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A novel, generic and effective method for the rapid purification of G protein-coupled receptors

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ABSTRACT

G protein-coupled receptors (GPCRs) constitute the largest family of membrane receptors and are of major therapeutic importance. Structure determination of G protein-coupled receptors and other applications require milligram quantities of purified receptor proteins on a regular basis. Recombinant GPCRs fused to a heterologous biotinylation domain were produced in the yeast *Pichia pastoris*. We describe an efficient method for their rapid purification that relies on the capture of these receptors with streptavidin immobilized on agarose beads, and their subsequent release by enzymatic digestion with TEV protease. This method has been applied to several GPCRs belonging to the class A rhodopsin subfamily, leading to high yields of purified proteins; it represents a method of choice for biochemical and biophysical studies when large quantities of purified GPCRs are needed.

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Considerable research is ongoing in the area of membrane protein structure and function, and in recent years many efforts have been, in particular, focused on G protein-coupled receptors (GPCRs).[1] These proteins share a conserved architecture based on seven transmembrane segments; they are the key players of signal transduction and constitute the major group of drug targets for the pharmaceutical industry, with already more than half of modern drugs and almost 25% of the top 200 best-selling drugs targeting them [1]. Despite these crucial biological implications, very little is known about the detailed molecular mechanisms by which these membrane proteins recognize their extracellular stimuli and transmit the associated messages. The speed of discovery of novel GPCR-active drugs is hampered by the lack of structural information about these proteins, which is reflected by the fact that, to date, only rhodopsin, the rod-cell GPCR involved in dim light vision and the β_2-adrenergic receptor, which plays an important role in cardiovascular physiology, have been crystallized and their structures solved at 2.2 and 2.4 Å, respectively [2,3].

For several protein families, including most of helical membrane proteins and in particular GPCRs, structural studies are still limited by low expression levels in heterologous systems and/or by difficulties in obtaining pure, homogeneous protein preparations [4].

During the last decade, the methylotrophic yeast *Pichia pastoris* has been increasingly used for heterologous GPCR expression [5–8]. This yeast is particularly suitable for these applications, combining multiple advantages: (i) the relatively low costs related to protein expression; (ii) the availability of protease deficient *P. pastoris* strains; (iii) the presence of a well-defined strong promoter (i.e. the alcohol oxidase promoter) for efficient overexpression; (iv) the eukaryotic nature of post-translational modifications that lack extensive glycosylation; (v) the addressing of proteins to the plasma membrane due to the presence of targeting sequences and (vi) the feasibility of large-scale fermentation culturing when substantial quantities of material are needed.

We are currently using the *P. pastoris* system to express a dozen of GPCRs. Expression of these proteins has been optimized in a previous work [9], but their purification presented several problems: despite the relatively good levels of expression, GPCRs expressed in *P. pastoris* were very difficult to purify to homogeneity and in large quantities when conventional methods such as metal affinity chromatography and/or flag-agarose chromatography were used.

We have developed a rapid, generic purification procedure for GPCRs expressed in *P. pastoris*. This method relies on the presence of a C-terminal biotinylation domain in the recombinant proteins, allowing the high affinity capture of solubilized proteins on streptavidin (SAV) coupled to agarose beads. We also used the highly

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[1] *Abbreviations used:* GPCRs, G protein-coupled receptors; SAV, streptavidin; TEV, tobacco etch virus; PBS, phosphate buffer saline; CHS, cholesteryl hemisuccinate.

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specific tobacco etch virus (TEV) protease for the removal of both N- and C-terminal tags and the concomitant release of the native protein. Scale-up of this purification protocol will allow large quantities of receptors to be purified, thus enabling three-dimensional crystallization attempts and other biophysical approaches.

Materials and methods

Strains and plasmids

Pichia pastoris strains expressing different GPCRs were obtained as previously described [9].

The plasmid for tobacco etch virus (TEV) protease overexpression in *Escherichia coli* was obtained from Gunter Stier (EMBL, Heidelberg).

The plasmid for streptavidin overexpression in *E. coli* was obtained from Prof. Arosio (University of Brescia) [10].

Growth of P. pastoris and protein expression

Cells were grown overnight in BMGY (1% yeast extract; 1% peptone; 1.34% yeast nitrogen base; 1% glycerol; 0.00004% biotin; 100 mM potassium phosphate pH 6.0). Next day, the cells were diluted in BMGY and grown to an OD_{600} of 10. The culture was centrifuged and the pellet resuspended in the same volume of BMMY (1% yeast extract; 1% peptone; 1.34% yeast nitrogen base; 0.5% methanol; 0.00004% biotin; 100 mM potassium phosphate pH 6.0; 2.5% DMSO; 0.4 mg/ml histidine) for the induction of protein production through the AOX1 promoter. After 18 h at 23 °C, the cells were spun down, washed with PBS (phosphate buffer saline: 20 mM sodium phosphate pH 7.4; 2 mM KCl; 150 mM NaCl) and either used immediately or frozen at −80 °C.

