Protein Engineering, Design & Selection vol. 22 no. 6 pp. 357–366, 2009
Published online April 22, 2009 doi:10.1093/protein/gzp011

Selection and characterization of DARPins specific for the neurotensin receptor 1

Peter Milovnik[1,4], Davide Ferrari[1,2,4], Casim A. Sarkar[1,3] and Andreas Plückthun[1,5]

[1]Biochemisches Institut, Universität Zürich, Winterthurerstrasse 190, 8057 Zurich, Switzerland
[2]Present address: Dipartimento di Biochimica e Biologia Molecolare, Università di Parma, Via Usberti 23/a, 43100 Parma, Italy
[3]Present address: Department of Bioengineering, University of Pennsylvania, 240 Skirkanich Hall, 210 S. 33rd St., Philadelphia, PA 19104-6321, USA

[4]These authors contributed equally to this work.

[5]To whom correspondence should be addressed.
E-mail: plueckthun@bioc.uzh.ch

We describe here the selection and characterization of designed ankyrin repeat proteins (DARPins) that bind specifically to the rat neurotensin receptor 1 (NTR1), a G-protein coupled receptor (GPCR). The selection procedure using ribosome display and the initial clone analysis required <10 μg of detergent-solubilized, purified NTR1. Complex formation with solubilized GPCR was demonstrated by ELISA and size-exclusion chromatography; additionally, the GPCR could be detected in native membranes of mammalian cells using fluorescence microscopy. The main binding epitope in the GPCR lies within the 33 amino acids following the seventh transmembrane segment, which comprise the putative helix 8, and additional binding interactions are possibly contributed by the cytoplasmic loop 3, thus constituting a discontinuous epitope. Since the selected binders recognize the GPCR both in detergent-solubilized and in membrane-embedded forms, they will be potentially useful both in co-crystallization trials and for signal transduction experiments.

Keywords: DARPins/GPCR/neurotensin/NTR1/ribosome display

Introduction

The neurotensin receptor 1 (NTR1) is a G-protein coupled receptor (GPCR) (Kroeze *et al*., 2003; Leifert *et al*., 2005) found in the central nervous system and in the gastrointestinal tract. Depending on the cell type and the ligand, it can couple, with varying efficiencies, to all three G-protein subtypes, G_s, G_i and G_q, generating a wide diversity of functional responses (Pelaprat, 2006). Its physiological ligand, the tridecapeptide neurotensin (NT), exerts a variety of behavioral and hormonal effects, including regulation of body temperature as well as inhibition of nociception and food intake. NTR1 ligands have potential therapeutic utility in the treatment of psychiatric and neurological disorders such as schizophrenia, Parkinson's disease and drug addiction (Boules *et al*., 2006). NT is, however, readily degraded by peptidases, and hence there is great interest in finding protease-resistant NT-analogs able to cross the blood–brain barrier (Boules *et al*., 2006). NT and its analogs also participate in the growth of cancer cells, and thus the receptor can be used for tumor imaging with NT analogs (Carraway and Plona, 2006). Despite the physiological and clinical relevance of NTR1, no structural information about this membrane-bound receptor is currently available.

Membrane proteins are intrinsically very difficult to crystallize and this is reflected by the small number of solved membrane protein structures, especially of mammalian proteins (www.mpibp-frankfurt.mpg.de/michel/public/memprotstruct.html, http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html). Unlike many other classes of membrane proteins, GPCRs have no bacterial homologs. The generation of well-diffracting crystals is primarily hindered by the large conformational flexibility of the detergent-solubilized membrane protein and the presence of rather small hydrophilic loops that connect the large hydrophobic segments surrounded by a detergent belt. These sparse polar regions limit the chances of generating productive intermolecular protein–protein interactions which mediate crystal growth.

For several years, the only GPCR crystal structure available was that of bovine rhodopsin (Palczewski *et al*., 2000), which can be isolated in fairly large amounts from its native source, the bovine retina. Only recently have the crystal structures of three other GPCRs, the closely related β_2- and β_1-adrenergic receptors (ARs) (Cherezov *et al*., 2007; Rasmussen *et al*., 2007; Warne *et al*., 2008) and the adenosine A_{2A} receptor (Jaakola *et al*., 2008), been determined. β_1AR and A_{2A}R were solved with the help of a soluble protein partner, either as a complex with a Fab fragment at 3.4 Å resolution (Cherezov *et al*., 2007) or as a fusion with T4 lysozyme at 2.4 Å resolution (Rasmussen *et al*., 2007; Jaakola *et al*., 2008). In both cases, most of the crystal contacts are provided by the soluble protein.

A complementary strategy to increase the conformational rigidity is to introduce mutations in the receptor itself. This has been carried out by directed evolution of NTR1 (Sarkar *et al*., 2008), which has been improved in thermal stability by this strategy and is the subject of the present study, and by alanine scanning on β_1AR (Serrano-Vega *et al*., 2008), through which a stabilized mutant could be crystallized (Warne *et al*., 2008).

Different studies have already shown the use of target-specific antibody fragments in the co-crystallization with other membrane proteins (Hunte and Michel, 2002). In all of these studies, classical immunization was used to generate the antibodies, with little control over the conformational state of the detergent-solubilized membrane proteins after injection into the animal.

In contrast to classical hybridoma technologies, synthetic libraries and *in vitro* selection technologies, such as phage display and ribosome display, can be used to generate such binding molecules in a fast and reliable way. Since the binding step is performed *in vitro*, selection conditions can be adapted to the need of the target protein (Huber *et al*., 2007). We have previously generated recombinant Fab fragments to the citrate transporter CitS (Röthlisberger *et al*., 2004).

Furthermore, recombinant technologies allow the use of protein classes other than antibodies. We have previously developed designed ankyrin repeat proteins (DARPins) (Binz *et al*., 2003) as an alternative binding molecule for co-crystallization (Binz *et al*., 2004; Kohl *et al*., 2005; Schweizer *et al*., 2007), because of their superior biophysical properties. Ankyrin repeat proteins mediate important protein–protein interactions in all cellular compartments (Li *et al*., 2006) and occur in all phyla. Their modular structure is built from stacked, 33-amino acid repeats, each forming a β-turn followed by two antiparallel α-helices and a loop connecting to the β-turn of the next repeat. A DARPin library, conserving the structure-determining residues and varying the potential interaction residues, was built (Binz *et al*., 2003) in which varying numbers of designed ankyrin repeats (typically 2 or 3), which carry the binding interface, are cloned in between specialized N- and C-terminal capping-repeats. These DARPins are termed N2C and N3C, where the number denotes how many internal repeats are present.

DARPins are obtained in very high yields (typically 100–200 mg soluble purified protein per liter *Escherichia coli* shake flask culture), and they tolerate the presence of reducing agents, as DARPins do not contain any disulfide bonds or free cysteines. They have a rather shallow groove-like binding surface, which is complementary to folded proteins. Therefore, both the molecular design and the *in vitro* selection strategy significantly increase the likelihood of obtaining binders against structural epitopes, rather than unstructured tails. Another very important advantage of DARPins is their high thermodynamic stability (Wetzel *et al*., 2008), which allows them to retain function even in harsh environments, such as detergent solutions (Huber *et al*., 2007).

DARPins can be selected both by ribosome display and by phage display (Binz *et al*., 2004; Steiner *et al*., 2008), and they have already been selected against integral membrane proteins using both methods (Huber *et al*., 2007) (A. Batyuk *et al*., unpublished results).

In this paper, we describe for the first time DARPins selected against a GPCR by ribosome display. The initial characterization of the selected NTR1-specific DARPin binders required only small amounts (<10 μg) of the membrane receptor. We show that the selected DARPins recognize the GPCR in solubilized form and in the membrane of mammalian cells.

