds to the C-terminal part of the fusion protein cut off by HRV 3C protease, i.e., the remaining HRV 3C protease site (GP), two linker residues (GG) and the NTR1 binding-epitope of NT, which is NT8-13 (RRPYIL).

Whenever quantitative size exclusion chromatographic analyses were performed, the NTR1 variants were concentrated by an Amicon-15 Ultra concentrator with 50 kDa cutoff to less than 500 µL (this cutoff was suitable for all tested detergents). The concentrate was transferred to an Eppendorf tube and centrifuged in a table-top centrifuge for 10 min at 10,000 × *g*. The supernatant was loaded on a Superdex-200 10/300 column that was pre-equilibrated with running buffer containing 10 mM HEPES, pH 8.0, 150 mM NaCl, 4 mM DTT, 0.28% (w/v) NG, and 100 nM NT1.

Identification of mutant pD-NT columns for antagonist-bound receptor purifications

The pD-NT constructs carrying various mutations in the NT moiety were expressed at the scale of 1 L *E. coli* cultures in 2 × YT (0.2% (w/v) glucose, 100 µg/ml ampicillin). After inoculation to OD_{600} of 0.05 from a saturated pre-culture, the cells were grown at 37 °C and they were induced by 1 mM IPTG for 4 h after reaching OD_{600} of 0.6. The cells were harvested by centrifugation and they were resuspended in 25 ml lysis buffer (50 mM HEPES, pH 8, 500 mM NaCl, 25 mM imidazole, pH 8, two tablets of complete protease inhibitor cocktail). The mutant pD-NT proteins were purified and coupled to NHS-activated Sepharose analogously to the above-mentioned protocol for the wild-type NT ligand construct, albeit at 10-fold smaller scale (using 5 ml slurry ligand column).

Small-scale TM86V purification tests were performed in parallel as follows: for all mutant pD-NT constructs (and wild-type pD-NT as control) 1 ml slurry ligand resin (equilibrated in NT wash buffer 1) was transferred to a 50 ml Falcon tube. Twenty milliliters of solubilized TM86V (corresponding approximately to one tenth of the preparation from 50 g of *E. coli* cells as described above) was applied to each resin and the mixtures were incubated overnight at 4 °C. The pD-NT resins were subsequently pelleted at 400 g in a swinging-bucket rotor and transferred to empty Micro Bio-spin columns. Each resin was washed by 10 ml NT wash buffer A (25 mM HEPES, pH 8, 600 mM NaCl, 10% (v/v) glycerol, 0.5% (w/v) DM), followed by 3 ml NT wash buffer B (25 mM HEPES, pH 7, 2 mM DTT, 150 mM NaCl, 10% (v/v) glycerol, 0.5% (w/v) DM). At this stage the pD-NT resin samples corresponding to Fig. 5A (see below) were collected. Subsequently, 500 µl of antagonist elution buffer (10 mM HEPES, pH 7, 2 mM DTT, 150 mM NaCl, 10% (v/v) glycerol, 0.5% (w/v) DM, 5 mM SR142948) was added to each resin, followed by incubation of the mixture for 30 min on a roller mixer (Stuart). The columns were subsequently drained (elution: samples