The induction conditions deviate slightly from standard ones: we showed previously that for most receptors, including DMSO in the induction medium and performing the induction at 23 °C led to an increase in the ratio of active versus total receptor [9].

Preparation of the cell membranes

All procedures were performed on ice. The cells were resuspended in cold lysis buffer (50 mM sodium phosphate pH 7.4; 100 mM NaCl; 5% glycerol; 5 mM EDTA; 1 mM PMSF). Cells were broken with glass beads (0.5 mm; 3 × 20 s), using a MP Biomedicals fast prep device. Cell debris and unbroken cells were removed by centrifugation at 5000*g* for 10 min and the turbid supernatant containing cell membranes was ultracentrifuged at 150,000*g* for 45 min. The resulting pellets were resuspended in membrane buffer (50 mM Tris; pH 8.0; 120 mM NaCl; 10% glycerol; 1 mM PMSF) and ultracentrifuged again. The membrane pellets were resuspended in membrane buffer, flash frozen in liquid nitrogen and stored at −80 °C. Protein concentrations were determined by bicinchoninic acid assay (Pierce) using bovine serum albumin as standard.

Western blotting and protein detection

Chemiluminescent Western blotting detection reagents were from Pierce. Mouse monoclonal anti-Flag M2 antibody was from Sigma. Peroxidase-linked anti-mouse IgG was purchased from GE Healthcare. Alkaline phosphatase-coupled streptavidin and the corresponding substrate tablets (NBT/BCIP) were purchased from Sigma.

Proteins were separated by SDS–PAGE (10% gel) and visualized with coomassie blue or transferred overnight at 30 V to a nitrocellulose membrane. The membrane was developed either with the anti-FLAG M2 antibody or with alkaline phosphatase conjugated streptavidin.

In vitro biotinylation

Membranes (50 μg) were mixed with 10 mM ATP, 10 mM MgOAc, 50 μM biotin, 50 mM bicine buffer (pH 8.3) with or without 12,500 U of biotin–protein ligase (BirA; Genecopoeia). The suspensions were incubated at 30 °C for 40 min, then submitted to SDS–PAGE and Western blotting. Biotinylated proteins were detected as described above.

Solubilization

Detergents were obtained from Anatrace except for CHAPS (Euromedex). Membranes were diluted to obtain a protein concentration of 1 mg/ml in the solubilization buffer (final concentrations: 50 mM sodium phosphate pH 7.4; 10% glycerol; 400 mM NaCl). Then the detergent *n*-dodecyl β-D-maltopyranoside (DDM) was added to a final concentration of 1% and the solution was stirred at room temperature for 15 min. After ultracentrifugation (150,000*g*, 45 min), the supernatant was directly used for purification.

Purification with streptavidin beads

The supernatant obtained after solubilization was mixed with streptavidin-coated beads (see below) at various ratios. This preparation was kept overnight at 4 °C with orbital shaking; then the suspension was centrifuged at 1000*g* for 5 min, the supernatant was removed and the beads were incubated for 1 h at 30 °C in solubilization buffer supplemented with 0.1% DDM and endoglycosidase (PNGase F, 1000 U). The beads were then sequentially washed with (i) solubilization buffer supplemented with 1% CHAPS, (ii) solubilization buffer supplemented with 0.1% DDM, (iii) solubilization buffer supplemented with NaCl (1.5 M final concentration) and 0.1% DDM, (iv) solubilization buffer supplemented with 1 M NaSCN and 0.1% DDM, (v) three times with solubilization buffer supplemented with 2 mM DTT and 0.1% DDM (TEV buffer). All washing steps were performed at room temperature with gentle orbital shaking. Immobilized receptor were then cleaved off their tags and therefore released from the beads by addition of TEV protease (1 μg of protease for 20 mg of starting membrane proteins) and shaking at 25 °C for 0.5–16 h.