Materials and methods

Materials

All detergents were obtained from Anatrace, except for cholesteryl hemisuccinate tris salt (CHS), which was from Sigma. PCR reactions between the ribosome display rounds were performed using the Phusion Polymerase from New England Biolabs (NEB). T7 RNA polymerase was from NEB. ThermoScript reverse transcriptase was from Invitrogen. Restriction enzymes were obtained either from NEB or from Fermentas. The radioligands [^{3}H]-neurotensin and [methoxy-^{3}H]-SR48692 were from PerkinElmer and GE Healthcare, respectively. [^{35}S]-Methionine was from Hartmann Analytic (Braunschweig, Germany). The NTR1 agonist NT (8–13) and the biotinylated neurotensin (biot-NT), connected via a 6-aminohexanoic linker, were from AnaSpec (San Jose, USA). Streptavidin–alkaline phosphatase (SA–AP) conjugate was from Roche. 5-Bromo-4-chloro-3′-indolylphosphate para-toluidine salt (BCIP) and nitro-blue tetrazolium chloride (NBT) were from Fluka. Neutravidin was from Pierce.

Protein expression and purification

The N-terminally truncated form of the rat NTR1 (rT43NTR1), missing the first 42 amino acids, was expressed and purified essentially as described previously (White *et al*., 2004), with the main difference that the *E.coli* strain XL1-Blue was used in place of DH5α, as it gave a slightly better yield (data not shown). For simplicity, the rT43NTR1 construct will be denoted as NTR1 throughout the paper.

The extract from 30 l of expression culture was loaded onto 40 ml of Ni-NTA material (Qiagen). For ligand affinity chromatography, biot-NT (0.8 ml, 100 μM) was immobilized on a monomeric avidin column (6 ml, Pierce). The preparative size-exclusion chromatography (SEC) was performed on a HiLoad 16/60 Superdex 200 prep grade column (GE Healthcare). Fractions from the gel filtration column were shock frozen in liquid nitrogen and stored at -80°C. The active fraction of the purified receptor was quantitated in a radioligand binding assay according to the manufacturer's instructions with Bio-Spin 30 spin-columns (Bio-Rad) to remove the excess of [^{3}H]-labeled ligand. The radioactive samples were analyzed by liquid scintillation counting. The total receptor concentration was measured using the DC-assay (Bio-Rad). We isolated ~0.3 mg of active solubilized wild-type NTR1 from 30 l of expression culture before a mutant evolved for expression and stability became available (Sarkar *et al*., 2008). Before an experiment, the gel filtration fractions were thawed on ice, and the active portion was determined to be ~50–70% of the total receptor concentration. The receptor was subsequently immobilized via its ligand (see below), ensuring that only active receptor is retained.

The biotinylated DARPins were obtained using the plasmid pAT223 (Amstutz *et al*., 2005) (GenBank™ accession number AY327138). The expressed polypeptide carries an N-terminal Avi-tag for *in vivo* biotinylation, followed by the phage λ protein D (pD) and a His_6-tag for purification and, at the C-terminus, the DARPin. *In vivo* biotinylation was performed according to protocols from Avidity (Denver, CO, USA) and Qiagen in *E.coli* XL1-Blue cells (Stratagene, La Jolla, CA, USA) transformed with both the pBirAcm (Avidity) and the pAT223-DARPin plasmids. Successful biotinylation of the IMAC purified proteins was confirmed by using the 4′-hydroxyazobenzene-2-carboxylic acid assay (Pierce), and by western blot using an SA–AP conjugate (Roche Applied Science). Non-biotinylated DARPins were produced using pAT223 and *E.coli* XL1-Blue while omitting the pBirAcm plasmid and biotin, reducing biotinylation (by genomically encoded BirA) to ~1%.

The DARPin constructs with an N-terminal $RGSH_6$-tag and 5 myc-tags at the C-terminus were expressed in *E.coli* XL1-Blue from a pQE30-based vector (Huber *et al*., 2007) and then purified using Ni-NTA affinity chromatography.

Ribosome display

The PCR-amplified DARPin N2C and N3C libraries were transcribed and the ribosome display selections were performed as previously described (Hanes and Plückthun, 1997; Huber *et al*., 2007; Zahnd *et al*., 2007) with a few modifications. The main difference is the presence of 0.5% CHAPS, 0.1% CHS, 0.1% DDM during the translation reactions as well as the binding and washing steps. According to previous studies (Tucker and Grisshammer, 1996), this detergent cocktail best preserves the receptor activity.

For a round of ribosome display, MaxiSorp plates/strips (Nunc) were coated with 66 nM neutravidin (100 μl/well) in TBS at 4°C overnight or for 1 h at 37°C. Wells were then blocked with 0.5% BSA in TBS for 1 h at 30–37°C. The receptor was immobilized by forming a complex between biot-NT and NTR1, and binding that to the immobilized neutravidin. This was achieved by preincubating 15–30 nM of purified NTR1 with 10–40 nM biot-NT in NTR-buffer (50 mM Tris, pH 7.4 at 4°C, 1 mM EDTA, 0.1% BSA, 0.2% CHAPS, 0.04% CHS, 0.1% DDM) for 30–60 min at 4°C. The solution was then transferred to the plate for 1 h at 4°C.

We used two alternative strategies to remove non-specific binders from the selection pools. The clones isolated using the first strategy, pre-panning of the translation mix in wells lacking the receptor (containing only biot-NT bound to neutravidin), are denoted as D-clones. The clones selected using the second strategy, addition of excess of neutravidin (500 nM) and biot-NT (40 nM) directly in the buffer used to stop the translation reactions, are denoted as P-clones.

The translation reactions were stopped with 3–4 volumes of cold wash buffer WB (50 mM Tris, pH 7.4, 50 mM $MgCl_2$, 0.5% BSA, 0.5% CHAPS, 0.1% CHS, 0.1% DDM), transferred to the wells and incubated for 20–30 min. The solution was then removed and the wells were washed with WB. The washing time was increased from round to round, reaching ~5 min in the last (fourth) round. After elution of the bound complexes with a buffer containing 50 mM Tris (pH 7.5 at 4°C), 200 mM NaCl and 50 mM EDTA, the mRNA was reverse transcribed using ThermoScript reverse transcriptase and the cDNA was amplified by PCR using Phusion DNA Polymerase.

Enzyme-linked immunosorbent assays

Neutravidin coating of the MaxiSorp plate and blocking with BSA was performed as described above. For receptor immobilization, a 30 nM solution of the NTR1/biot-NT complex was used. For testing specificity, 30 nM solutions of biotinylated MBP, biotinylated TrxA and the full construct MBP-NTR1-TrxA (complexed to biot-NT) were immobilized. Free biotin-binding sites were blocked with 3–5 μM biotin. Excess biotin was removed by extensive washing. In a separate reaction, a mixture of 4 nM biotinylated DARPin and 5 nM SA–AP was incubated for 45–60 min at 4°C in WB. The mixture was then transferred to the plate and incubated for 15 min. For competition experiments, 50–70 nM NTR1 saturated with its (non-biotinylated) agonist NT was included. In a control experiment, 750 nM free NT had not caused dissociation of the immobilized receptor (data not shown). Unbound DARPin/SA–AP complexes were washed away, and the bound complexes were detected by following the time course of absorbance at 405 nm upon conversion of the AP substrate 4-nitrophenyl-phosphate (*p*NPP).

Size-exclusion chromatography

SEC experiments were performed using a Superdex 200 GL 10/300 column (GE Healthcare). The runs were performed in buffer GF (25 mM Tris/pH 7.5 at 4°C, 300 mM NaCl, 0.15% CHAPS, 0.03% CHS, 0.3% DDM, 5% glycerol) at a flow rate of 200 μl/min at 4°C on an Äkta Explorer system (GE Healthcare). Protein elution was monitored at 280 nm. Samples of 200 μl containing 15 μM receptor and/or 50 μM DARPin with 5 myc-tags at the C-terminus were injected. pD (17 kDa), SHP (49 kDa) and β-amylase (200 kDa) were used to calibrate the column.

