Appl Biochem Biotechnol (2010) 160:2175–2186
DOI 10.1007/s12010-009-8715-8

The N-Terminal End Truncated Mu-Opioid Receptor: from Expression to Circular Dichroism Analysis

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Received: 17 March 2009 / Accepted: 9 July 2009 /
Published online: 28 July 2009

Abstract In order to evaluate the biochemical, biophysical, and pharmacological implication of the N-terminal domain of the human mu-opioid receptor (HuMOR), deletion mutants lacking 64 amino acids from the amino terminus of HuMOR were constructed and expressed in the yeast *Pichia pastoris*. The recombinant proteins differed with respect to the presence of the *Saccharomyces cerevisiae* α-factor prepropeptide and the enhanced green fluorescent protein fused to the N terminus of the receptor. Pharmacological studies indicated that deletion of the N-terminal domain produced little effect on ligand affinities. The N-terminal end truncated and c-myc/6his-tagged receptor was subsequently purified to homogeneity and a yield of 5 mg/l was obtained after purification. The N-terminal end truncated receptor was further characterized by circular dichroism in trifluoroethanol and showed a characteristic pattern of α-helical structure. A pH effect on the structure of the receptor was observed when it was solubilized in sodium dodecyl sulfate micelles, with an increase of helicity at low pH.

Keywords G-protein coupled receptor · Solubilization · Purification · *Pichia pastoris* · Mu-opioid receptor

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Introduction

The mu-opioid receptor which belongs to the G-protein coupled receptors (GPCRs) superfamily [1] is responsible for specific interactions with endogenous opioid peptides such as enkephalins [2] and is therefore involved in several fundamental biological processes like pain perception, stress, and emotions. This receptor is also the receptor for morphine and heroin [3] and is consequently responsible for drug addictions. The mu-opioid receptor is an integral membrane protein and belongs to the GPCR rhodopsin class in the glutamate–rhodopsin–adhesion–frizzled/taste2–secretin classification [4]. The rhodopsin family is the largest family of GPCR in human and displays several characteristics including the NSxxNPxxY motif in the seventh transmembrane domain (TM7), the DRY motif or D (E)-R-Y (F) at the border between TM3 and intracellular loop (IL) 2. Contrary to glutamate, adhesion, frizzled, and secretin receptors, the rhodopsin family receptors have, in general, short N-termini [4]. At this time, high-resolution crystallographic structures have been obtained for only four receptors, and they all belong to the rhodopsin family. The first structure of a GPCR was determined in 2000, and it was the one of bovine rhodopsin itself [5]. The recent determination of high-resolution crystal structures of human beta2 adrenergic receptor [6, 7], turkey beta1 adrenergic receptor [8], and human A2A adenosine receptor [9] has shown that several obstacles need to be overcome before GPCR structural biology becomes routine: overexpression, solubilization, purification, and refolding of milligram quantities of active and stable receptors. Moreover, if we except rhodopsin, it is still a challenge to get the structure of an unmodified GPCR.

In this perspective, we have developed over the last years a very efficient strategy to over express GPCRs in the methylotrophic yeast *Pichia pastoris* [10–14] using the human mu-opioid receptor (HuMOR) as a model. This receptor displays a N-terminal domain which is assumed to have minor effects on receptor function [15]. Nevertheless, the presence of this domain could considerably complicate structural biology experiments due to peptide flexibility and glycosylation heterogeneity. Indeed, smaller proteins are more suited for structural studies, and the availability of a pure N-terminal truncated form of the mu-opioid receptor could provide a good model for structural studies. In this context, we report here the expression, solubilization, purification assays, pharmacological characterization, and circular dichroism (CD) analysis of recombinant mu-opioid receptors lacking 64 amino acids at the N-terminal end.

Materials and Methods

Construction of the ΔN64 Mu-Opioid Receptors

ΔN64-HuMOR-cmyc-6his was generated by polymerase chain reaction (PCR) using the 5′-forward primer: 5′GGGGTACCTTCGAAACGATGCCCTCCATGATCACGGCCATCAC GATCATG3′, the 3′-reverse primer 5′TCCTTTTCTTTGGAGCCAGAGAGCATGCGGA CACT CTTGAGGCGCAAGAT3′, and the pPICZ-mutBstBI-HuMOR-cmyc-6his vector [14] as a template. The double-stranded PCR product was purified and cloned in a TOPO vector (Invitrogen, Carlsbad, CA, USA). After enzymatic cleavage with BstBI and SphI, the mutated fragment was isolated and inserted in the pPICZ-mutBstBI-HuMOR-cmyc-6his vector replacing the original sequence and generating the pPICZ-ΔN64-mutBstBI-HuMOR-cmyc-6his vector. For the construction of the pPICZ-αMF-enhanced green fluorescent protein (EGFP)-ΔN64-HuMOR-cmyc-6his plasmid, the first 64 amino acids from the amino terminus of the human MOR were deleted by PCR using the following 5′-