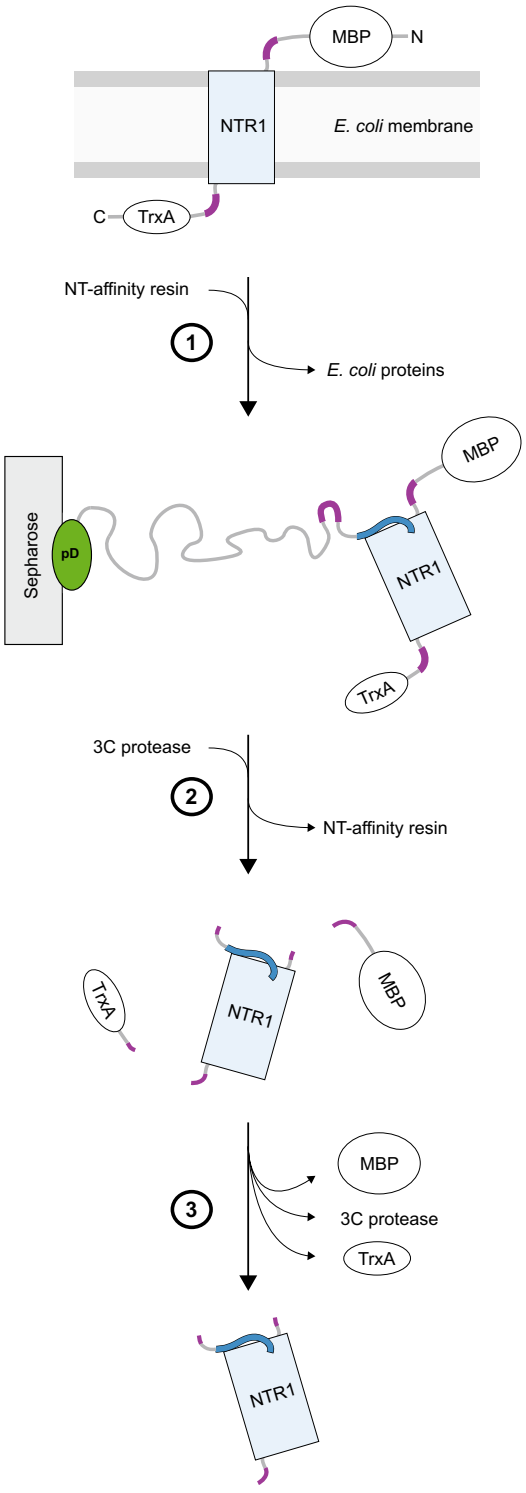

Fig. 3. Protocol overview for the large-scale preparation of NT-bound NTR1 variants from whole *E. coli* cells. Step 1, ligand-mediated pull-down of receptors directly from solubilized *E. coli* cells and washing of the NT-affinity resin in batch. The ligand is shown in blue. Various detergents can be applied in the wash buffer at this stage, in case a detergent exchange is required. Step 2, elution of the agonist-bound receptors and of the fusion proteins from the NT-affinity resin via cleavage at the three 3C rhinovirus protease sites (magenta). Step 3, removal of the cleaved off fusion proteins by cation-exchange chromatography in batch (gravity flow). The purified receptor/NT8-13 complexes were routinely analyzed by SEC (Fig. 4B and C). This procedure is completed in less than one working day (including analytical or quantitative SEC) and allowed the isolation >10 mg of pure receptor/agonist complexes. The ligand-mediated pull-down and the gravity-flow format allowed processing of several NTR1 variants in parallel.