Affinity determination on the BioVeris system

The affinities of selected NTR1 binders were determined in solution in a competition electrochemiluminescence (ECL)-based assay on a BioVeris M1M Analyzer (Witney, UK), essentially as described (Huber *et al*., 2007). The assay relies on the light emission of the 'BV-tag', a tris(2,2′-bipyridine)ruthenium(II) complex conjugated to the protein to be determined, upon electro-oxidation in the presence of aliphatic amines, such as 3-propylamine (Miao *et al*., 2002). Neutravidin modified with the BV-tag (neutravidin-BV) was prepared by reacting the protein with a 5-fold excess of BV-tag *N*-hydroxysuccinimide ester (BV-tag-NHS) in PBS at 25°C, pH 7.8, for 1 h. Unreacted BV-tag-NHS was removed with an Amicon 15 (30 kDa MWCO) concentrator (Millipore). The labeling stoichiometry was found to be 1:1 based on protein concentration determined by using the DC-assay (BioRad) and on the BV-tag concentration determined by using an ϵ_{455} of 15 400 M^{-1} cm^{-1}.

The assay is based on immobilizing one DARPin, P28, to magnetic beads, and detecting the binding of the GPCR–ligand complex, itself bound to BV-tag-neutravidin via the biotinylated ligand, in the presence of varying amounts of DARPin competitor. Since the DARPins of interest all competed for the same epitope, they could all be measured in competition with the same immobilized DARPin P28.

To prepare DARPin P28 immobilized on streptavidin magnetic beads, 150 nM enzymatically biotinylated DARPin biot-P28 (biotinylated to ~60%) was incubated with 1 mg/ml of MyOne T1 streptavidin beads (Roche) in TBS (pH 7.5 at 4°C), 0.5% BSA at 4°C for 2 h. Unbound DARPin was washed away and remaining biotin binding sites were blocked with 3 μM biotin. The competition solutions contained, in a total volume of 280 μl, 0.5 nM DARPin biot-P28 immobilized on 5.5 μg/ml streptavidin magnetic beads, 10 nM NTR1, 13 nM biot-NT, 25 nM neutravidin-BV-tag and varying concentrations of the non-biotinylated DARPins as competitors (0–1500 nM) in 50 mM Tris (pH 7.5 at 4°C), 0.5% CHAPS, 0.1% CHS and 0.1% DDM.

The competition solutions were incubated for 2 h at 4°C, then 130 μl was transferred to the BioVeris plate and the ECL-based signal was measured in the BioVeris workstation. The standard deviation between duplicate measurements was <2%. The signal of NTR1/NT/SA-BVtag complex bound to

an immobilized DARPin is:

$$\mathrm{ECL} = (\mathrm{ECL}_{\max} - \mathrm{ECL}_{\mathrm{BG}}) \cdot \frac{C_{\mathrm{b}}}{C_{\mathrm{b,max}}} + \mathrm{ECL}_{\mathrm{BG}} \qquad (1)$$

where ECL is the measured signal, $\mathrm{ECL}_{\max}$ the maximal signal in the absence of competitor, and $\mathrm{ECL}_{\mathrm{BG}}$ the signal due to background binding. C_{b} is the bound receptor–DARPin complex on the beads in the presence of competing DARPin in solution and $C_{\mathrm{b,max}}$ the bound receptor–DARPin complex on the beads in the absence of competing DARPin.

The data of P28 itself were first analyzed by fitting the experimental values to an equation accounting for both the solution and the solid phase equilibria (assuming identical K_{D} values for both the immobilized and the free DARPin), essentially as derived previously (Huber *et al*., 2007). When both equilibria are accounted for, Eq. (1) can be rewritten in terms of total concentrations of the relevant species:

$$\mathrm{ECL} = (\mathrm{ECL}_{\max} - \mathrm{ECL}_{\mathrm{BG}}) \cdot \frac{1}{S_{\mathrm{t}}/I_{\mathrm{t}} + 1} \cdot \frac{(R_{\mathrm{t}} + S_{\mathrm{t}} + I_{\mathrm{t}} + K_{\mathrm{D}}) - \sqrt{(R_{\mathrm{t}} + S_{\mathrm{t}} + I_{\mathrm{t}} + K_{\mathrm{D}})^2 - 4(S_{\mathrm{t}}/I_{\mathrm{t}} + 1) I_{\mathrm{t}} R_{\mathrm{t}}}}{(R_{\mathrm{t}} + I_{\mathrm{t}} + K_{\mathrm{D}}) - \sqrt{(R_{\mathrm{t}} + I_{\mathrm{t}} + K_{\mathrm{D}})^2 - 4 I_{\mathrm{t}} R_{\mathrm{t}}}} \qquad (2)$$

where R_{t} is the total concentration of receptor added, S_{t} the total concentration of soluble non-biotinylated competitor DARPin added, I_{t} the total concentration of the DARPin immobilized and K_{D} the dissociation constant.

When different DARPins were tested, the affinities of the immobilized (P28) and the free DARPins (P4 and D14) could no longer be assumed to be identical, so a simplified equation [Eq. (5)] was used. This derivation neglects receptor depletion due to binding to the immobilized DARPin, which represents a maximum of 5% of the total NTR1 in solution and thus only marginally disrupts the equilibrium in solution when the solution-phase K_{D} is not extremely low. It is thus assumed that the ECL signal is proportional to the fraction of free receptor in solution.

$$\mathrm{ECL} = (\mathrm{ECL}_{\max} - \mathrm{ECL}_{\mathrm{BG}}) \cdot \frac{R_{\mathrm{free}}}{R_{\mathrm{t}}} + \mathrm{ECL}_{\mathrm{BG}} \qquad (3)$$

or, after rearrangement,

$$\mathrm{ECL} = \mathrm{ECL}_{\max} - \frac{\mathrm{ECL}_{\max} + \mathrm{ECL}_{\mathrm{BG}}}{R_{\mathrm{t}}} \cdot C_{\mathrm{sol}} \qquad (4)$$

where C_{sol} is the receptor–DARPin complex in solution. In the simplified equation,

$$\mathrm{ECL} = \mathrm{ECL}_{\max} - \frac{(\mathrm{ECL}_{\max} - \mathrm{ECL}_{\mathrm{BG}})}{2R_{\mathrm{t}}} \cdot \left[(S_{\mathrm{t}} + R_{\mathrm{t}} + K_{\mathrm{D}}) - \sqrt{(S_{\mathrm{t}} + R_{\mathrm{t}} + K_{\mathrm{D}})^2 - 4 S_{\mathrm{t}} R_{\mathrm{t}}} \right] \qquad (5)$$

the symbols have the same meaning as in Eq. (2). To first compare this simplified treatment for the measurement of P28 itself, a value of 75 nM is obtained, as opposed to 71 nM from the more complete treatment of Eq. (2), an acceptable error considering the many other experimental uncertainties.

Epitope mapping

Different fragments of NTR1 were inserted between the *Bam*HI and *Hin*dIII sites of the vector pAT223 (see above), resulting in a C-terminal fusion to pD. The constructs span the following amino acids of the receptor: construct C1 [intracellular loop 3 (IL3)] Asn215-Gly264, construct C2 [IL3 to the end] Asn215-Tyr382 (plus His_6-tag), construct C3 [IL3 to extracellular loop 3 (EL3)] Asn215-Tyr309, construct C4 [EL3 to the end] Cys290-Tyr382 (plus His_6-tag), construct C5 [IL3 to helix 8] Asn215-Arg356 and construct C6 [C-terminus] Pro324-Tyr382 (plus His_6-tag). Constructs C1, C3 and C5 carried an additional tripeptide KLN before the stop codon, originating from the *Hin*dIII site of the vector.