Preparation of streptavidin, streptavidin-coated agarose beads (SAV beads) and TEV protease

Streptavidin was overproduced in *E. coli* and purified as described [10]. The protein was dialyzed against 0.1 M $NaHCO_3$ pH 8.3 containing 0.5 M NaCl and then concentrated on a vivaspin concentrator (30 kDa cut-off). The protein (0.8 mg/ml final concentration) was then coupled to cyanogen bromide-activated sepharose 4B (Sigma) according to the manufacturer's instructions, except that the sepharose beads were pre-hydrolyzed for 2 h at pH 8.3 before adding streptavidin. The beads were kept at 4 °C in PBS supplemented with 0.02% sodium azide (four volumes of PBS/azide added to one volume of wet beads), and no decrease in the GPCR capture efficiency was noticed after 6 months of storage. In some experiments, used streptavidin beads were recycled by denaturation with 8 M guanidine–HCl in 50 mM citrate pH 3.0 followed by renaturation with a decreasing guanidine concentration gradient. The capacity of these recycled beads to immobilize receptors was decreased by 40–60%, when compared with unused beads.

TEV protease was overexpressed in *E. coli* BL21(DE3)pLysS (overnight induction in LB at 23 °C with 0.5 mM IPTG) and then

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purified on an IMAC column (His Trap, GE healthcare) followed by a desalting step and a cation exchange chromatography at pH 6.1 on a Mono S column (GE healthcare). After elution with a 0–1.0 M NaCl gradient, protease containing fractions, which eluted at 750 mM NaCl, were aliquoted and stored at −80 °C.

Ligand binding experiments

Binding assays were performed as previously described [9], using 10 nM tritiated ligands CP55940 and rauwolscine for CNR2_HUMAN and ADA2B_HUMAN, respectively. The binding buffer used for the CNR2 receptor contained 50 mM Tris–HCl pH 7.4, 2.5 mM EDTA, 5 mM $MgCl_2$ and 0.5 mg/ml bovine serum albumin.

MALDI-TOF mass spectrometry

SDS–PAGE separated protein bands were excised from the gel and the proteins were digested according to the Shevchenko protocol [11]. The gel plugs were washed three times with 100 µl of 25 mM ammonium hydrogen carbonate (NH_4HCO_3) and 100 µl of acetonitrile. The cysteine residues were reduced by 50 µl of 10 mM dithiothreitol at 56 °C and alkylated by 50 µl of 55 mM iodoacetamide. After dehydration with acetonitrile, the proteins were cleaved with 15 µl of a 12.5 ng/µl solution of proteomics grade trypsin (Sigma) in 25 mM NH_4HCO_3. The digestion was performed overnight at room temperature. The resulting peptides were extracted with a 65% acetonitrile solution containing 5% acid formic and 0.05% SDS for 1 h. MALDI-TOF mass measurements were carried out on an AutoFlex II TOF/TOF (Bruker Daltonik GmbH, Bremen, Germany) operating in positive reflectron mode. The samples were prepared by standard dried droplet preparation on stainless steel MALDI targets using α-cyano-4-hydroxycinnamic acid as matrix. The samples were washed twice with 5% formic acid. External calibration of MALDI mass spectra was carried out using singly charged monoisotopic peaks of a mixture of bradykinin 1–7 (m/z = 757.400), human angiotensin II (m/z = 1046.542), human angiotensin I (m/z = 1296.685), substance P (m/z = 1347.735), bombesin (m/z = 1619.822), ACTH 1–17 (m/z = 2093.087) and ACTH 18–39 (m/z = 2465.199). Monoisotopic peptide masses were automatically annotated using Flexanalysis 3.0.

Results

GPCRs were overexpressed in *P. pastoris* as previously described [9]. The recombinant proteins comprise an N-terminal α-factor signal sequence from *Saccharomyces cerevisiae* followed by a Flag tag, a deca-histidine-tag, a tobacco etch virus (TEV) protease site, the GPCR open reading frame, a second TEV site and finally the *Propionibacterium shermanii* biotinylation domain at the C-terminus.

Our earlier purification schemes relied on the presence of the N-terminal deca-histidine and Flag tags, but this strategy gave unsatisfactory results because of the poor specificity and efficiency of these tags. In these recombinant proteins, the C-terminal transcarboxylase biotinylation domain had been added mainly to detect the protein throughout the purification steps and to maintain its stability [12,13]. As an alternative to the first purification strategy, we decided to take advantage of the biotinylation domain to efficiently capture the GPCRs on SAV–agarose beads. The protein is immobilized on the beads through high affinity interaction, and can undergo subsequent washing treatment and might also be reconditioned. The final step relies on exploiting the TEV protease sites flanking the receptor to release the purified protein from the beads (Fig. 1).