forward primer: 5′GGCGGTACCTCACTAGTCACGGCC ATCACGATCATGGCC3′ and 3′-reverse primer: 5′CTCTCTGAAGGCTAGCTTGA AGTTTTC 3′ and the pPICZ-αMF-EGFP-HuMOR-cmyc-6his [13] plasmid as a template. The PCR product was purified, digested with KpnI and SphI, and finally introduced in the pPICZ-αMF-EGFP-HuMOR-cmyc-6his vector digested with the same enzymes. To construct the pPICZ-EGFP-ΔN64-HuMOR-cmyc-6his plasmid, the pPICZ-EGFP-HuMOR-cmyc-6his plasmid was digested with KpnI and Sph1 and the KpnI to Sph1 fragment was introduced into the pPICZ-αMF-EGFP-ΔN64-HuMOR-cmyc-6his plasmid digested with the same enzymes.

Strains and Expression

The *Escherichia coli* Top 10 F′ strain used for plasmid propagation, the SMD1163 *P. pastoris* strain, and the conditions employed for receptor expression were as described [12, 13].

Crude Extracts Preparation

All operations were carried out at 4 °C. After induction of expression with 0.5% MeOH, cells were harvested and broken during 30 min with glass beads in a breaking buffer (Tris-HCl 10 mM, pH7.5) supplemented with protease inhibitors (benzamidine 20 μg/ml, pepstatin A 1 μg/ml, leupeptin 1 μg/ml, antipain 1 μg/ml, aprotinin 1 μg/ml). The cell lysate was then centrifuged at 1,000×*g* for 15 min to remove unbroken cells and particulate matter. The supernatant was further centrifuged at 10,000×*g* and 100,000×*g* for 30 min to harvest crude fractions. The resulting pellets were then stored at −80 °C in the breaking buffer. Protein quantization was performed as described previously [12, 13].

Solubilization

For the GFP-tagged receptors, solubilization conditions were determined by taking advantage of the fluorescence emitted by EGFP. The 100,000×*g* membrane fraction containing the αMF-EGFP-ΔN64-HuMOR-cmyc-6his proteins was washed three times with an ice-cold solubilization buffer (SB; 10 mM Tris-HCl, pH7.5) containing antiproteases and without detergent. The membrane pellet was suspended in SB containing various concentrations of detergents or chaotropic agent for 4 h at 4 °C (Table 2). The fluorescence ratio at 508 nm (Quantamaster spectrofluorometer, Photon Technology International, South Brunswick, NJ, USA) between the supernatant resulting from an ultracentrifugation step at 100,000×*g* (30 min, 4 °C) and the initial fluorescence of the samples was used to assess the efficiency of each solubilization condition tested.

In some cases, a crude fraction obtained after centrifugation at 10,000×*g* was solubilized in 100 mM NaH_2PO_4, 10 mM Tris-HCl, 20 mM β-mercapto-ethanol, pH8 with 8 M urea, and 0.1% sodium dodecyl sulfate (SDS) [14].

Purification

After solubilization, samples were centrifuged at 100,000×*g* in order to remove unsolubilized matter. Solubilized receptors were then incubated for 1–2 h at 4 °C or room temperature with Ni-NTA resin (Qiagen SA, Courtaboeuf, France) or chelating sepharose (GE Healthcare, UK) charged with 300 mM Ni-acetate. The resin was washed with 50 ml of the solubilization buffer with detergent, and proteins bound to the resin were eluted with a step imidazole gradient.

SDS-PAGE and Western Blot Analysis

Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) by using 10% acrylamide gels and visualized by silver nitrate staining. For immunoblot analysis, proteins were transferred to an Immun-Blot membrane (Bio-Rad Laboratories, Hercules, CA, USA) after SDS-PAGE. Antigens were probed as described [12, 13].

Radioligand Binding Assays

Saturation binding assays were performed for 1 h at 25 °C in 500 µl of binding buffer (50 mM Tris-HCl, 10 mM EDTA, pH7.5) containing 100,000×*g* or 10,000×*g* crude membrane fractions or solubilized membranes and varying concentrations (0.05 to 3 nM) of [^{3}H]diprenorphine ([^{3}H]DPN; 50 Ci/mmol, PerkinElmer). Nonspecific binding was determined in parallel test tubes in the presence of nonlabeled diprenorphine (0.05–3 µM). For competition studies, various concentrations of unlabelled opioid ligands (Table 2) and 1 nM [^{3}H]DPN were used. Data were analyzed with the PRISM program (GraphPad software Inc., San Diego, CA, USA).