A single colony of *E.coli* XL1-Blue, transformed with the corresponding pAT223 construct, was grown at 37°C overnight in 2YT with 1% glucose (glc) and 100 μg/ml ampicillin, resulting in an OD_{600} of 5–6. One milliliter of the overnight cultures was diluted to 20 ml in 2YT with 0.1% glc and 100 μg/ml ampicillin and grown to an OD_{600} of 0.6–0.7; expression was induced with 0.5 mM IPTG for ~2 h. The resulting OD_{600} for the constructs (defined in Results), pD-thioredoxin A (pD-TrxA) and no plasmid (*E. coli* XL1-Blue alone), were as follows: 2.55 (C1), 0.55 (C2), 0.70 (C3), 0.45 (C4), 0.95 (C5), 2.60 (C4), 3.30 (pD-TrxA) and 3.0 (no plasmid). An aliquot of the expression culture, corresponding to $\sim 5 \times 10^8$ cells, was centrifuged for 5 min at 7000*g* and the pellet resuspended in 100 μl of a reducing loading buffer with 100 mM β-mercaptoethanol, 1% SDS, 2 mM MgCl_2 and 125 U/ml benzonase (Novagen). The samples (25 μl) were analyzed by SDS–PAGE and western blot after an overnight incubation at room temperature. The biotinylated DARPin P28, complexed with the SA–AP used for detection, was incubated for 15 min at a concentration of 75 and 37.5 nM, respectively. Free biotin binding sites were then blocked with 1 μM biotin by incubation for at least 15 min and the biot-P28/SA–AP complex solution was incubated with the blotting membrane for 45 min. After washing the membrane, the biot-P28/SA–AP bound to the expressed receptor fragments was detected with BCIP/NBT.

Fluorescence microscopy

COS-7 cells were grown in Dulbecco's modified Eagle's medium in the presence of 10% (v/v) heat-inactivated fetal calf serum (FCS) and 5% CO_2 at 37°C in a humidified incubator. One day before transient transfection, 1.2×10^5 cells were seeded on poly-L-lysine-coated glass coverslips (18 × 18 mm) in a 6-well culture plate. Cells covered with 1 ml medium were transfected with 1 μg plasmid DNA using FuGENE HD (Roche) at 5 μl per μg DNA according to the manufacturer's instructions. Four to six hours later, the transfection solution was exchanged for 2 ml medium containing 10 μM SR48692 (Sanofi-Aventis), an antagonist for NTR1 (Gully *et al*., 1993), or naloxone, an antagonist for the κ-opioid receptor (KOR). Twenty-four hours after the transfection, the cells were fixed with 4% (w/v) paraformaldehyde

in PBS (pH 8.0) for 10 min at room temperature and blocked/permeabilized with PBS, 10% FCS, 0.1% saponin for 30 min. The Myc-tagged receptors were detected with a mouse anti-Myc antibody (Cell Signalling Technology, #2276, 1:3000) and stained with an AlexaFluor-555-labeled goat anti-mouse antibody (Invitrogen, A-21422, 1:10 000), both for 1 h in PBS, 1% FCS, 0.1% saponin. DARPins fused to the superfolder GFP (sfGFP) (Pedelacq *et al*., 2006) were used at 1 μM concentration during the second incubation step with the AlexaFluor-555-labeled antibody where indicated. After washing three times with PBS for 5–10 min, the nuclei were stained with 0.8 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in PBS for 2–3 min. The coverslips were quickly washed with PBS, mounted with Fluoromount G (Interchim) and sealed with nail varnish before microscopy. Fluorescence images were acquired using a Leica Leitz DMRXE microscope equipped with a DFC350FX camera at ×40 magnification. Colors, contrast and brightness of the whole image were adjusted in Adobe Photoshop.

Results

Ribosome display

DARPins that bind to functional, solubilized NTR1 were selected by ribosome display. We chose to use the detergent-solubilized GPCR as a target for selection over the use of whole cells or reconstituted proteoliposomes, since both extracellular and intracellular epitopes are accessible, and this would be the form of relevance for structural studies. Nevertheless, the detergent may also shield certain epitopes and the GPCR might, at least in principle, adopt a non-functional conformation. In order to ensure that the GPCR is immobilized in functional form, we complexed the receptor to a biotinylated agonist, NT, which was then bound to coated neutravidin.

For these experiments, we used either the wild-type rat NTR1 or a mutant, denoted D03, which had been obtained by a novel directed evolution approach and which expresses up to 10-fold better in *E.coli*, *Pichia pastoris* and mammalian cells and which is also significantly more stable in detergent-solubilized form (Sarkar *et al*., 2008).

The receptor was expressed in *E.coli* XL1-Blue essentially as previously described (White *et al*., 2004). The efficient assembly of NTR1 in the cytoplasmic membrane of *E.coli* requires a low expression temperature (20°C) and the use of two fusion partners: maltose-binding protein (MBP) at the N-terminus and thioredoxin (TrxA) at the C-terminus (White *et al*., 2004). The purification of the solubilized active receptor was performed in three chromatographic steps: (i) IMAC using a Ni-NTA column, (ii) affinity chromatography on a monomeric avidin column with immobilized biot-NT and (iii) gel filtration, which separates the receptor moiety from MBP, TrxA and TEV protease, added to cleave the fusion partners.

The ribosome display selections from the DARPin library (Binz *et al*., 2003) were performed essentially as described previously (Hanes and Plückthun, 1997; Huber *et al*., 2007; Zahnd *et al*., 2007) and are explained in more detail in Materials and methods.

In order to save material, the GPCR target was immobilized through its biot-NT ligand using a protein concentration an order of magnitude lower than recommended in the standard ribosome display protocol (Hanes and Plückthun, 1997; Huber *et al*., 2007; Zahnd *et al*., 2007). An important feature of our immobilization strategy is the preservation of GPCR activity, as the target protein must remain active in order to bind to the biot-NT immobilized on the neutravidin-coated plate. The same would not be true for other types of immobilization (e.g. through a tag on the receptor), which would not discriminate between active and inactive forms of the receptor. Receptor immobilization via biot-NT will, however, favor the selection of binders against the intracellular side of the receptor since the peptide linker is short and, therefore, the extracellular face of the bound receptor is constrained to be in close proximity to the solid phase and probably less accessible.

Two separate setups were tested for minimizing the selection of unspecific binders (denoted as P and D), as described in Materials and methods. Four rounds of ribosome display selection were sufficient (not shown) to obtain DARPins that could be further analyzed.

Pool and single clone RIA

After four rounds of ribosome display, the enrichment of binders for the receptor was determined by testing the binding of *in vitro* translated [^{35}S]Met-labeled DARPins to immobilized NTR1. The percentage of binders recognizing NTR1 in the pools increases from round to round, reaching ~50% after the fourth round (not shown). Sixty clones, 30 from each of the two different selection setups (P and D) described in Materials and methods, were randomly picked from the last selection round and sequenced. Among them we found 24 unique sequences which were further analyzed in the *in vitro* translation assay (Fig. 1). The majority of the selected DARPin clones specifically bind to the receptor. Comparison of the D and P selection strategies shows that the pre-panning step leads to more efficient selection of specific clones (Fig. 1A), whereas using excess neutravidin and biot-NT in solution to eliminate the unspecific binders resulted in a slightly higher background binding (Fig. 1B).

Among the 24 unique sequences, the 12 best NTR1-specific binders (according to the ELISA experiments) and two negative controls, presumably recognizing streptavidin and/or biot-NT, were chosen for further analysis.