In vivo biotinylation of recombinant GPCRs expressed in Pichia pastoris

A prerequisite for the development of such a purification method is that the receptors are efficiently biotinylated *in vivo*. Several studies conducted on membrane proteins fused with biotinylation tags and expressed in *P. pastoris* have shown that this yeast indeed possesses a biotin ligase (ortholog of the *E. coli* BirA protein) which biotinylates not only several low abundance *Pichia* proteins but also heterologous proteins [14,15].

Thus, we first verified that the *Pichia* strains used in this study effectively contained a biotin ligase activity and that the level of *in vivo* biotinylation was sufficient to directly undertake purification on streptavidin–agarose. For this purpose, yeast cells overexpressing four different GPCRs or yeast cells transformed with an empty vector were grown in induction conditions. Then, membrane proteins were extracted, submitted to SDS–PAGE and transferred to a nitrocellulose membrane prior to the detection

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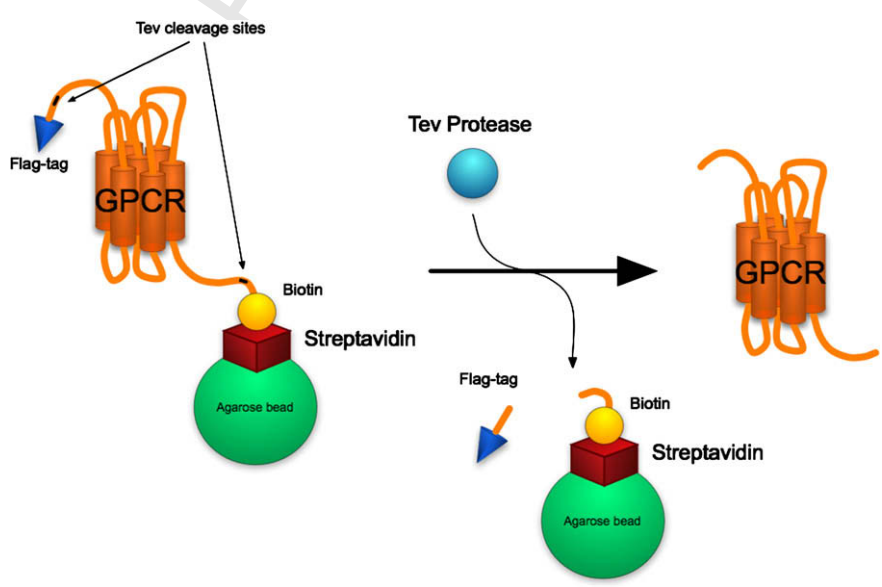

Fig. 1. Schematic representation of the method. A solubilized receptor is captured on immobilized streptavidin. The TEV protease then cleaves the N- and C-terminal tags, thus releasing the receptor and the N-terminal tag. The C-terminal tag remains bound to streptavidin.

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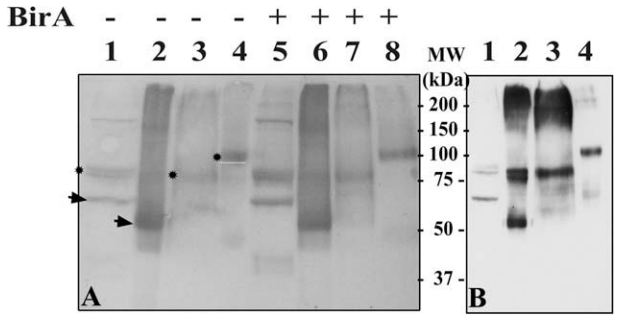

Fig. 2. Detection of four GPCRs expressed in *Pichia pastoris.* Membrane proteins were subjected to SDS–PAGE, and transferred to nitrocellulose. The receptors were detected with (A) Alkaline phosphatase-linked streptavidin or (B) anti-Flag M2 monoclonal antibody. Lanes 1 and 5: ADA2B_HUMAN; lanes 2 and 6: CNR2_HUMAN; lanes 3 and 7: OPRK_HUMAN; lanes 4 and 8: NK3R_HUMAN. Membranes from lanes 5 to 8 were subjected to *in vitro* biotinylation with the BirA protein–biotin ligase prior to SDS–PAGE. Arrows point to receptor bands at the expected size. Asterisks show receptors for which the signal sequence was not processed, leading to molecular masses higher than expected.