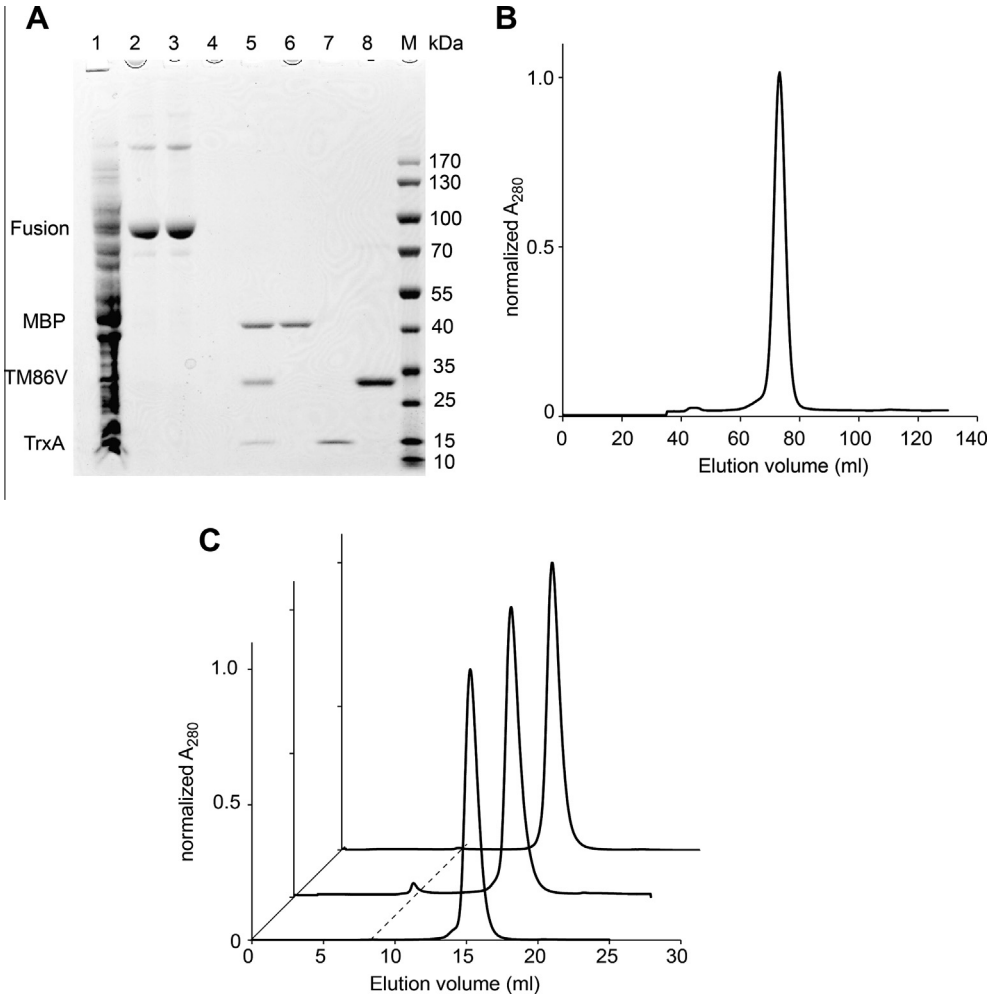

Fig. 4. Analysis of large-scale purifications of NTR1 variants in the NT-bound state in harsh glucoside detergents. (A) SDS–PAGE analysis of a typical purification of TM86V in OG. Lane 1, DM/CHAPS/CHS-solubilized whole *E. coli* cells. Lane 2, NT-affinity resin after pull-down and wash with DM-containing buffer. Lane 3, NT-affinity resin after detergent exchange to 1% (w/v) OG. Lane 4, second wash of NT-affinity resin using OG-containing buffer (detergent exchange). Lane 5, elution from NT-affinity resin by 3C protease. Lane 6, flow-through of cation-exchange column. Lane 7, wash of cation-exchange column. Lane 8, elution from cation-exchange column. (B) Quantitative SEC (S200 HiLoad 16/600) after elution from the cation-exchange column (lane 8 of the gel) using a buffer containing 1% (w/v) OG. (C) Semi-quantitative SEC of HTGH4 (front), OGG7 (middle) and TM86V (back) in NG-containing buffer. In these cases, the detergent was exchanged to 0.3% (w/v) NG on the NT-affinity resin. The dashed line depicts the void volume of the SEC column (S200 10/300 GL).

C in Fig. 5) and the resins were washed with another 2 ml of antagonist elution buffer. Further samples were collected from the washed resins after elution (samples B in Fig. 5).

Large-scale antagonist-bound TM86V purification

Solubilization of TM86V, receptor immobilization at the pD-NT-P10A resin and washing of the resin by NT wash buffer 1 was performed analogously to the purification protocol for the NT-bound NTR1 variants. The ligand resin was subsequently washed by 30 ml of pD-NT-P10A wash buffer 2 (25 mM HEPES, pH 7, 10% (v/v) glycerol, 150 mM NaCl, 4 mM DTT and 0.5% (w/v) DM). One bed volume of antagonist elution buffer (21 mM HEPES, pH 7, 8.5% glycerol, 128 mM NaCl, 4.3 mM antagonist SR142948, 0.85% DM) and 500 µl of HRV 3C protease (0.7 mg) was added to the washed resin. The column was closed, the resin was resuspended and the mixture was incubated for 2 h on a roller mixer (Stuart). Note that the antagonist stock solution for the elution buffer contained 5 mM SR142948 in 1% (w/v) DM (the antagonist was insoluble in H_2O above 1 mM). The column was subsequently drained and further eluted by pD-NT-P10A wash buffer 2 to give a total of 10 ml elution. Subsequently, 1 ml of 10% (w/v) NG was added to the eluted antagonist-bound TM86V and the solution was diluted by 19 ml SP binding buffer (10 mM HEPES, pH 7, 10% (v/v) glycerol, 4 mM DTT, 0.3% (w/v) NG). All subsequent steps were performed analogously to the purification protocol for agonist-bound NTR1 variants (exception: all buffers contained 300 nM antagonist and NT1 was omitted).

Results and discussion

Purification strategy and ligand column design

Many GPCR purification protocols rely on ligand-affinity chromatography to separate functional from non-functional protein. At the same time, ligand-binding usually increases receptor stability [15,22–26]. Therefore, it is apparent that a ligand-affinity column would in principle be most effectively applied as the *initial* purification step in a protocol, since this would combine the benefits of an activity-based separation with an early stabilization.

Moreover, many GPCR ligands exhibit nanomolar affinities, hence significantly stronger interactions than standard histidine tags display towards nitrilotriacetic acid-immobilized nickel ions, which have micromolar affinity [27]. This can be an additional

advantage during an initial purification step, particularly in the frequent case of low expression levels, when large amounts of membrane need to be solubilized in large buffer volumes, which leads to dilute GPCR concentrations and high quantities of competing impurities. On the other hand, a tight-binding ligand would normally require a harsh elution step from the affinity column, which may jeopardize receptor integrity, thus potentially eliminating any gain from such an affinity column.

In order to effectively isolate functional NTR1 variants from solubilized *E. coli* cell lysates, we generated a very inexpensive ligand-affinity resin, which allows mild receptor elution by cleaving the binary receptor-ligand complex off the column as a whole. The receptor takes the cleaved off ligand along all of the purification and as a result, it is maximally stable during the entire procedure. The ligand component consists of the minimal receptor-binding NT epitope (amino acids 8–13), which is fused to a major part of protein D from phage lambda (pD) for reasons described below. pD is C-terminally connected to NT8-13 via a long and flexible linker, which encodes an HRV 3C protease cleavage site in close proximity to NT (Fig. 1). Neither the linker nor NT contain lysine residues. The construct can thus be coupled to NHS-activated Sepharose *specifically* via pD, which contains 5 lysines and the free N-terminal amino group – leaving NT fully accessible for receptor binding and for proteolytic elution.