As mentioned above, the purification of unfused NTR1 from *E.coli* requires a protease cleavage step and a separation of the mixture on a size exclusion column. Analyzing the purified NTR1-fractions used for the selection by silver-stained SDS–PAGE, only cleaved NTR1 was found, and neither uncut full length protein (MBP-rT43NTR1-TrxA) nor partially cleaved constructs nor the fusion partners MPB and TrxA alone were detected in the NTR1 fraction (not shown). Nonetheless, the selection of binders against the fusion partners and/or the NTR1-fusion constructs resulting from some minor contamination in the purified receptor could not be excluded. We thus analyzed the [^{35}S]Met-labeled DARPins as single clones for binding to MBP and TrxA alone, to the full fusion construct and to the receptor alone. Importantly, no binding to purified MBP and TrxA could be detected (Fig. 2). The signal arising from binding to the uncleaved fusion protein is lower than that from the cleaved and

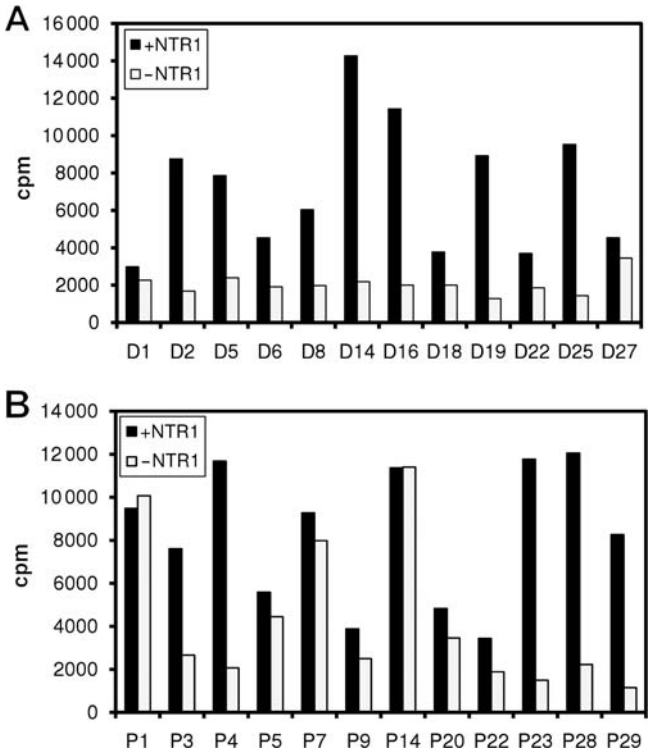

Fig. 1. Radioimmunoassay using 24 of the unique clones selected after four rounds of ribosome display. NTR1/biot-NT (black bars) or biot-NT alone (grey bars) was immobilized on a neutravidin-coated ELISA plate. The binding of the DARPins was detected by measuring the radioactivity of the *in vitro* expressed and [^{35}S]-Met-labeled binders. (**A**) Clones selected using a pre-panning step to minimize non-specific binding (strategy D). (**B**) Clones selected using an excess of neutravidin and biot-NT in solution to minimize unspecific binding (strategy P).

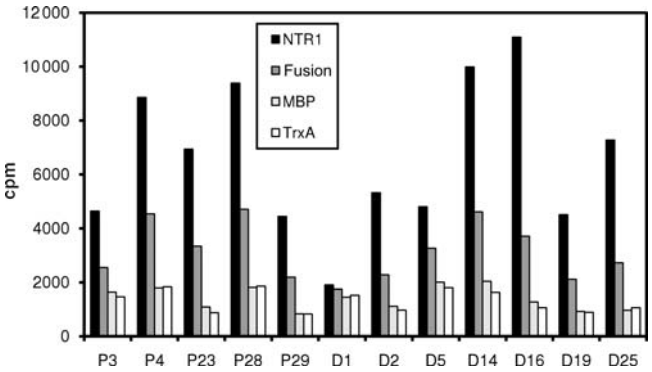

Fig. 2. Specificity of the selected binders. Twelve selected DARPin binders were purified and tested for binding to NTR1 (black bars), the full construct MBP-NTR1-TrxA (dark grey bars), MBP (light grey bars) and TrxA (white bars). The targets were immobilized on a neutravidin-coated ELISA plate. TrxA and MBP were biotinylated directly, whereas NTR1 and MBP-NTR1-TrxA were immobilized via biot-NT, as described in Materials and methods.

purified receptor alone. We assume that steric hindrance of DARPin binding or a less efficient receptor immobilization, both due to the presence of the fusion proteins, might cause the reduced signal. This result is consistent with the binding epitope being localized to the beginning of C-terminus after helix 7 and IL3, since TrxA might shield these regions (see Epitope mapping below).

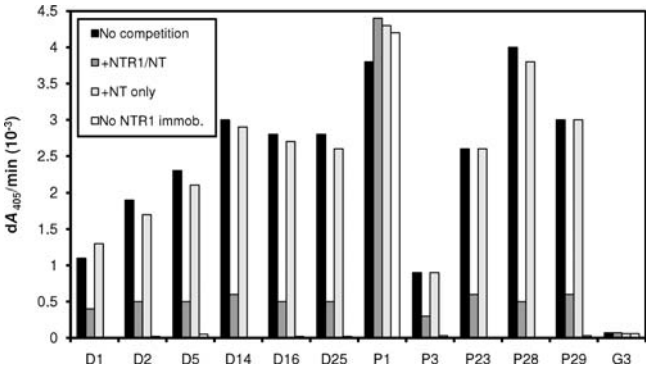

Fig. 3. Competition ELISA experiment. Black bars: direct binding of the DARPins to the immobilized NTR1 (*no competition*). Dark grey bars: binding of the DARPins in the presence of an excess of NTR1 and NT in solution (*+NTR1/NT*). Light grey bars: binding of the DARPins in the presence of an excess of NT alone in solution (*+NT*). White bars: background binding of the DARPins in the absence of the immobilized receptor (*no NTR1 immob.*). For details, see Materials and methods.

Specificity and selectivity of the binders

DARPins were expressed in *E.coli* in the presence or absence of the biotinylation plasmid expressing BirA and subsequently purified by IMAC. To analyze the requirements for complex formation with the GPCR, especially with regard to crystallization, selected DARPins were tested for receptor binding in solution in a competition experiment. The receptor was bound to immobilized ligand, as in ribosome display selections, and the binding of the biotinylated DARPins was detected using a streptavidin-AP conjugate (Fig. 3). Addition of an excess of the receptor to the DARPin solution (see Materials and methods) significantly decreased the signal for all of the binding clones, consistent with specific binding to the GPCR. The reduction in signal was not caused by the dissociation of the immobilized receptor from the plate during the incubation steps, as the addition of a large excess of the agonist NT alone did not significantly lower the signal (Fig. 3). No signal was observed when NTR1 was not immobilized. As a negative control, the signal due to the clone P1 could neither be decreased by competition with the receptor in solution nor by omitting the receptor during the immobilization, consistent with the direct ELISA binding data (Fig. 1). Another control DARPin, G3, specific for HER2, gave just a background signal (Fig. 3).

The biotinylated DARPins could capture all three forms of the receptor from solution—the agonist-bound form, the antagonist-bound form and the ligand-free form—as determined in experiments with the radiolabeled ligands [^{3}H]NT and [^{3}H]SR48692 (not shown). Thus, the binders do not seem to differentiate between receptor conformations. However, as expected, the selected binders are specific for NTR1, as only using NTR1 and not a control GPCR (adenosine A_{2A} receptor) was a binding signal detected in a BIAcore experiment with the DARPins immobilized on a streptavidin chip (not shown).

