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Stability study of the human G-protein coupled receptor, Smoothened

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ABSTRACT

Smoothened is a member of the G-protein coupled receptor (GPCR) family responsible for the transduction of the Hedgehog signal to the intracellular effectors of the Hedgehog signaling pathway. Aberrant regulation of this receptor is implicated in many cancers but also in neurodegenerative disorders. Despite the pharmacological relevance of this receptor, very little is known about its functional mechanism and its physiological ligand. In order to characterize this receptor for basic and pharmacological interests, we developed the expression of human Smoothened in the yeast *Saccharomyces cerevisiae* and Smoothened was then purified. Using Surface Plasmon Resonance technology, we showed that human Smoothened was in a native conformational state and able to interact with its antagonist, the cyclopamine, both at the yeast plasma membrane and after purification. Thermostability assays on purified human Smoothened showed that this GPCR is relatively stable in the classical detergent dodecyl-β-D-maltoside (DDM). The fluorinated surfactant C_8F_{17}TAC, which has been proposed to be less aggressive towards membrane proteins than classical detergents, increased Smoothened thermostability in solution. Moreover, the replacement of a glycine by an arginine in the third intracellular loop of Smoothened coupled to the use of the fluorinated surfactant C_8F_{17}TAC during the mutant purification increased Smoothened thermostability even more. These data will be very useful for future crystallization assays and structural characterization of the human receptor Smoothened.

1. Introduction

G-protein coupled receptors (GPCRs) are transmembrane proteins that mediate most of their intracellular actions through pathways involving activation of G-proteins and arrestin [1,2]. G-proteins transmit intracellular signals to effector proteins such as enzymes and ion channels, resulting in rapid changes in the concentration of intracellular signaling molecules, cAMP, cGMP, inositol phosphates, diacylglycerol, arachidonic acid, and cytosolic ions [3,4]. The superfamily of GPCRs comprises receptors for many hormones, paracrines (local hormones), neurotransmitters, and neuromodulators with important physiological functions. GPCRs represent about 30% of current drug targets and their dysfunction causes many human diseases. In the GPCR database [5], GPCRs are divided into six classes. These are the Class A Rhodopsin-like receptors, which account for over 80% of all GPCRs, Class B Secretin-like receptors, Class C Metabotropic glutamate receptors, Class D Pheromone receptors, Class E cAMP receptors and the Class F Frizzled/Smoothened family. This latter class comprises Frizzled and Smoothened receptors, which are key regulators of animal development that signal through the Wnt and Hedgehog signaling pathways, respectively. The Hedgehog (Hh) signaling pathway regulates body patterning and organ development during embryogenesis. In adults, the Hh pathway is mainly quiescent, with the exception of roles in tissue maintenance and repair, and its inappropriate reactivation has been linked to several disparate human cancers such as basal cell carcinomas and medulloblastomas [6]. The Hh pathway involves two integral membrane proteins, Patched (Ptc) and Smoothened (Smo). Ptc, a 12-transmembrane domain protein, is the receptor of the secreted protein Hedgehog (Hh). In the absence of Hh, Ptc inhibits the activity of Smo through an unknown mechanism. Hh binding to Ptc releases Smo inhibition. Activated Smo is stabilized on the plasma membrane and transduces the Hh signal to the intracellular components of the pathway, culminating in the activation of the transcription factor Gli in mammals or Ci in *Drosophila*. Despite the important pharmacological interest of this receptor, very little is known about its functional mechanism or its physiological ligand and partners in mammals [7].

In order to better understand signaling via Smo in mammals, structural data are essential. Smo, like many other GPCRs, is present in very low amounts in native sources. In order to undergo biochemical and structural characterization, we over expressed this receptor in

Abbreviations: GPCR, G-Protein Coupled Receptor; hSmo, Smoothened; MAP, Multitag Affinity Purification; DDM, dodecyl-β-D-maltoside; FS, fluorinated surfactant; SPR, surface plasmon resonance; RU, resonance unit; Hh, Hedgehog; HA, hemagglutinin; CPN, cyclopamine

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several heterologous systems [8,9]. Heterologously overproduced and purified proteins provide a starting point for biochemical, biophysical and structural studies, but the lack of sufficient quantities of functional and stable membrane proteins is frequently a bottleneck hindering such studies. Recurring problems include i) recovering adequate amounts of functional protein from the heterologous expression system used, ii) solubilizing and purifying these proteins, iii) maintaining stability and functionality in solution after extraction from membranes and purification.