of biotinylated proteins with alkaline phosphatase-linked streptavidin. For yeast transformed with an empty vector, several endogenous biotinylated proteins were detected in the soluble fractions, but none could be detected in the membrane fractions (results not shown). Fig. 2A (lanes 1–4) shows that all four receptors tested, respectively, ADA2B_HUMAN, CNR2_HUMAN, OPRK_HUMAN and NK3R_HUMAN are biotinylated in *P. pastoris*. The sizes of the detected proteins are in good agreement with those expected for ADA2B (observed Mr: 65 kDa) and CNR2 (55 kDa), taking into account the size contribution of the N- and C-terminal tags. For OPRK (85 kDa) and NK3R (100 kDa), the observed sizes are higher than expected. This is likely to be due to a lack of processing of the N-terminal α-factor signal sequence by internal Kex2 endopeptidase of *P. pastoris*. The same probably applies to the additional ~85 kDa doublet observed for the ADA2B receptor (Fig. 2, lanes 1 and 5). This absence of signal sequence processing was reported for the mouse $5HT_{5A}$ receptor also expressed in *P. pastoris* [14].

When the membranes were treated with exogenously added biotin–protein ligase (BirA), only a slight increase in the level of biotinylation could be observed (Fig. 2A, compare lanes 1–4 and 5–8), indicating that, *in vivo*, the biotinylation of the proteins was almost complete. Fig. 2B shows the same receptors detected with an anti-flag M2 monoclonal antibody; the bands observed are essentially the same as those detected with streptavidin-phosphatase. Taken together, these results indicate that both the N- and C-terminal parts of the proteins are intact since they are detected by the anti-flag antibody and streptavidin-phosphatase, respectively. Dimers and higher order oligomers are also detected, especially for CNR2 and OPRK (Fig. 2A, lanes 2, 3, 6 and 7; Fig. 2B, lanes 2 and 3). Such SDS-resistant dimers/higher order oligomers have been reported for an ever-increasing number of GPCRs [16].

These results along with the efficient *in vivo* biotinylation of these receptors prompted us to perform further purification tests. With the aim of developing a generic method (see Fig. 1), the human peripheral cannabinoid receptor (CNR2_HUMAN) was used as a prototypical GPCR.

CNR2_HUMAN capture onto streptavidin-coated beads and its release by TEV protease

Membrane proteins (1 mg/ml) containing CNR2_HUMAN were solubilized with 1% DDM (*n*-dodecyl β-D-maltopyranoside) and then mixed with SAV–agarose beads. Commercial beads were tested in preliminary experiments, but the results were not as good as with our in-house made SAV beads prepared as described in Experimental section. The difference is most likely due to the different levels of substitution of the agarose beads with SAV: the pre-hydrolysis of the beads which we included prior to streptavidin coupling most probably leads to a lower density of SAV on the beads and thus reduces the steric hindrance problems that could be associated with a higher SAV density.

After solubilization, receptors were captured on SAV-coated beads in a batch mode. Surprisingly, very small quantities of the receptors bound to the beads within the first hours, although the streptavidin–biotin interaction is the strongest non-covalent biological interaction ($K_a = 10^{15}\ M^{-1}$) between a protein and its ligand known to date. The bond formation between SAV and biotin is normally rapid and essentially non-reversible, suggesting that most probably, in our case, steric hindrance due to the interaction of the biotinylated C-terminal part of the tagged GPCR with neighboring parts of the protein prevented the rapid capture of biotin by SAV. However, the quantity of bound protein increased with time, and an overnight contact at 4 °C proved to be optimum. Longer incubation times, even at higher temperatures (20–37 °C), did not increase the yield (data not shown). The optimal amount of home-made SAV–agarose resin necessary for the purification, starting from 20 mg of membrane proteins, was calculated to be 1.2 ml of resin. Increasing the resin quantity did not improve the yield of purified protein but actually slightly increased the amount of contaminants.

After capture of CNR2 on SAV, the beads were incubated in presence of an endoglycosidase (PNGase F). They were further extensively washed to remove non-specific contaminants. Several washing buffers were tested, including high and low pH buffers, high detergent and high salt buffers. The best results in terms of final receptor purity consisted in sequential washes with buffers containing (i) 1% CHAPS for removal of contaminants interacting through hydrophobic bonds, (ii) 1.5 M NaCl for contaminants bound through hydrophilic interactions and in some cases and (iii) the chaotropic agent NaSCN (1.0 M) for very strongly bound contaminants. Then, the beads were equilibrated in TEV buffer containing 2 mM DTT which is an essential component to maintain the functionality of the TEV protease over long periods of time. The TEV protease cleaves off the receptors from the SAV-coated beads to which the receptors are bound by their C-terminal biotinylated tag, and also releases the N-terminal tags from the receptors. After addition of the protease, a time-course was performed to estimate the optimal incubation time: Fig. 3 shows that the cleaved receptor can be detected after 1 h of incubation (lane 2). Its quantity increased from 1 to 3 h (lanes 1–4). However, the maximum amount of receptor was obtained either after 3 h (lane 4) or after overnight incubation (lane 5). Two bands can be seen, the main one at 36 kDa and a more hazy band at ~40 kDa. Both were identified as the cannabinoid receptor by mass spectrometry (see below). The 40 kDa band most likely represents a post-translationally modified version of the receptor.