Ligand production and coupling efficiency

Fusing the NTR1 ligand to the carrier protein pD was expected to have two beneficial effects on the ligand column production: first, N-terminal pD fusions were previously described to enhance expression levels of soluble proteins significantly in *E. coli* [28], thus permitting ligand production at large quantities. Second, peptides, such as the NT ligand, are frequently degradation-prone, when overexpressed by themselves in *E. coli* and the linkage to a folded domain was expected to minimize this problem. This is far more economical than using a synthetic peptide. Up to 100 mg pD-NT could be expressed per liter of *E. coli* culture and 700–900 mg were typically obtained per purification run, using standard bench-top IMAC procedures (Fig. 2, lane 1).

The IMAC-purified pD-NT appeared to be pure on SDS–PAGE. However, it was initially not possible to achieve high coupling efficiencies to the NHS-activated Sepharose (initial yields: 40–70%). We suspected that small molecules containing primary amines might co-purify with pD-NT to some extent, and that these potential contaminants act as competitors in the coupling reaction. In order to prevent this, we introduced a dialysis step after the IMAC, which indeed improved the coupling efficiency to approximately 100% (Fig. 2).

In a typical coupling reaction, 12 mg pD-NT was immobilized per milliliter of NHS-Sepharose bed volume, corresponding to a theoretical column capacity of 35 mg/ml for full-length NTR1. Ligand expression (in a fermenter), purification of a small fraction of the expressed ligand and NHS-coupling of less than half of the purified pD-NT (350 mg) required typically less than 3 days and yielded 25 ml ligand resin (bed volume), which was suitable for more than 10 large-scale GPCR purifications.

Large-scale preparation of functional NTR1 variants

Our NTR1 expression construct encoded an N-terminal MBP for membrane targeting and a C-terminal TrxA fusion [18]. Unlike for previously described protocols, the fusion proteins were separated from the receptor by linkers with 3C rhinovirus protease sites in close proximity to the GPCR (Fig. 3, purple).

Extraction of NTR1 variants from whole *E. coli* cells was performed without prior membrane preparation using a mixture of DM, CHAPS and CHS. Subsequently, a small amount of ligand-affinity resin (2.5 ml bed volume per 200 ml solubilization reaction, cf. Materials and Methods) was applied to efficiently pull down milligram quantities of functional receptor from the soluble fraction. The ligand-affinity resin and the bound GPCRs were first washed by a DM-containing buffer and in a second step by a buffer containing a detergent of choice (bench-top column in a cold room). As observed by SDS–PAGE analysis, the receptor purity was close to 100% after this first purification step (Fig. 4A, lane 2 and 3). The subsequent addition of catalytic amounts of 3C protease allowed quantitative elution of the NT-bound NTR1 via ligand cleavage, and concomitantly of its cleaved fusion proteins MBP and TrxA (Fig. 4A, lane 5). The fusion proteins and the 3C protease were subsequently separated from the receptors by a cation-exchange step using a simple gravity-flow column format (bench-top), and the pure NTR1 variants were typically analyzed by quantitative SEC (Fig. 4B).

This purification principle by ligand-mediated immobilization was successfully applied to the three evolved NTR1 variants TM86V, OGG7 and HTGH4 [15,16,29] (Fig. 4C) using several different detergents (see Fig. S1 for a compilation of further SEC profiles using alternative detergents). Choosing the ligand-affinity step for detergent exchange was advantageous for two reasons: first, the column format allowed direct and early monitoring of NTR1 integrity in the presence of the new detergent by UV-absorbance measurements in the wash fractions; and second, the NTR1 variants were expected to be maximally stable at this stage, due to the bound ligand, which increased the range of tolerated detergents for these intrinsically stable GPCR variants even further [15].