Circular Dichroism Experiments

To prepare CD samples in trifluoroethanol (TFE), purified receptors were prepared by extended dialysis of the samples against pure water. After lyophilization, receptors were solubilized in 100% TFE and filtrated. Protein concentration was determined by UV absorbance spectroscopy using an extinction coefficient of ε_{280}=74,113 $M^{-1}cm^{-1}$, which was determined experimentally by the procedure of Gill and von Hippel [16]. For CD in SDS micelles, samples were concentrated after purification. Buffer was exchanged, on a vivaspin 15R concentrator (Sartorius, Germany), against a buffer devoid of imidazole and containing the same concentration of detergent (0.1% SDS) at different pH. NaH_2PO_4 concentration in the buffer was also reduced to 10 mM. Protein concentration was determined with a BCA protein assay kit (Interchim, Les Ulis, France). CD spectra were recorded at room temperature by using Jobin-Yvon Mark VI circular dichroism apparatus at a scan speed of 0.2 nm/s and an integration time of 1 s. Total absorbance was maintained lower than 1.0 to ensure sufficient light transmission. Corresponding blanks were realized for each assay and substracted from the raw data. Two spectra were recorded and averaged to increase the signal-to-noise ratio. Protein concentrations were 50 to 300 µg/ml. The data were recorded in ΔA units and then converted into normalized $\Delta\varepsilon$ values on the basis of an amino acid mean residue mass of 112 Da. The CD data were analyzed with the three programs available in the CDPro software package [17]: CDSSTR [18], ContinLL [19], and Selcon3 [20] using a reference set of 56 proteins including 13 membrane proteins (SMP56) [21]. The fractions of regular and distorted α structures from CDPro were combined to obtain α-helix fraction. The secondary structure fractions are presented as averages with standard deviation of the results given by the three programs.

Results and Discussion

Binding Affinities of Ligands for the N-Terminal Truncated Mu-Opioid Receptors

The human mu-opioid receptor is composed of three main peptidic domains: an extracellular N-terminal domain, a hydrophobic domain composed of seven α-helices and

an intracellular C-terminal domain, which lengths were determined after hydrophobicity [22] and computational analysis [23]. The amino terminus of the human mu-opioid receptor contains five consensus amino acid sequences for asparagine-linked glycosylation, Asn-X-Ser/Thr, where X is any amino acid. These potential glycosylation sites are located in position 9, 12, 33, 40, and 48 of the peptidic sequence. In the course to access to 3-D structure of GPCRs, it is essential to work on a single homogenous polypeptide chain. The complex microheterogeneity introduced by the presence of *N*-glycans at the N terminus of the mu-opioid receptor can introduce difficulties during biophysical analysis of the protein. Moreover, this N-terminal flexible peptide may account for the occurrence of sharp resonances devoid of any nuclear Overhauser effects in the case of nuclear magnetic resonance experiments thus making the interpretation difficult. This flexibility can also prevent the growth of diffracting crystals suitable for X-ray crystallography. Thus, the N-terminal part of the turkey β1 adrenergic receptor was deleted in the construct used for crystallography [8], and the electron density was uninterpretable in the extracellular domain of the β2 adrenergic receptor [7]. In the present work, we wanted not only to examine the role of the N terminus part of the human mu-opioid receptor on the expression, solubilization, and purification of the receptor but also to analyze the pharmacological and biophysical properties of the truncated receptor. The mu-opioid receptor was expressed in the methylotrophic yeast *P. pastoris*, as different recombinant proteins. The N-terminal end truncated receptor was expressed in the following ways (Fig. 1): (1) in fusion with the *Saccharomyces cerevisiae* α-factor prosequence and EGFP at the N terminus (αMF-EGFP-ΔN64-HuMOR-cmyc-6his), (2) in fusion with the enhanced green fluorescent protein (EGFP-ΔN64-HuMOR-cmyc-6his), and (3) without N-terminal tags (ΔN64-HuMOR-cmyc-6his). In a first attempt, we used GFP constructs to develop methods as this fluorescent protein is a good reporter of the following events: (1) selection of over-expressing clones on plates, (2) determination of total recombinant protein expression versus active expression, (3) direct determination of optimal solubilization conditions prior to purification, and (4) direct quantification of purified fractions. For the rest, it can be used in molecular replacement which is a method for solving the phase problem in X-ray crystallography.

All these receptors present at their C terminus, 6-histidine, and c-myc epitopes to make downstream purification and detection. A 64-amino acid deletion was chosen since mRNA for this truncated form was naturally present in rat brain and the expressed protein displayed a typical mu-opioid receptor profile [22]. The results were compared with data

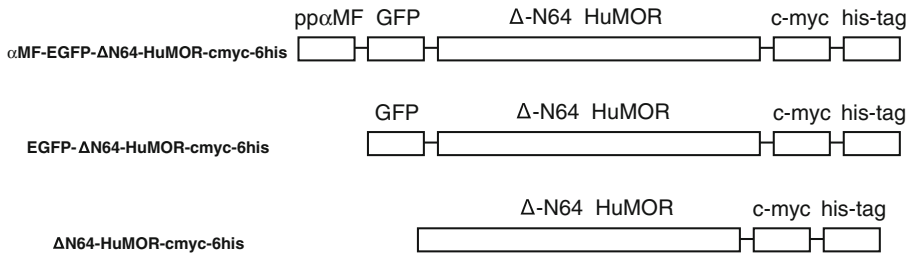

Fig. 1 Schematic representation of various *P. pastoris* expression constructions used for heterologous production of the human ΔN64 mu-opioid receptor. *ppαMF* coding region for the prepropeptide of the *S. cerevisiae* mating type factor α, *GFP* coding region for the enhanced green fluorescent protein, *Δ-N64 HuMOR* coding region for the N-terminal end truncated human mu-opioid receptor, *c-myc* coding region for the c-myc epitope (EQKLISEEDL), *his-tag* sequence consisting of six consecutive histidine codons