Analytical size-exclusion chromatography

We used analytical gel filtration chromatography as a second test for complex formation in solution (Fig. 4). An evolved mutant of NTR1, denoted D03, which expresses up to 10-fold better than the wild type in *E.coli* and is more stable

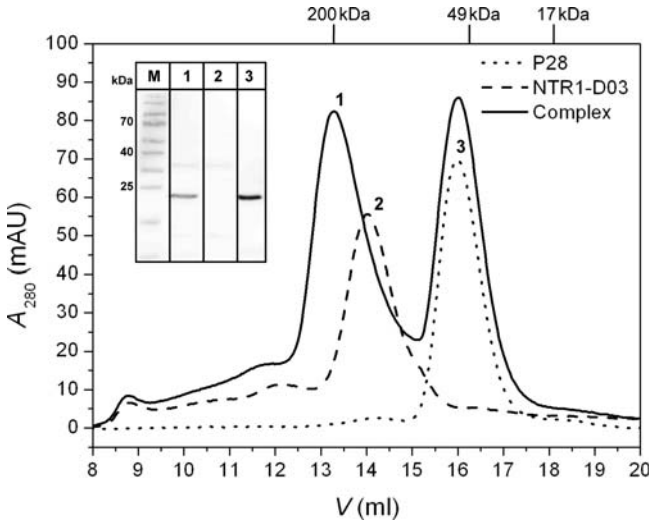

Fig. 4. SEC using the NTR1 mutant D03 (Sarkar *et al*., 2008) and the selected DARPin P28. *Solid line*: elution profile of a mixture of D03 (3 nmol) and the DARPin P28 (10 nmol); *dashed line*: elution profile of the receptor alone; *dotted line*: elution profile of the DARPin P28 alone. Inset: western blot analysis of TCA-precipitated fractions of the corresponding peaks. Lane 1 corresponds to peak (1) of the mixture, lane 2 to the peak labeled (2) of the GPCR and lane 3 to the DARPin run alone, peak (3). The proteins were separated by SDS–PAGE, transferred to a PVDF membrane and detected by a mixture of an anti-rNTR1 (R-20) antibody (Santa Cruz Biotechnology, #sc-7598) and an anti-$RGSH_4$ antibody (for DARPin detection). It should be noted that the DARPin carries a 65-aa C-terminal 5-fold Myc-tag which is highly charged and presumably disordered. We have previously observed that this significantly increases the hydrodynamic radius of these already elongated molecules.

in detergents (Sarkar *et al*., 2008), was used in this experiment. Control ELISA experiments demonstrated that the selected DARPins recognize the D03 mutant and the wild-type receptor equally well (not shown).

Both the GPCR and the DARPin elute mainly as single peaks from a Sephadex 200 column at the expected molecular weight when injected separately. When the receptor is pre-incubated with a ~3-fold molar excess of the DARPin, two elution peaks are observed. In the first peak, clearly shifted to a higher molecular weight, both the GPCR and the DARPin could be detected on a western blot (Fig. 4, inset), whereas the second peak consists of the excess unbound DARPin. The change in the elution volume corresponds well to the size of the DARPin protein, as calculated from the elution volume of the DARPin and the standard proteins.

Determination of the affinities in solution

The affinities in solution were assessed by equilibrium titration. Since previous experiments had shown that the best binding DARPins all compete for the same or overlapping epitopes, equilibrium affinities could be measured by competition with binding to one immobilized DARPin. The binding of the receptor to the biotinylated DARPin, P28, immobilized on streptavidin-coated magnetic beads was detected by ECL (Huber *et al*., 2007) as described in Materials and methods. The inhibition of GPCR binding to immobilized P28 by different DARPins in solution was used to calculate their affinities for the receptor in solution (Fig. 5). The negative control DARPin P14 did not compete for the binding of the receptor even at 1 μM concentration (not shown). The K_D values calculated as described in

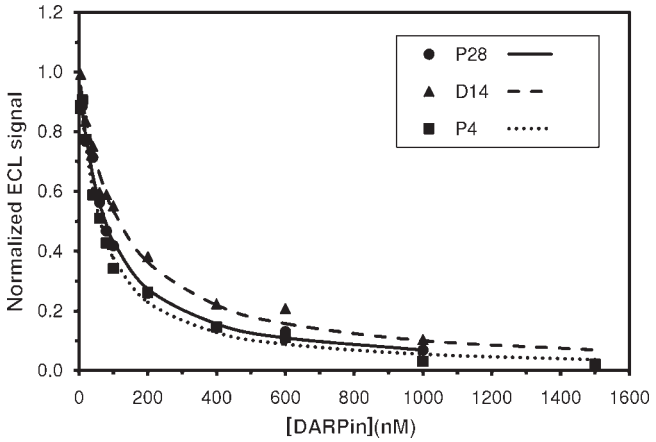

Fig. 5. Determination of the affinities in solution of three selected DARPins for NTR1. Binding of the NTR1 to the DARPin P28 immobilized on streptavidin beads was competed for by the DARPins present in solution at concentrations ranging from 0 to 1500 nM. The fits shown were calculated as described in Materials and methods.

Materials and methods were 75 nM for P28, 58 nM for P4 and 112 nM for D14 (Fig. 5). Using numerical fits with the program DYNAFIT (Kuzmic, 1996) to the two equilibria underlying Eq. (2), values of 67 nM for P28, 50 nM for P4 and 96 nM for D14 were obtained. It should be stressed that no attempt had been made in these selection experiments to maximize affinity, either by a selection strategy that emphasizes affinity or by an affinity maturation approach (Zahnd *et al*., 2007).

Epitope mapping

In order to determine the binding epitope recognized by the selected DARPins, we produced fragments of NTR1 as C-terminal fusions to the well expressed and soluble pD and probed them with one of the best binders, the selected DARPin P28, on a western blot (Fig. 6). Control samples, with cells carrying no expression plasmid or only expressing pD-TrxA at high level, gave no signal in western blots (data not shown). Receptor fragments composed of unpaired transmembrane helices, and thus probably exposing continuous hydrophobic patches (Fig. 6B,C, lanes 3 and 4), were very poorly expressed and/or were causing cell death, despite being fused to pD (data not shown). Good expression levels, i.e. clear bands on a Coomassie-stained gel, were obtained only when parts of the receptor normally exposed to solvent (IL3 or the C-terminal region) were fused to pD (Fig. 6B, lanes 1 and 6). The C-terminal fragment of the receptor gave a very strong signal in the western blot and a strong band in the Coomassie-stained gel, whereas the IL3 alone produced a weak signal, however, clearly distinguishable from the background (Fig. 6B,C lanes 1 and 6), and a clear Coomassie-stained band. Fragment C5 (IL3 to helix 8, missing the last 24 C-terminal amino acids) was visible on the Coomassie-stained gel as a rather weak band (Fig. 6B, lane 5); yet, it produced a fairly strong signal on the western blot (Fig. 6C, lane 5). Among the fragments expressed at levels not detectable in the Coomassie-stained gel, only fragment C2 (IL3 to the end of the receptor) gave a clear signal in the western blot (Fig. 6C, lane 2). The addition of the C-terminal part of the receptor to the IL3 reduced the expression of the fragments to very low levels (compare