A typical purification of TM86V in OG yielded 10 mg of ligand-bound receptor (after SEC), which corresponds to 3.6 mg per liter

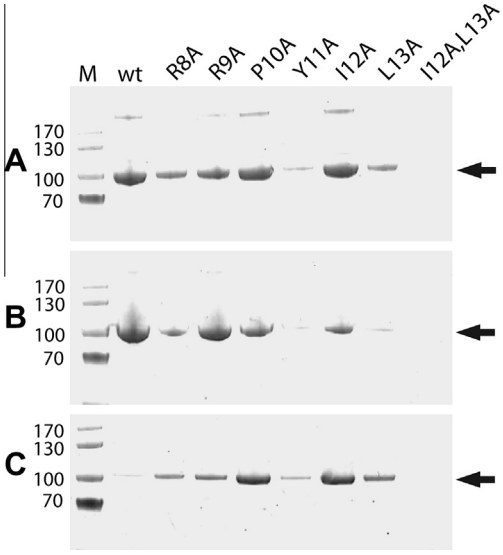

Fig. 5. SDS–PAGE analysis guiding the identification of NT8-13 mutations that allow TM86V-binding at lower affinity and thus permit elution by competition with excess antagonist. In preparation of this experiment, seven different pD-NT variants were generated and coupled to NHS-activated Sepharose beads. Each variant encoded either a single alanine substitution within NT8-13 or the double substitution I12A/L13A. The lanes are labeled by the substituted NT8-13 amino acids of the immobilized pD-NT8-13 constructs. Each lane represents an individual resin-binding experiment with the full TM86V expression construct from solubilized *E. coli* cells. (A) pD-NT beads after wash. (B) pD-NT beads after antagonist-mediated elution. (C) Elution by excess antagonist. The band corresponding to the receptor construct is depicted by an arrow. Note that samples (A) and (B) consisted of beads (pD-NT resin) that were resuspended in SDS sample buffer. The presence of SDS disrupted the receptor-ligand interaction and thus permitted the analysis on the gel.

of *E. coli* culture (Fig. 4B). Purifications of OGG7 yielded similar amounts of agonist-bound receptors, whereas the same procedure with HTGH4 resulted typically in even higher yields (12–16 mg).

The described purification protocol could be completed within one working day and the simplicity of the ligand-mediated pull-down and of the gravity-flow format allowed processing of several receptor variants in parallel.

Purification of antagonist-bound TM86V

Given the potential of NTR1 antagonists for medical applications [30,31], atomic-resolution structural insights about their binding mode are of high interest. We previously demonstrated that the evolved and signaling-active NTR1 variant TM86V binds to the antagonist SR142948 with high apparent affinity. TM86V was extensively characterized regarding its function and, as it yielded well diffracting crystals in complex with neurotensin, it is a promising candidate for the establishment of a purification procedure in complex with SR142948 and for subsequent crystallization trials.

In order to benefit from the ligand-mediated receptor isolation principles, we intended to adapt the agonist-based purification protocol in such a way that immobilized TM86V was no longer eluted from the pD-NT resin via proteolytic ligand cleavage, but instead by competition with excess antagonist (5 mM SR142948; note, [pD-NT] < 1 μmol/ml resin). Unfortunately, even though trace amounts of antagonist-bound TM86V could be eluted using this strategy, the receptor off-rate from the pD-NT construct appeared to be too slow for quantitative TM86V preparations.

In order to identify a more suitable immobilized ligand, we tested several Sepharose resins in parallel, each displaying a different version of pD-NT with alternative mutations in the C-terminal NT. The SDS–PAGE analysis of the bound, eluted and non-eluted TM86V fractions implied that the pD-NT resins carrying the mutations P10A and I12A exhibit a reasonably reduced affinity that allows for both efficient receptor binding and antagonist-mediated elution (Fig. 5).

We then chose to implement the pD-NT-P10A resin and the antagonist competition step in the previously developed large-scale purification protocol (Fig. 6A) – i.e., all other steps were performed analogously to the protocol for the receptor/agonist complex isolation described in Fig. 3. This purification procedure resulted in monodisperse SEC profiles and yielded typically about 3 mg of antagonist-bound TM86V per liter expression culture (0.2 mg per gram of wet cells), thus confirming the results of the small-scale ligand column screen and the feasibility of this purification strategy for crystallization trials (Fig. 6B and C). Since the antagonist exhibits a characteristic absorbance spectrum in the UV-range, the antagonist-bound state of purified TM86V was confirmed spectroscopically (Fig. S2).

It is noteworthy that, in contrast to the protease-mediated pD-NT cleavage for the agonist-bound purification, elution by antagonist competition does not irreversibly remove the ligand from the column. But in order to prevent reproducibility problems and due