obtained previously on the full-length receptor (HuMOR-cmyc-6his) [14], the N-terminal EGFP-tagged full-length receptor (EGFP-HuMOR-cmyc-6his) [14], and on the N-terminal α-mating factor EGFP- and α-cmyc-6his-tagged full-length receptor (αMF-EGFP-HuMOR-cmyc-6his) [12]. All pharmacological studies were realized on the membrane fraction. As for the αMF-EGFP-HuMOR-cmyc-6his protein [13], the total amount of the αMF-EGFP-ΔN64-HuMOR-cmyc-6his receptor, detected by EGFP fluorescence, was found to be 14-fold higher than that detected by diprenorphine binding on the 100,000-g crude membrane fraction. The EGFP fluorescence experiments thus show that a major fraction of the receptor present in the membranes is unable to bind diprenorphine. It implies that a significant part of the receptor is either not fully folded, aggregated, or in a low affinity state due to an unfavorable membrane environment. As determined previously for the EGFP-tagged full-length mu-opioid receptor [14], no binding was observed for the EGFP-tagged N-terminal truncated receptor when the antagonist diprenorphine was used. This lack of specific binding was also seen for other receptor types such as the β1, β2, β3 adrenergic receptors fused to EGFP [24]. When receptors are tagged with EGFP alone at the N terminus (EGFP-ΔN64-HuMOR-cmyc-6his) and expressed in *P. pastoris*, they are detected in a special fraction obtained after cell breakage and differential centrifugation. The fraction was obtained after centrifugation at 10,000×*g* and was called *P. pastoris* inclusion bodies-like fraction [14]. This fraction contains the overexpressed recombinant receptor in an aggregated form, thus explaining the lack of specific binding. On the contrary, fluorescence from αMF-EGFP-ΔN64-HuMOR-cmyc-6his proteins was mainly detected in the 100,000-g membrane fraction and exhibited pharmacological profiles that were in the range of the expected values (Table 1; Fig. 2). Nevertheless, if the pharmacological constants for αMF-EGFP-ΔN64-HuMOR-cmyc-6his were similar to those reported for αMF-EGFP-HuMOR-cmyc-6his [13], we observed a decrease of agonists affinities for ΔN64-HuMOR-cmyc-6his compared to HuMOR-cmyc-6his [14]. This was particularly true for the peptidic agonist [D-Ala2,MePhe4,Gly(ol)5]enkephalin (DAGO) for which a sevenfold reduction in affinity was observed. In general, deletion of the N-terminal domain of the mu-opioid receptor produced little effect on receptor function when it was expressed in higher eukaryotic cells; however, decreased affinities have also been observed for specific ligands with a more stamped effect for agonists and without effect on signal transduction [15, 22, 25–27]. In addition, analysis of *N*-glycosidase F-treated membranes indicated that *N*-glycan chains within the amino-terminal domain of MOR did not contribute significantly to ligands affinities [27]. Thus, amino acid residues within the amino-terminal domain of MOR appear to play a role in the organization of the binding pocket for mu-opioid agonist ligands [27]. It was also suggested that the N-terminal region of MOR undergoes conformational changes following receptor activation that can be selectively detected by region-specific antibodies [28]. The B_{max} values determined for ΔN64-HuMOR-cmyc-6his was in the same range (0.22 pmol/mg proteins) than the value determined for the full-length mu-opioid receptor (0.45 pmol/mg). In the same way, no difference was observed between αMF-EGFP-ΔN64-HuMOR-cmyc-6his and αMF-EGFP-HuMOR-cmyc-6his B_{max} values. Since deletion of the N-terminal domain of the mu-opioid receptor expressed in *P. pastoris* results in little influence on the binding constants, we decided to perform more in-depth studies on this truncated form of the receptor.

Solubilization and Purification of αMF-EGFP-ΔN64-HuMOR-cmyc-6his Receptors

In the present study, solubilization assays were performed on the 100,000×*g* membrane fraction where the αMF-EGFP-tagged receptor was mainly found, by using a set of

Table 1 Affinity values of agonists and antagonists

	αMF-EGFP-ΔN64-MOR-cmyc-6his[a]	αMF-EGFP-MOR-cmyc-6his [13]	ΔN64-MOR-cmyc-6his[a]	MOR-cmyc-6his [14]
Agonists				
Morphine	400±20	410±20	997±133	585
DAGO	100±20	169±20	750±50	112
Antagonists				
DPN (K_D)	0.3±0.04	0.45±0.04	1.1±0.2	0.77
Naloxone	6±2	5±2	19±3	16
B_{max} (pmol/mg)	0.7	0.65	0.22	0.45