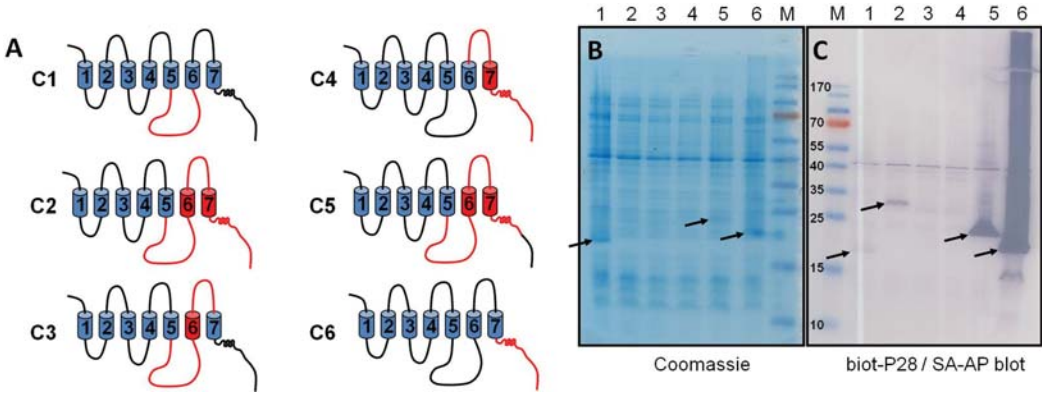

Fig. 6. Mapping of the binding epitope on the NTR1. Receptor fragments were expressed in *E.coli* and detected using SDS–PAGE and western blot. (**A**) Schematic drawings of the receptor fragments 1–6. Parts of the receptor fused to pD are highlighted in red. Theoretical amino acid length of the expressed constructs (including pD): C1, 155 aa; C2, 275 aa; C3, 188 aa; C4, 200 aa; C5, 247 aa and C6, 166 aa. MW, PageRuler marker (Bio-Rad). (**B**) Coomassie-stained gel of the whole cell extracts. (**C**) Western blot after a transfer of the cell extract proteins to a PVDF membrane. Detection of the expressed fragments was performed by using biot-P28/streptavidin-AP (SA–AP). Constructs C1–C6 correspond to lanes 1–6 in the Coomassie-stained gel and on the western blot. The arrows denote bands discussed in the text.

fragments C1 and C2, i.e. lanes 1 and 2), but the detection with the DARPin is still much stronger than that of the well-expressing IL3 construct alone.

Fluorescence microscopy

Fluorescence microscopy was used to test the binding of the selected DARPins in mammalian cells (Fig. 7). NTR1 and, as a control, KOR were expressed in COS7 cells. In order to optimize the assay, we first used a well-characterized anti-MBP DARPin (Binz *et al*., 2004), denoted off7, and generated a cognate GPCR target by fusing MBP to the C-terminus of KOR, in the cytoplasm. The expression of the Flag-KOR-MBP-Myc-His_6 construct could be detected, after cell permeabilization, with an anti-Myc antibody, stained with an AlexaFluor-555-labeled antibody in the red channel as seen in Fig. 7A2. The image shows that only a subset of the COS-7 cells had been transfected and hence produced the GPCR-MBP fusion protein. Some of the expressing cells showed only diffuse red staining whereas, in other cells, a clear punctate staining was seen as well, which is likely to correspond to an endoplasmic reticulum, Golgi or endosomal localization of the receptor fusion. Direct staining with the MBP-specific DARPin off7 fused to sfGFP only led to punctate staining and thus the recognition of GPCR fusion localized in internal stores (Fig. 7A3). The lack of co-staining of the more diffuse receptors, presumably at the cell surface, with the off7-sfGFP construct might be due to some proteolytic cleavage of the KOR-MBP fusion, or differences in the degree of PFA-crosslinking in the different cell compartments. In contrast to the anti-Myc antibody, which recognizes a linear epitope, the DARPin off7 recognizes a structured epitope within MBP (Binz *et al*., 2004) and might thus be more sensitive to the extent of crosslinking performed during the fixation step.

Following optimization of the control (KOR-MBP-Myc-His with the anti-MBP DARPin off7-sfGFP), the DARPins P28 and P14 fused to sfGFP were used for the detection of the D03 mutant of NTR1. The control DARPin P14,

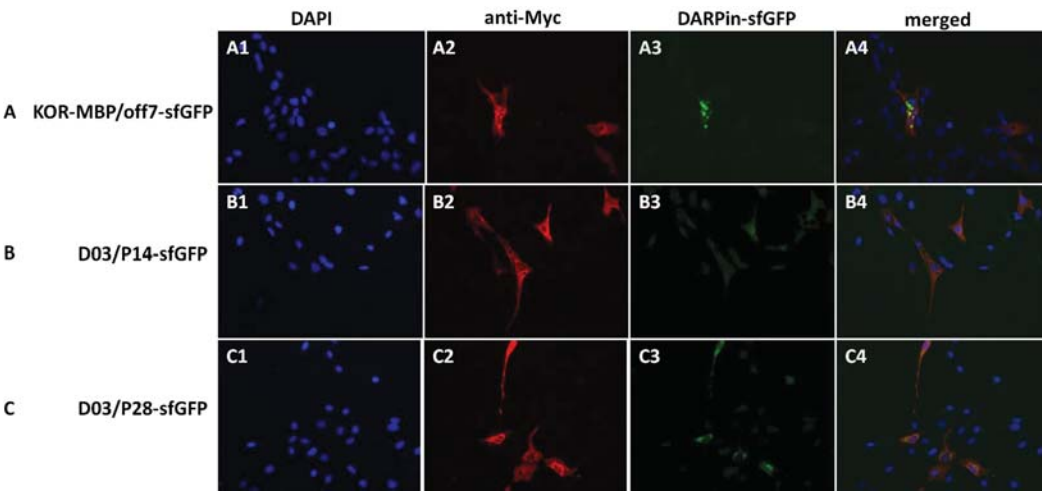

Fig. 7. Binding of the selected DARPins to NTR1 expressed in mammalian cells detected using fluorescence microscopy. COS-7 cells were transfected with (**A**) pcDNA3.1-Flag-KOR-MBP-Myc-His_6, or (**B** and **C**) pcDNA3.1-D03-Myc-His_6, as described in Materials and methods. In (B) and (C), two different DARPins were tested, in (B) the negative control DARPin P14 and in (C) the specific DARPin P28. (A1–C1) Staining of the cell nuclei with DAPI, (A2–C2) staining of the receptors using mouse anti-Myc and goat anti-mouse-AlexaFluor-555-labeled antibodies, (A3–C3) detection of the receptors using the DARPin-sfGFP fusions indicated and (A4–C4) merged pictures from the blue, red and green channels.

identified previously as not binding to the GPCR, but probably to neutravidin/biot-NT (Fig. 1), showed only background staining in the GFP channel, i.e. it stained both the transfected and the non-transfected cells at equally low levels (Fig. 7B3) and hence failed to co-localize with the red signal originating from the Myc-tagged receptor. In contrast, the P28 binder, shown *in vitro* to bind to the GPCR, exhibited mostly perinuclear staining, clearly distinguishable from the background signal, which co-localized with the anti-Myc receptor staining (Fig. 7C2 and C4). The staining of the D03 receptor with P28-sfGFP was, however, weaker than the KOR-MBP staining with the off7-sfGFP. The reasons for this might be the lower affinity of P28 to the GPCR (~70 nM) than of off7 to MBP (~4 nM) (Binz *et al*., 2004) and the fact that the binding epitope on the NTR1 receptor is close to the membrane, which might decrease affinity due to steric effects. Nonetheless, while the DARPin was selected in detergent-solubilized form, binding in whole cells can be detected.

Discussion

To the best of our knowledge, this is the first work to describe the generation of protein binders to a GPCR that are derived neither from antibodies nor from ligands or natural binding partners. Although Fab fragments are larger than DARPins (50 versus 18 kDa) and can protrude further out and potentially leave more space in the crystal for the membrane-bound detergent molecules, they are built from several domains and therefore display more conformational flexibility than the very rigid and stable ankyrin fold, which has been successfully used in co-crystallization of several targets (Binz *et al*., 2004; Kohl *et al*., 2005; Schweizer *et al*., 2007). In DARPins, the binding residues are located in stable secondary structure elements and short β-turns, leading to a rather shallow binding surface with an ideal shape complementary to folded proteins. This preference may also limit the dynamics of the receptor, potentially leading to greater conformational homogeneity, which is a major factor in successful crystallization of GPCRs (Cherezov *et al*., 2007; Rasmussen *et al*., 2007; Warne *et al*., 2008).