Several purified GPCRs

The one step purification method described above was tested out on several other GPCRs belonging to the rhodopsin-related class A subfamily. Fig. 4 shows the results obtained with five of these receptors, the human neurokinin-3 receptor (NK3R_HUMAN, theoretical molecular weight 52 kDa), the human purinergic receptor type-1 (P2RY1_HUMAN, 42 kDa), the human κ-opioid receptor (OPRK_HUMAN, 43 kDa), the human histamine H_1 receptor (HRH1_HUMAN, 56 kDa), the human α_{2B}-adrenergic receptor (ADA2B_HUMAN, 50 kDa). All of these receptors were obtained in a relatively pure state (Fig. 4). NK3R (lane 1) and ADA2B (lane 5) migrate at the expected molecular weights, but P2RY1 (lane 2), OPRK (lane 3) and HRH1 (lane 4) migrate slightly faster than

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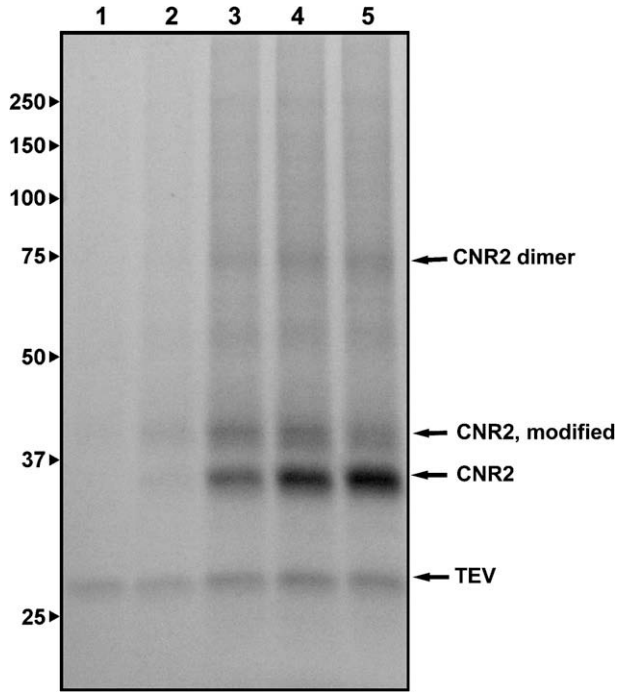

Fig. 3. Time course of CNR2 release by the TEV protease. CNR2 was solubilized and bound to streptavidin–agarose beads; the beads were washed and samples were taken at different time points after addition of the TEV protease and denatured with SDS sample buffer. Lane 1, time 0 after addition of the TEV protease; lane 2, 1 h after addition; lane 3, 2 h; lane 4, 3 h; lane 5, 14 h (overnight).

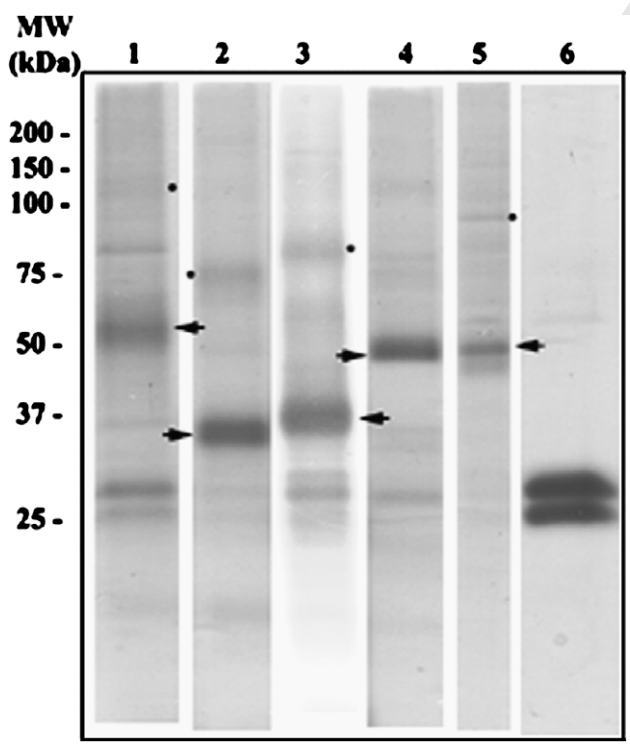

Fig. 4. Several purified GPCRs. Proteins purified with streptavidin beads were separated by SDS–PAGE and stained with coomassie blue. Lane 1, NK3R_HUMAN; lane 2, P2Y1_HUMAN; lane 3, OPRK_HUMAN; lane 4, HRH1_HUMAN; lane 5, ADA2B_HUMAN; lane 6, TEV protease. Arrowheads indicate purified GPCRs and asterisks indicate probable dimers.