K_D (nM) values were estimated by analysis of saturation isotherms of [^{3}H]DPN to membranes from *Pichia* cells expressing the receptors. Values in the table are expressed as ligand dissociation constants (K_i, nM) and were determined by nonlinear regression of competition curves using [^{3}H] diprenorphine as radioligand

DPN diprenorphine, *MOR* human mu-opioid receptor

[a] This work

detergents and conditions. The fluorescence ratio (at 508 nm) between the supernatant resulting from the 100,000×*g* ultracentrifugation step (containing the solubilized proteins) and the initial fluorescence of the samples was used to assess the efficiency of each solubilization condition tested. Each detergent was used at a concentration which was about twice its critical micellar concentration. A set of 23 different detergents (neutral, ionic, zwitterionic) were tested for their ability to solubilize the receptor (Table 2). None of them was able to keep the receptor active after solubilization. Nevertheless, in this tedious work, EGFP fluorescence represented an easy and very fast tool which enabled us to select Triton X100 for the purification trials. In fact, a 2% concentration of this neutral detergent, used at 4°C for 4 h was sufficient to solubilize 70% of the fluorescent receptor. Extending the solubilization period did not ameliorate the solubilization yield. As an efficient solubilizer, triton X-100 was also used to purify the receptor. Thus, membranes containing the αMF-EGFP-ΔN64-HuMOR-cmyc-6his receptor were solubilized with 2% Triton X100 in 100 mM NaH_2PO_4 and 10 mM Tris-HCl (pH 8.9). After a centrifugation step at 100,000×*g*, the supernatant containing the 6his-tagged receptor was charged on a Ni^{2+} column. The column was washed with a 20-mM imidazole containing buffer to remove unspecific bound proteins while bound receptors were eluted using an imidazole step gradient. Nevertheless, no enrichment was observed after SDS-PAGE, and we have consequently focused on the purification of the ΔN64-MOR-cmyc-6his which is highly expressed in the *P. pastoris* yeast cells.

Solubilization and Purification of ΔN64-HuMOR-cmyc-6his Receptors

Contrary to the α-mating factor-fused receptor (αMF-HuMOR-cmyc-6his or αMF-GFP-HuMOR-cmyc-6his), the untagged receptor (HuMOR-cmyc-6his) is mainly found in a 10,000×*g* centrifugation fraction under an aggregated form [14]. A similar preparation process was performed for ΔN64-HuMOR-cmyc-6his and 10,000×*g* and 100,000×*g* fractions were prepared. Receptor contents into the fractions were compared by Western blotting, and a considerable enrichment in receptor was found in the 10,000×*g* fraction (data not shown). Presumably, this 10,000-g fraction, unlike the 100,000×*g* fraction, is not

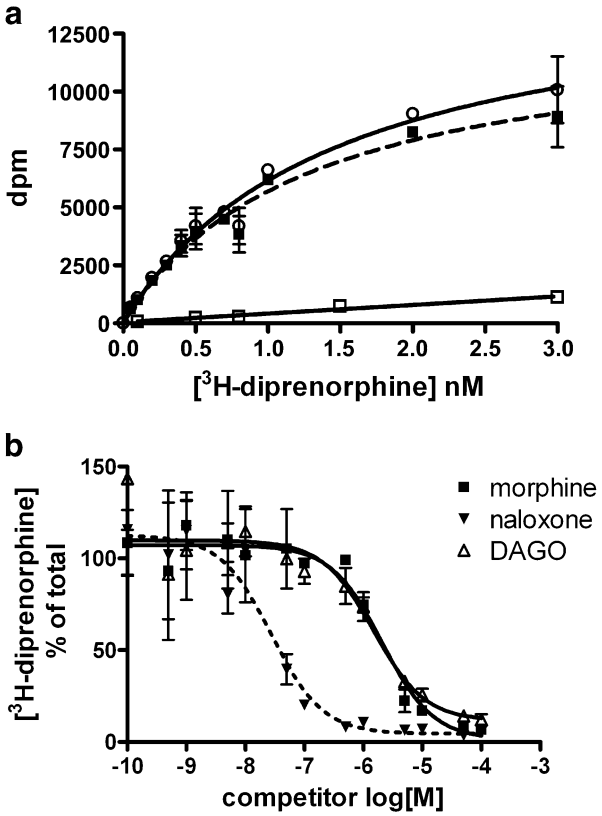

Fig. 2 **a** Saturation binding of [^{3}H] diprenorphine to ΔN64-HuMOR-cmyc-6his in SMD1163 *P. pastoris* membranes. Total binding (*open circle*), specific binding (*closed square*), nonspecific binding (*open square*). **b** Displacement binding profile for ΔN64-HuMOR-cmyc-6his when located in SMD1163 membranes. K_i values were calculated for each drug from the experimentally IC50 values and are recorded in Table 1. *Closed square* morphine, *inverted closed triangle* naloxone, *open triangle* DAGO