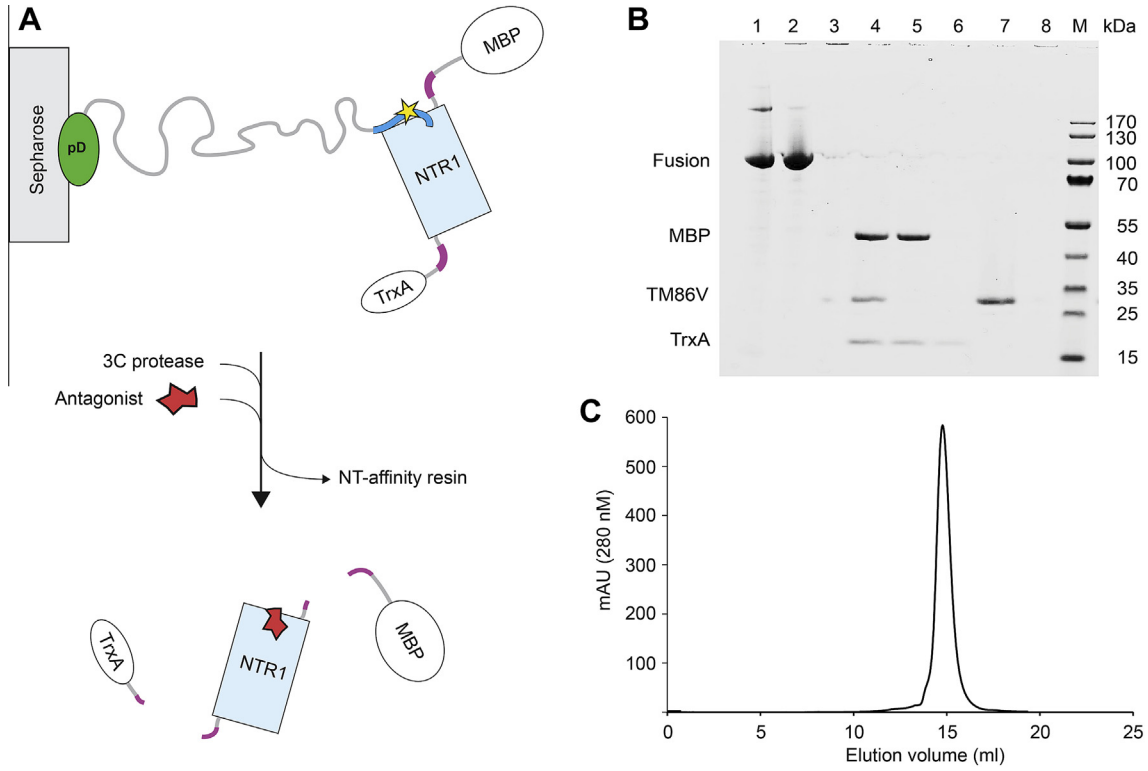

Fig. 6. Large-scale preparation of antagonist-bound TM86V by pD-NT-P10A Sepharose. (A) Schematic representation of the key step. The ligand-affinity resin exhibits a slightly reduced interaction strength with TM86V, due to the mutation P10A (yellow star) in NT8-13 (dark blue). Quantitative elution is possible using excess antagonist (SR142948). Note that no 3C rhinovirus protease site is encoded in the pD-NT-P10A construct. This allows simultaneous fusion protein removal and competitive elution without co-elution of free NT-P10A. The solubilization step and the removal of the cleaved fusion proteins by cation-exchange chromatography (in batch) were carried out as described for the purification of agonist-bound receptors. (B) SDS–PAGE analysis of a typical purification of antagonist-bound TM86V. Lane 1, NT-P10A-affinity resin after pull-down and wash with DM-containing buffer. Lane 2, NT-P10A-affinity resin after detergent exchange to 0.3% (w/v) NG. Lane 3, NT-P10A-affinity resin after competitive elution. Lane 4, elution from NT-P10A-affinity resin by antagonist competition. Lane 5, flow-through of cation-exchange column. Lane 6, wash of cation-exchange column. Lane 7, elution from cation-exchange column. Lane 8, cation-exchange resin after elution. (C) Semi-quantitative SEC (S200 10/300 GL) of the combined elution fractions from the cation-exchange column (lane 7 in B). The complete purification was performed in 0.3% (w/v) NG after detergent exchange.

to the simplicity of ligand resin production in large quantities, it is preferred to apply the pD-NT-P10A resin in single use as well.

Generalizability of purification principle

A large number of proteins were reported to exhibit high-affinity interactions with peptides [32,33]. Many of these ligands are devoid of lysines, hence similar economic NHS immobilization strategies by means of pD-peptide fusions may be feasible for ligand column production. In the case where a peptide-binding protein of interest benefits from ligand-mediated stabilization, or when efficient removal of aggregates is crucial, comparable purification strategies may likely improve the quantity and quality of purified proteins critically.

Conclusions

Ligand columns are very rarely used as a first step in large-scale GPCR purifications, in spite of their potential advantages, such as ligand-mediated receptor stabilization and high affinity interactions. There are usually several practical reasons that preclude the use of such columns.

Directly applying the soluble fraction to a ligand column would often prevent the repeated usage of the resin, as ligands can be unstable under these conditions (e.g., degraded by co-purified proteases) or because residual cellular debris leads to incomplete column recovery. The use of synthetic ligands can be very expensive and prohibitive for single-use columns. Also, harsh elution conditions from a tight-binding column with ensuing receptor denaturation might abrogate the effect of having captured active receptor in the first place.