expected (34, 37 and 50 kDa, respectively). This behavior is relatively frequent with membrane proteins and particularly among GPCRs [17]. They may bind more SDS than other proteins of similar size, due to a higher level of hydrophobicity and thus migrate faster during SDS–PAGE.

Table 1
Tryptic fragments observed by MS

Receptor	Measured masses	Sequence
OPRK	1086.536	157–165
	1176.577	362–371
	1389.618	340–349
	1431.763	360–371
CNR2	758.386	229–235
	971.483	345–352
	1045.551	229–237
	1243.616	322–333
	1333.612	307–317
	1542.740	215–228
	2075.997	334–352
	2109.026	14–32
ADA2B	854.459	58–65
	980.569	23–30
	1055.676	140–150
	1186.689	254–264
	1320.670	163–174
	1342.804	254–265
	1395.967	241–263
	1722.032	175–191
	1822.928	158–174
	2202.289	117–139
	2441.589	126–150

Tryptic fragments were obtained and analyzed by MALDI mass spectrometry, as described in Methods section, for the following receptors: OPRK_HUMAN, CNR2_HUMAN and ADA2B_HUMAN.

In order to confirm the identities of the purified receptors, the SDS–PAGE bands of purified OPRK, CNR2 and ADA2B, respectively, were excised and submitted to in-gel digestion followed by MALDI-TOF mass fingerprinting. As shown in Table 1, a total of 4, 8 and 11 peptides were identified for OPRK, CNR2 and ADA2B, respectively. Their masses were in concordance with the calculated molecular masses of theoretical tryptic peptides derived either from the cytoplasmic loops or from the N- and C-terminal domains. These analyses provided an unambiguous confirmation of the identities of the purified receptors. In the case of the CNR2 receptor, that gives two bands on SDS–PAGE (Fig. 3), both bands gave a similar pattern, thus confirming that the upper band likely represents a post-translationally modified version of the receptor.

Ligand binding capacity of purified CNR2 and ADA2B receptors

Binding assays were realized for two purified, SAV-agarose bound receptors, CNR2_HUMAN and ADA2B_HUMAN, using specific ^{3}H-labelled ligands (CP 55940 and rauwolscine, respectively). Two purification conditions were tested, the first including 0.1% DDM in all buffers, the second with 0.1% DDM and 0.02% cholesteryl hemisuccinate (CHS), a well-known GPCR stabilizer. For CNR2, the binding level was 9 nmol/mg in the first condition and 10.5 nmol/mg in the second, corresponding, respectively, to 36% and 42% of active receptors in the preparation. For the ADA2B receptor, the values were much lower: 0 and 0.2 nmol/mg in the two conditions tested, respectively, corresponding to only 0% and 1% of active receptors.

Yields of purification

The yields obtained for several receptors are displayed in Table 2. They were mainly determined by two methods. The first consisted of protein measurements made after the removal of the TEV protein and the N-terminal free tags over a gel filtration column (Superose

Table 2
Purification yields for 10 GPCRs

Receptor	Yield (mg)
CNR2_HUMAN	0.9
NK1R_HUMAN	0.05
NK3R_HUMAN	0.4
ADA2B_HUMAN	0.2
ADA1B_MESAU	0.1
HRH1_HUMAN	0.5
D2DR_HUMAN	0.05
P2Y1_HUMAN	0.5
OPRD_HUMAN	0.7
OPRK_HUMAN	0.8
ADA2C_RAT	<0.02
PAR1_HUMAN	<0.02

The yields are expressed in mg of purified GPCR, obtained from 1 L of induced culture.