a membrane fraction and could be closer to the inclusion bodies observed during overexpression of proteins in *E. coli*. Moreover, this 10,000×*g* fraction is solubilized only by anionic detergents such as SDS [14]. Thus, the truncated receptor found in the 10,000-g fraction was fully solubilized in 8 M urea and 0.1% SDS, and these conditions were further used for the purification of ΔN64-HuMOR-cmyc-6his. For the purification of ΔN64-HuMOR-cmyc-6his, the 10,000×*g* pellet fraction was solubilized in 8 M urea and 0.1% SDS and charged on a Ni-chelating sepharose column. Samples eluted from the column with an imidazole step gradient were analyzed by SDS-PAGE (Fig. 3a, b). Following elution, ΔN64-MOR-cmyc-6his appeared as a single band in the 50-mM imidazole fraction of the silver-stained gel (Fig. 3a) and exhibited an apparent molecular weight of 38–39 kDa. This value correspond well to the theoretical size of ΔN64-MOR-cmyc-6his (40.9 kDa), and the band was detected with an anti c-myc antibody (Fig. 3b), supporting the fact that the protein is the expressed opioid receptor. An additional protein was detected in the 100- and 300-mM fractions with an apparent molecular weight of 65 kDa, suggesting that a dimer form of the receptor was purified.

Table 2 Solubilization of the αMF-EGFP-ΔN64-HuMOR-cmyc-6his proteins

	Solubilizing agent (*w*/*v*)	Efficiency (%)
Nonionic detergent		
Triton X100	0.1%	7
	1%	60
	2%	70
Triton N101	2%	70
Hecameg	1.3%	35
Mega 8	3.6%	49
Mega 9	1.3%	48
Mega 10	0.49%	54
Digitonin	2%	40
Dodecylmaltoside	0.015%	25
Octylglucoside	1.47%	40
Nonylglucoside	2%	48
Octylthioglucoside	2%	47
Heptylthioglucoside	2%	35
Tween 20	0.012%	31
C12E8	0.009%	17
Saponin	2%	40
Ionic detergents		
Cholic acid	2%	54
C6DAO	2%	21
C8DAO	2%	29
C10DAO	2%	58
BigChap	0.58%	32
DeoxyBigChap	0.18%	32
Chaps	0.8%	31
Chapso	1%	38
Chaotropic agents		
Urea	8 M	10
Guanidine	6 M	Not determined

Membranes were incubated for 4 h at 4°C with the solubilizing agent at the concentration indicated. After ultracentrifugation at 100,000 g, the EGFP fluorescence was measured

Higher molecular weight receptors were also detected by Western blot but represent minor forms and were not detected after silver staining. Finally, immobilized metal ion affinity chromatography purification was efficient and resulted in the production of 5 mg of pure ΔN64-HuMOR-cmyc-6his per liter of yeast culture.

Circular Dichroism of ΔN64-HuMOR-cmyc-6his Receptors

After purification and extended dialysis against water, the N-terminal end truncated mu-opioid receptor was lyophilized and finally solubilized in the organic solvent trifluoroethanol. As emphasize in our recent study on the full-length receptor [29],

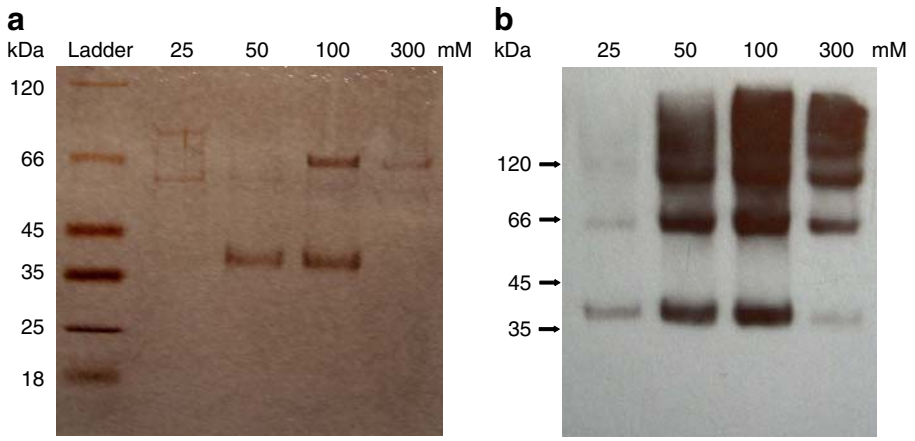

Fig. 3 Purification of ΔN64-HuMOR-cmyc-6his. The SDS-PAGE gel was stained with silver nitrate (**a**). *Lane 25* 25 mM imidazole, *lane 50* 50 mM imidazole, *lane 100* 100 mM imidazole, *lane 300* 300 mM imidazole, *ladder* molecular weight marker (Bio-Rad, 116, 66, 45, 35, 25, 18 kDa; the calculated molecular weight of ΔN64-HuMOR is 40.9 kDa). Western blotting (**b**); the band appearing around 66 kDa is assigned to a dimer of receptors