However, in this work we have overcome these problems for the case of a peptide-binding GPCR by developing a high capacity ligand column, from which the receptor-ligand complex can be cleaved off. The ligand resin is so efficiently and inexpensively produced that the obtained column material is suitable for single use, thus rendering column recovery problems irrelevant.

The pD-NT Sepharose resins described in this work allowed time-efficient and highly reproducible receptor purifications directly from whole *E. coli* cell lysates. The outlined method was not only the basis for the first crystal structures of signaling-active NTR1 variants expressed in a prokaryote, it will also be key to various novel *in vitro* studies on these receptors. As *E. coli* is the preferred expression host for isotope-labeled protein production, the way is now paved for an array of NMR studies that were not feasible using functionally expressed GPCRs to date. Future experiments based on these purification principles will likely contribute to an improved understanding of GPCR dynamics and thus facilitate drug development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pep.2014.10.006.

References

[1] N. Vita, P. Laurent, S. Lefort, P. Chalon, X. Dumont, M. Kaghad, D. Gully, G. Le Fur, P. Ferrara, D. Caput, Cloning and expression of a complementary DNA encoding a high affinity human neurotensin receptor, FEBS Lett. 317 (1993) 139–142.
[2] G. Bissette, C.B. Nemeroff, P.T. Loosen, A.J. Prange Jr., M.A. Lipton, Hypothermia and intolerance to cold induced by intracisternal administration of the hypothalamic peptide neurotensin, Nature 262 (1976) 607–609.
[3] R.M. Schimpff, C. Avard, G. Fenelon, A.M. Lhiaubet, L. Tenneze, M. Vidailhet, W. Rostene, Increased plasma neurotensin concentrations in patients with Parkinson's disease, J. Neurol. Neurosurg. Psychiatry 70 (2001) 784–786.
[4] M. Alifano, F. Souaze, S. Dupouy, S. Camilleri-Broet, M. Younes, S.M. Ahmed-Zaid, T. Takahashi, A. Cancellieri, S. Damiani, M. Boaron, P. Broet, L.D. Miller, C. Gespach, J.F. Regnard, P. Forgez, Neurotensin receptor 1 determines the outcome of non-small cell lung cancer, Clin. Cancer Res. 16 (2010) 4401–4410.
[5] G. Griebel, F. Holsboer, Neuropeptide receptor ligands as drugs for psychiatric diseases: the end of the beginning?, Nat Rev. Drug Discov. 11 (2012) 462–478.
[6] D. Skrzydelski, A.M. Lhiaubet, A. Lebeau, P. Forgez, M. Yamada, E. Hermans, W. Rostene, D. Pelaprat, Differential involvement of intracellular domains of the rat NTS1 neurotensin receptor in coupling to G proteins: a molecular basis for agonist-directed trafficking of receptor stimulus, Mol. Pharmacol. 64 (2003) 421–429.
[7] K. Tanaka, M. Masu, S. Nakanishi, Structure and functional expression of the cloned rat neurotensin receptor, Neuron 4 (1990) 847–854.
[8] P.R. Dobner, Multitasking with neurotensin in the central nervous system, Cell. Mol. Life Sci. 62 (2005) 1946–1963.
[9] D. Pelaprat, Interactions between neurotensin receptors and G proteins, Peptides 27 (2006) 2476–2487.
[10] J.I. Hwang, D.K. Kim, H.B. Kwon, H. Vaudry, J.Y. Seong, Phylogenetic history, pharmacological features, and signal transduction of neurotensin receptors in vertebrates, Ann. N. Y. Acad. Sci. 1163 (2009) 169–178.
[11] J.F. White, N. Noinaj, Y. Shibata, J. Love, B. Kloss, F. Xu, J. Gvozdenovic-Jeremic, P. Shah, J. Shiloach, C.G. Tate, R. Grisshammer, Structure of the agonist-bound neurotensin receptor, Nature 490 (2012) 508–513.
[12] C.A. Sarkar, I. Dodevski, M. Kenig, S. Dudli, A. Mohr, E. Hermans, A. Plückthun, Directed evolution of a G protein-coupled receptor for expression, stability, and binding selectivity, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 14808–14813.
[13] I. Dodevski, A. Plückthun, Evolution of three human GPCRs for higher expression and stability, J. Mol. Biol. 408 (2011) 599–615.
[14] K.M. Schlinkmann, A. Honegger, E. Tureci, K.E. Robison, D. Lipovsek, A. Plückthun, Critical features for biosynthesis, stability, and functionality of a G protein-coupled receptor uncovered by all-versus-all mutations, Proc. Natl. Acad. Sci. U.S.A. 109 (2012) 9810–9815.
[15] K.M. Schlinkmann, M. Hillenbrand, A. Rittner, M. Kunz, R. Strohner, A. Plückthun, Maximizing detergent stability and functional expression of a GPCR by exhaustive recombination and evolution, J. Mol. Biol. 422 (2012) 414–428.
[16] P. Egloff, M. Hillenbrand, C. Klenk, A. Batyuk, P. Heine, S. Balada, K.M. Schlinkmann, D.J. Scott, M. Schütz, A. Plückthun, Structure of signaling-competent neurotensin receptor 1 obtained by directed evolution in *Escherichia coli*, Proc. Natl. Acad. Sci. U.S.A. 111 (2014) 655–662.
[17] R.A. Venters, B.T. Farmer, C.A. Fierke, L.D. Spicer, Characterizing the use of perdeuteration in NMR studies of large proteins C-13, N-15 and H-1 assignments of human carbonic anhydrase II, J. Mol. Biol. 264 (1996) 1101–1116.
[18] J.F. White, L.B. Trinh, J. Shiloach, R. Grisshammer, Automated large-scale purification of a G protein-coupled receptor for neurotensin, FEBS Lett. 564 (2004) 289–293.
[19] J.F. White, R. Grisshammer, Automated large-scale purification of a recombinant G-protein-coupled neurotensin receptor, Curr. Protoc. Protein Sci. (2007). Chapter 6, Unit 6 8.
[20] R. Grisshammer, J.F. White, L.B. Trinh, J. Shiloach, Large-scale expression and purification of a G-protein-coupled receptor for structure determination – an overview, J. Struct. Funct. Genomics 6 (2005) 159–163.
[21] J. Tucker, R. Grisshammer, Purification of a rat neurotensin receptor expressed in *Escherichia coli*, Biochem. J. 317 (1996) 891–899.
[22] A.M. Ring, A. Manglik, A.C. Kruse, M.D. Enos, W.I. Weis, K.C. Garcia, B.K. Kobilka, Adrenaline-activated structure of beta2-adrenoceptor stabilized by an engineered nanobody, Nature 502 (2013) 575–579.
[23] T. Ohtaki, K. Ogi, Y. Masuda, K. Mitsuoka, Y. Fujiyoshi, C. Kitada, H. Sawada, H. Onda, M. Fujino, Expression, purification, and reconstitution of receptor for pituitary adenylate cyclase-activating polypeptide. large-scale purification of a functionally active G protein-coupled receptor produced in Sf9 insect cells, J. Biol. Chem. 273 (1998) 15464–15473.
[24] H.M. Weiss, R. Grisshammer, Purification and characterization of the human adenosine A(2a) receptor functionally expressed in *Escherichia coli*, Eur. J. Biochem. 269 (2002) 82–92.
[25] T. Warne, J. Chirnside, G.F. Schertler, Expression and purification of truncated, non-glycosylated turkey beta-adrenergic receptors for crystallization, Biochim. Biophys. Acta 1610 (2003) 133–140.
[26] J.F. White, R. Grisshammer, Stability of the neurotensin receptor NTS1 free in detergent solution and immobilized to affinity resin, PLoS One 5 (2010).
[27] L. Nieba, S.E. Nieba-Axmann, A. Persson, M. Hamalainen, F. Edebratt, A. Hansson, J. Lidholm, K. Magnusson, A.F. Karlsson, A. Plückthun, BIACORE