We show in the present study that human Smo could be expressed in the plasma membrane of the yeast *Saccharomyces cerevisiae* and purified in a native and stable conformation, as assessed by antagonist-binding and Surface Plasmon Resonance studies. Our results indicate that human Smo is relatively stable in solution with the classical detergent dodecyl-β-D-maltoside (DDM), and that the fluorinated surfactant C_8F_{17}TAC increases Smo thermostability in solution. We also observed a synergistic effect of the fluorinated surfactant C_8F_{17}TAC and the replacement of a glycine by an arginine in the Smo third intracellular loop on the receptor thermostability.

2. Materials and methods

2.1. Construction of expression vectors

For expression in *S. cerevisiae*, the cDNAs from human Smo and a mutant form of human Smo in which the guanine in position 1303 was replaced by an adenine (giving the mutation G435R in the polypeptide sequence) were inserted in the YEpPMA-MAP vector containing the constitutive plasma membrane proton ATPase promoter and the Multitag Affinity Purification sequence as described in [8], giving the expression vectors YEpPMA-hSmo-MAP and YEpPMA-hSmoG435R-MAP respectively.

For expression in *Drosophila* Schneider 2 cells, the wild type and the mutant human Smo cDNAs were inserted in the pAc-MAP vector containing the strong constitutive actine promoter and the Multitag Affinity Purification sequence as described in [9], giving the expression vectors pAc-hSmo-MAP and pAC-hSmo-G435R-MAP respectively.

2.2. Yeast culture

S. cerevisiae strain K699 (Mata, ura3, and leu 2–3), which was generously given by R. Arkowitz, was transformed with YEpPMA-hSmo-MAP or YEpPMA-hSmo-G435R-MAP expression vector by the lithium acetate procedure [10] and grown on plates containing minimal medium and an amino-acid mixture lacking leucine. Clones were grown at 30 °C to an OD_{600} of 2 in minimal medium (0.67% yeast nitrogen base without amino acids, 0.3 mM adenine, 0.5 mM uracil, 0.3 mM tyrosine and an amino acid mixture lacking leucine) supplemented with 2% D-glucose. Yeasts were diluted to an OD_{600} of 0.1 in complete YEP medium (1.1% yeast extract, 2.2% bactopeptone, 0.3 mM adenine) supplemented with 2% D-glucose, grown at 18 °C under 200 rpm shaking until the OD_{600} was 7, and centrifuged for 10 min at 2000 *g* and 4 °C (see detailed protocol in [11].

Large-scale production of hSmo-MAP was carried out in a 40-L fermentor (BIOSTAT C-PLUS Sartorius BBI Systems, Unité de fermentation IMM/IFR88 CNRS Marseille France). One liter of yeast with an OD_{600} of 1.5 served to inoculate 30 L of YEP medium. Yeasts were grown at 18 °C for 24 h (2.5 OD_{600}) under 200 rpm shaking with 30 L of air per minute. The culture was then oxygenated with 45 L of air per minute, kept for 6 h at 18 °C and 250 rpm shaking until the OD_{600} was 7, and centrifuged in a Sharples AS16 centrifuge (100 L/h).