TFE is a lipomimetic solvent which induces and stabilizes the formation of α-helices in peptides that have the propensity to form α-helix such as transmembrane domains. The mu-opioid receptor exhibits, as a GPCR, a pattern of hydrophobic peptide segments that are believed to be in an α-helix conformation. The solubilized ΔN64-HuMOR-cmyc-6his sample in 100% TFE exhibit far-UV-CD spectra characteristic of predominantly alpha helical structure, as seen by the presence of a positive band at ~190 nm and negative bands at ~222 and ~208 nm (Fig. 4a). Secondary structural analysis of the ΔN64-HuMOR-cmyc-6his spectrum showed an α-helical content (47±3%) lower than the one calculated (64%) by using the Jpred3 secondary structure prediction server [30]. For the full-length mu-opioid receptor, a good accordance was observed between experimental (57.5±3%) and calculated (54%) α-helical contents [29]. Far-UV-CD spectra were also realized on the receptor solubilized in the anionic detergent SDS at various pHs (Fig. 4b). A pH effect was observed on the secondary structure with higher helical contents at acidic pH. Helical contents were 36.1±3.3% at pH9, 41.7±2.4% at pH8, 41.1±2.7% at pH7, and 50.5±3.8% at pH6. Indeed, this pH effect was also observed for the full-length receptor in SDS [29]. The increase of helicity in acidic solutions observed both for the full-length and for the N-terminal end truncated receptors correlates well with a dual electrostatic and hydrophobic effect of acidic SDS on the secondary structure of the receptor [29]. Helix contents observed in 0.1% SDS buffer at pH6 and in 100% TFE are in accordance (~50%) but are lower than the predicted value (64%). This discrepancy could be due to the formation of smaller helices than calculated by the Jpred3 secondary structure prediction program.

Conclusion

In this work, we have compared the expression and purification of N-terminal truncated forms of the mu-opioid receptor. Pharmacological studies using specific ligands demonstrated a typical opioid profile for the N-terminal end truncated

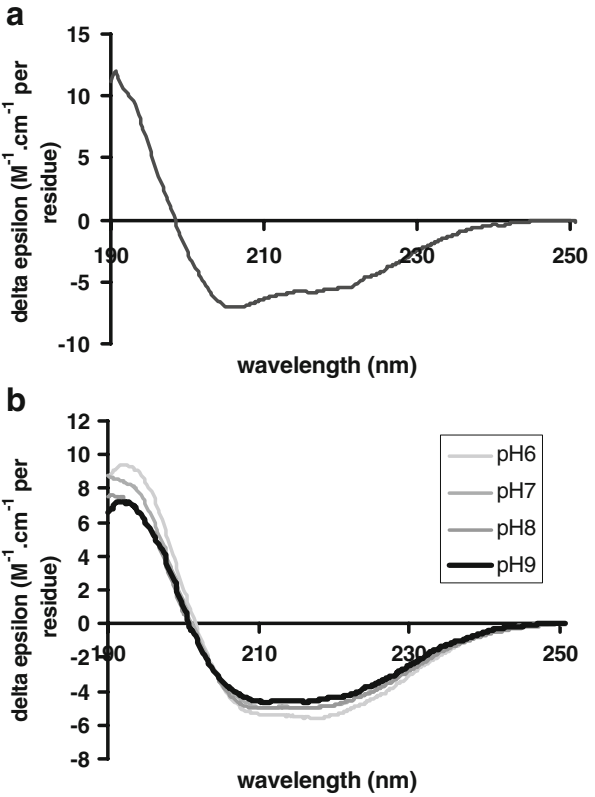

Fig. 4 Circular dichroism spectra of ΔN64-HuMOR in 100% TFE (**a**) and in 0.1% SDS buffer at several pH (**b**)

proteins. The EGFP- and cmyc-6his-tagged receptors were mainly detected in the cell membrane fraction since it was fused to the α-mating factor signal sequence of *S. cerevisiae* which is known to help this protein import. The untagged receptor was mainly found in yeast "inclusion bodies-like" structures and was mostly inactive. The use of a panel of 23 different detergents for the solubilization of αMF-EGFP-ΔN64-HuMOR-cmyc-6his demonstrated the impossibility to preserve the functionality of this receptor under the conditions tested. Purification by nickel affinity did not allow producing αMF-GFP- and cmyc-6his-tagged pure receptors. On the contrary, we were able to produce milligram quantities of the pure untagged ΔN64-HuMOR-cmyc-6his. This "shorter" version of the receptor presents the advantage to be devoid of any *N*-glycosylation site and of a presumably unstructured domain, while keeping its opioid binding activity in the membrane fraction. Since the purification procedure from inclusion bodies leads to a loss of binding activity, we are currently attempting to refold this receptor as it was realized in the group of J.L. Baneres for the BLT1 [31] and 5-HT4 [32] receptors.

Acknowledgments This work was supported by the "Centre National de la Recherche Scientifique" and by the University Paul Sabatier (Toulouse III).