analysis of histidine-tagged proteins using a chelating NTA sensor chip, Anal. Biochem. 252 (1997) 217–228.
[28] P. Forrer, R. Jaussi, High-level expression of soluble heterologous proteins in the cytoplasm of *Escherichia coli* by fusion to the bacteriophage lambda head protein D, Gene 224 (1998) 45–52.
[29] D.J. Scott, A. Plückthun, Direct molecular evolution of detergent-stable G protein-coupled receptors using polymer encapsulated cells, J. Mol. Biol. 425 (2013) 662–677.
[30] W. Rostene, M. Azzi, H. Boudin, I. Lepee, F. Souaze, M. Mendez-Ubach, C. Betancur, D. Gully, Use of nonpeptide antagonists to explore the physiological roles of neurotensin. Focus on brain neurotensin/dopamine interactions, Ann. N. Y. Acad. Sci. 814 (1997) 125–141.
[31] T.W. Moody, Peptide hormones and lung cancer, Panminerva Med. 48 (2006) 19–26.
[32] Y. Okuno, J. Yang, K. Taneishi, H. Yabuuchi, G. Tsujimoto, GLIDA: GPCR-ligand database for chemical genomic drug discovery, Nucleic Acids Res. 34 (2006) 673–677.
[33] C. Reichen, S. Hansen, A. Plückthun, Modular peptide binding: from a comparison of natural binders to designed armadillo repeat proteins, J. Struct. Biol. 185 (2013) 147–162.