Our epitope mapping experiments (Fig. 6) are consistent with the observation that DARPin P28 recognizes both wild-type NTR1 and the D03 mutant equally well, since none of the mutated amino acids is located in the epitope. The mapping experiments also show that the minimal epitope must lie within the sequence corresponding to the intersection of fragments C2, C5 and C6, i.e. within the 33 amino acids after the helix 7. This part of the receptor includes the putative helix 8. However, the recognition of C1 and C2, relative to their low expression levels, also hints at the recognition of IL3. This weak but detectable signal arising from IL3 suggests that the binders might recognize a discontinuous epitope composed of both structural elements, potentially stabilizing this flexible region in the folded receptor. As seen in the X-ray structures, stabilization of this region may be important in generating high-quality crystals (Cherezov *et al*., 2007; Rasmussen *et al*., 2007; Warne *et al*., 2008).

We tested whether the DARPins can distinguish among different conformations of the receptor. The competition ELISA (Fig. 3), based on detection of only the active receptor complexed to its biotinylated agonist, showed that this form is recognized. A further ELISA, in which DARPins were coated, receptor was bound, and finally radioactive agonist and antagonist were added, showed no systematic differences that would indicate that DARPins differentiate between both forms (data not shown). SEC experiments (Fig. 4) demonstrate the binding of a selected DARPin to a ligand-free receptor. Additional chromatography experiments carried out at small scales show that the DARPins bind both to ligand-free and to agonist-bound receptor (data not shown). Taken together, these experiments showed that the binders recognize the receptor bound to agonist, antagonist and in free form, although we have not determined whether subtle differences in affinity exist.

The selected DARPin binders recognize NTR1 in different environments—in detergent-solubilized form (either free in solution or immobilized on a microtiter plate or beads) and in the native membrane of mammalian cells—as shown by various immunoassays, fluorescence microscopy and SEC. Owing to this versatility and reasonable affinity for the receptor, the binders can be used for a variety of applications such as co-crystallization and *in vivo* signal transduction assays.

A frequently encountered obstacle when studying GPCRs is the low amount of receptor available and the limited stability in detergent-solubilized form. Having evolved the receptor to higher expression levels and greater stability in detergents (Sarkar *et al*., 2008), this problem could be overcome. In the present study, we show that by using our DARPin technology and ribosome display, we could isolate functional binders using <10 μg NTR1. We believe that these technologies will allow the study of other GPCRs that have been refractory to structural and functional studies until now, but are nevertheless of great biomedical interest.

Acknowledgements

We thank Daniela Bukatz for the help with NTR1 purification, and Esin Kutluay and Igor Dodevski for fruitful discussions.

Funding

This work was supported by the NCCR Structural Biology. C.A.S. was supported by a postdoctoral fellowship from the National Institutes of Health (F32 GM069267). D.F was supported by an EMBO long-term fellowship (ALTF-129-2005).

References

Amstutz,P., Binz,H.K., Parizek,P., Stumpp,M.T., Kohl,A., Grütter,M.G., Forrer,P. and Plückthun,A. (2005) *J. Biol. Chem.*, **280**, 24715–24722.
Binz,H.K., Stumpp,M.T., Forrer,P., Amstutz,P. and Plückthun,A. (2003) *J. Mol. Biol.*, **332**, 489–503.
Binz,H.K., Amstutz,P., Kohl,A., Stumpp,M.T., Briand,C., Forrer,P., Grütter,M.G. and Plückthun,A. (2004) *Nat. Biotechnol.*, **22**, 575–582.
Boules,M., Fredrickson,P. and Richelson,E. (2006) *Peptides*, **27**, 2523–2533.
Carraway,R.E. and Plona,A.M. (2006) *Peptides*, **27**, 2445–2460.
Cherezov,V., *et al*. (2007) *Science*, **318**, 1258–1265.
Gully,D., *et al*. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 65–69.
Hanes,J. and Plückthun,A. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 4937–4942.
Huber,T., Steiner,D., Röthlisberger,D. and Plückthun,A. (2007) *J. Struct. Biol.*, **159**, 206–221.
Hunte,C. and Michel,H. (2002) *Curr. Opin. Struct. Biol.*, **12**, 503–508.

Jaakola,V.P., Griffith,M.T., Hanson,M.A., Cherezov,V., Chien,E.Y., Lane,J.R., Ijzerman,A.P. and Stevens,R.C. (2008) *Science*, **322**, 1211–1217.
Kohl,A., Amstutz,P., Parizek,P., Binz,H.K., Briand,C., Capitani,G., Forrer,P., Plückthun,A. and Grütter,M.G. (2005) *Structure*, **13**, 1131–1141.
Kroeze,W.K., Sheffler,D.J. and Roth,B.L. (2003) *J. Cell Sci.*, **116**, 4867–4869.
Kuzmic,P. (1996) *Anal. Biochem.*, **237**, 260–273.
Leifert,W.R., Aloia,A.L., Bucco,O., Glatz,R.V. and McMurchie,E.J. (2005) *J. Biomol. Screen.*, **10**, 765–779.
Li,J., Mahajan,A. and Tsai,M.D. (2006) *Biochemistry*, **45**, 15168–15178.
Miao,W., Choi,J.P. and Bard,A.J. (2002) *J. Am. Chem. Soc.*, **124**, 14478–14485.
Palczewski,K., *et al*. (2000) *Science*, **289**, 739–745.
Pedelacq,J.D., Cabantous,S., Tran,T., Terwilliger,T.C. and Waldo,G.S. (2006) *Nat. Biotechnol.*, **24**, 79–88.
Pelaprat,D. (2006) *Peptides*, **27**, 2476–2487.
Rasmussen,S.G., *et al*. (2007) *Nature*, **450**, 383–387.
Röthlisberger,D., Pos,K.M. and Plückthun,A. (2004) *FEBS Lett.*, **564**, 340–348.
Sarkar,C.A., Dodevski,I., Kenig,M., Dudli,S., Mohr,A., Hermans,E. and Plückthun,A. (2008) *Proc. Natl Acad. Sci. USA*, **105**, 14808–14813.
Schweizer,A., *et al*. (2007) *Structure*, **15**, 625–636.
Serrano-Vega,M.J., Magnani,F., Shibata,Y. and Tate,C.G. (2008) *Proc. Natl Acad. Sci. USA*, **105**, 877–882.
Steiner,D., Forrer,P. and Plückthun,A. (2008) *J. Mol. Biol.*, **382**, 1211–1227.
Tucker,J. and Grisshammer,R. (1996) *Biochem. J.*, **317**, 891–899.
Warne,T., Serrano-Vega,M.J., Baker,J.G., Moukhametzianov,R., Edwards,P.C., Henderson,R., Leslie,A.G., Tate,C.G. and Schertler,G.F. (2008) *Nature*, **454**, 486–491.
Wetzel,S.K., Settanni,G., Kenig,M., Binz,H.K. and Plückthun,A. (2008) *J. Mol. Biol.*, **376**, 241–257.
White,J.F., Trinh,L.B., Shiloach,J. and Grisshammer,R. (2004) *FEBS Lett.*, **564**, 289–293.
Zahnd,C., Amstutz,P. and Plückthun,A. (2007) *Nat. Methods*, **4**, 269–279.

Received February 8, 2009; revised March 6, 2009; accepted March 15, 2009

Edited by Paul Carter