Declaration of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Marinissen, M. J., & Gutkind, J. S. (2001). *Trends in Pharmacological Sciences, 22*, 368–376.
2. Terenius, L. (2000). *Upsala Journal of Medical Sciences, 105*, 1–15.
3. Matthes, H. W., Maldonado, R., Simonin, F., Valverde, O., Slowe, S., Kitchen, I., et al. (1996). *Nature, 383*, 819–823.
4. Schioth, H. B., & Fredriksson, R. (2005). *General and Comparative Endocrinology, 142*, 94–101.
5. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., et al. (2000). *Science, 289*, 739–745.
6. Cherezov, V., Rosenbaum, D. M., Hanson, M. A., Rasmussen, S. G., Thian, F. S., Kobilka, T. S., et al. (2007). *Science, 318*, 1258–1265.
7. Rasmussen, S. G., Choi, H. J., Rosenbaum, D. M., Kobilka, T. S., Thian, F. S., Edwards, P. C., et al. (2007). *Nature, 450*, 383–387.
8. Warne, T., Serrano-Vega, M. J., Baker, J. G., Moukhametzianov, R., Edwards, P. C., Henderson, R., et al. (2008). *Nature, 454*, 486–491.
9. Jaakola, V. P., Griffith, M. T., Hanson, M. A., Cherezov, V., Chien, E. Y., Lane, J. R., et al. (2008). *Science, 322*, 1211–1217.
10. Talmont, F., Sidobre, S., Demange, P., Milon, A., & Emorine, L. J. (1996). *FEBS Letters, 394*, 268–272.
11. Massou, S., Puech, V., Talmont, F., Demange, P., Lindley, N. D., Tropis, M., et al. (1999). *Journal of Biomolecular NMR, 14*, 231–239.
12. Sarramegna, V., Demange, P., Milon, A., & Talmont, F. (2002). *Protein Expression and Purification, 24*, 212–220.
13. Sarramegna, V., Talmont, F., Seree de Roch, M., Milon, A., & Demange, P. (2002). *Journal of Biotechnology, 99*, 23–39.
14. Sarramegna, V., Muller, I., Mousseau, G., Froment, C., Monsarrat, B., Milon, A., et al. (2005). *Protein Expression and Purification, 43*, 85–93.
15. Surratt, C. K., Johnson, P. S., Moriwaki, A., Seidleck, B. K., Blaschak, C. J., Wang, J. B., et al. (1994). *Journal of Biological Chemistry, 269*, 20548–20553.
16. Gill, S. C., & von Hippel, P. H. (1989). *Analytical Biochemistry, 182*, 319–326.
17. Sreerama, N., & Woody, R. W. (2000). *Analytical Biochemistry, 287*, 252–260.
18. Johnson, W. C. (1999). *Proteins, 35*, 307–312.
19. Provencher, S. W., & Glockner, J. (1981). *Biochemistry, 20*, 33–37.
20. van Stokkum, I. H., Spoelder, H. J., Bloemendal, M., van Grondelle, R., & Groen, F. C. (1990). *Analytical Biochemistry, 191*, 110–118.
21. Sreerama, N., & Woody, R. W. (2004). *Protein Science, 13*, 100–112.
22. Wang, J. B., Imai, Y., Eppler, C. M., Gregor, P., Spivak, C. E., & Uhl, G. R. (1993). *Proceedings of the National Academy of Sciences of the United States of America, 90*, 10230–10234.
23. Fowler, C. B., Pogozheva, I. D., LeVine, H., 3rd, & Mosberg, H. I. (2004). *Biochemistry, 43*, 8700–8710.
24. Talmont, F. (2009). *Biotechnology Letters, 31*, 49–55.
25. Shahrestanifar, M., Wang, W. W., & Howells, R. D. (1996). *Journal of Biological Chemistry, 271*, 5505–5512.
26. Dietrich, G., Gaibelet, G., Capeyrou, R., Butour, J. L., Pontet, F., & Emorine, L. J. (1998). *Journal of Neurochemistry, 70*, 2106–2111.
27. Chaturvedi, K., Shahrestanifar, M., & Howells, R. D. (2000). *Brain Research. Molecular Brain Research, 76*, 64–72.
28. Gupta, A., Decaillot, F. M., Gomes, I., Tkalych, O., Heimann, A. S., Ferro, E. S., et al. (2007). *Journal of Biological Chemistry, 282*, 5116–5124.
29. Muller, I., Sarramegna, V., Renault, M., Lafaquiere, V., Sebai, S., Milon, A., et al. (2008). *Journal of Membrane Biology, 223*, 49–57.
30. Cole, C., Barber, J. D., & Barton, G. J. (2008). *Nucleic Acids Research, 36*, W197–W201.
31. Baneres, J. L., Martin, A., Hullot, P., Girard, J. P., Rossi, J. C., & Parello, J. (2003). *Journal of Molecular Biology, 329*, 801–814.
32. Baneres, J. L., Mesnier, D., Martin, A., Joubert, L., Dumuis, A., & Bockaert, J. (2005). *Journal of Biological Chemistry, 280*, 20253–20260.