12). The second was obtained by comparing the band intensities of purified receptors on a coomassie blue stained gel with known quantities of BSA loaded on the same gels. The yields vary between 0.05 and 0.9 mg of purified receptor per liter of culture. Since several liters of *P. pastoris* culture can be easily handled and processed, especially using the easy purification method we describe here, most of the receptors are available in sufficient quantities for biophysical studies or for other studies requiring large amounts of purified proteins. A few receptors could not be purified, e.g. the human serotonin $5HT_{1B}$ receptor (5HT1B_HUMAN) or only purified in low amounts, e.g. the human proteinase-activated receptor 1 precursor (PAR1_HUMAN), the mesau α_{1B}-adrenergic receptor (ADA1B_MESAU), the human neurokinin-1 receptor (NK1R_HUMAN) and the rat α_{2C}-adrenergic receptor (ADA2C_RAT). In addition, the human neurokinin-2 receptor (NK2R_HUMAN) could only be purified as a low molecular weight, breakdown product, due to the presence of a cryptic TEV site in its sequence, as was the case for the human neurotensin receptor [18].

Discussion

The GPCRs are the largest protein family known to date, comprised of an estimated 600–1000 members involved in various types of stimulus-response pathways, ranging from intercellular communication pathways to determination of physiological senses. GPCRs are involved in a wide range of disorders, including allergies, cardiovascular dysfunction, depression, obesity, cancer, pain, diabetes and various central nervous system disorders. The GPCR family of receptors is well-established as the premier target family for drug development: about half of the drugs on the market today are targeted towards GPCRs. Thus, taking into consideration the pharmacological importance of GPCRs, it is crucial to be able to produce pure and functional GPCRs on a custom basis to assist in the development of new GPCR targeting drugs.

Methods in use for GPCR purification from mammalian, insect, yeast or bacterial cells usually include numerous, lengthy, high-cost purification steps [4,19]. Recent advances have been made with cell-free translational systems and the results obtained seem to be promising. However, the number of GPCRs expressed in this way is still low and the experiments are still costly and require a lot of development and optimization [20–22].

Purification methods for membrane proteins that use the avidin–biotin interaction have been developed in a few cases [23,24]. Usually, monomeric avidin bound to agarose has been used, but some problems, mainly concerning elution of the proteins, have been frequently reported [24]. The streptavidin–biotin is not generally used because of the very high strength of this interaction, making it virtually impossible to elute the protein of interest in mild conditions. We turned this drawback to our advantage by actually conserving the N-terminal tags in position on the streptavidin protein and exploiting TEV elution to liberate the protein of interest. The strength of the streptavidin–biotin interaction also allows, when necessary, the use of harsh washing conditions such as very high or low pH, or high concentrations of denaturants such as urea or guanidine.

The method described here is highly efficient and allows the rapid purification of GPCRs: the purification itself can be performed within 18 h, and only 3 days are necessary to obtain the purified protein from a starter culture. Preliminary tests can be performed, when a new GPCR or a mutated GPCR is being studied, with only 20–50 mg of membrane proteins, thus limiting the amount of handwork needed. The costs involved are very low because of the use of the yeast *P. pastoris*, but also because no expensive purification resins or apparatuses are needed, since the method relies on the sole streptavidin–agarose resin and batch method.

The method allows easy exchange of buffers, salts and detergents, as well as addition of stabilizing additives early on in the purification process, or only at the last step involving elution by TEV. In our hands, no strong inhibition of the TEV protease could be observed, whatever the additives (specific ligands and/or CHS) or detergents used. In a previous study, the protease was shown to be inhibited by several detergents, however, the detergent concentrations used were much higher, at least by an order of magnitude, than the concentrations used in our study [25].

For most GPCRs used in this study, substantial amounts of tag-free proteins have been purified in quantities and at purity levels sufficient to initiate structural studies. Among the receptors tested, one could not be purified and a few were obtained only in low amounts. Most of these refractory receptors were nevertheless expressed and biotinylated to levels comparable to receptors purified with good yields, indicating that the problem most probably lies in the organization of the C-terminal part of the proteins, where the biotin moiety could be hindered. A possible remedy would be to add a linker between the GPCR and the biotinylation domain, thus rendering the biotin able to interact with SAV.

Besides structural studies, a possible application of the method would be to use streptavidin–agarose bound receptors to go "fishing" for receptor-interacting proteins in soluble/membrane extracts of various types of cells or tissues, in the presence or absence of added ligands. We recently obtained preliminary results showing that β-arrestin-1, a protein involved in GPCRs internalization, can be retrieved with SAV–agarose bound CNR2 receptor from an *E.* coli extract containing arrestin expressed in low amounts (results not shown). The method described here would allow (i) a direct biochemical confirmation of interactions detected by other methods such as immunoprecipitation, (ii) the identification of novel interacting partners and (iii) the possibility to release the GPCR-interacting partner complexes with the TEV protease and to realize a biochemical study of such complexes.

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