2.3. Yeast membrane preparation

All steps were performed at 4 °C. Yeast cells were washed in cold water, re-suspended in ice-cold disintegration buffer (50 mM Tris–HCl [pH 7.4], 500 mM NaCl, 2.5 mM EDTA, 4 mM Benzamidine and 1 mM PMSF), and broken with glass beads (425–600 μm, Sigma) for 15 min in a shaker at 2000 rpm (Heidolph Multi Reax shaker). Unbroken cells and nuclei were pelleted for 10 min at 3000 *g* and crude extract was obtained from the supernatant. Plasma membranes were prepared by centrifugation of crude extract for 1 h at 18,000 *g*. The membrane fraction was then washed twice with purification buffer (50 mM Tris–HCl pH (7.4), 500 mM NaCl, 10% glycerol, 4 mM Benzamidine and 1 mM PMSF). Finally, the membrane fraction was re-suspended in purification buffer and frozen at −80 °C (for a detailed protocol, see [11]).

2.4. Solubilization and purification

Membrane fractions were thawed and simultaneously diluted to 3 mg/mL with ice-cold purification buffer supplemented with 20 mM of the non-ionic detergent DDM (dodecyl-β-D-maltoside, CMC = 0.15 mM). Solubilization of hSmo from the membrane was performed at 4 °C with gentle agitation for 1 h. The solution was centrifuged at 100,000 *g* for 1 h to pellet unsolubilized material. For SPR experiments, Smo-MAP was purified on a calmodulin column. The supernatant containing solubilized hSmo was incubated with a pre-equilibrated calmodulin column in the presence of 2 mM $CaCl_2$ overnight at 4 °C on a rotating wheel. Non-specifically bound material was removed from the resin with several washes with purification buffer supplemented with 2 mM DDM and 2 mM $CaCl_2$. In order to exchange DDM with the fluorinated surfactants $C_6F_{13}C_2H_4$-S-poly-*Tris*-(hydroxymethyl) aminomethane (C_6F_{13}TAC) (CMC = 0.3 mM), or $C_8F_{17}C_2H_4$-S-poly-*Tris*-(hydroxymethyl) aminomethane (C_8F_{17}TAC) (CMC = 0.03 mM) [12,13], the resin was washed 3 times with 5-column volumes of purification buffer supplemented with 2 mM $CaCl_2$ and either 2 mM C_6F_{13}TAC or 2 mM C_8F_{17}TAC. Elution was carried out with purification buffer supplemented with 5 mM EGTA and the appropriate surfactant. Aliquots were collected throughout the purification process, and analyzed by western-blot. For mass spectrometry analysis, hSmoG435R-MAP was purified on Ni/NTA agarose (Qiagen) and on Streptavidin sepharose (Amersham). The solubilized fraction supplemented with 10 mM imidazole was incubated overnight at 4 °C on Ni/NTA resin equilibrated with purification buffer containing 2 mM of DDM and 10 mM of imidazole. The column was washed 3 times with purification buffer containing 50 mM of imidazole, and hSmoG435R-MAP was eluted with a buffer containing 400 mM of imidazole. Eluted fractions were pooled and loaded on the streptavidin column, for 1 h at 4 °C. Finally, hSmoG435R-MAP was eluted and loaded on SDS-PAGE for western-blotting and Coomassie blue staining. The band corresponding to hSmoG435R-MAP was cut and analyzed by mass spectrometry (MALDI-TOF-TOF 4800 Applied Biosystems, IPMC Sophia Antipolis).

2.5. MAP peptide expression and purification

The MAP sequence was inserted into the *Xho*I and *Nhe*I restriction sites of the pBAD vector containing the inducible arabinose promoter (kind gift from P. Demange). The *Escherichia coli* strain MC1061 was transformed with the vector pBAD-MAP. Bacteria were grown in LB medium supplemented with ampicillin at 37 °C. At an OD_{600} of 0.8, 0.4% of L-arabinose was added to the culture for 4 h at 37 °C to induce MAP peptide expression. The bacteria were then centrifuged and the pellet was washed with ice-cold water and then with ice-cold PBS buffer supplemented with 1 mM PMSF and 4 mM Benzamidine. The bacteria were sonicated for 5 min and centrifuged. $CaCl_2$ was then added to the supernatant to a final concentration of 2 mM prior to incubation for 1 h with calmodulin resin pre-equilibrated in PBS buffer supplemented with 1 mM PMSF, 4 mM benzamidine and 2 mM $CaCl_2$. After several washes with equilibration buffer, the MAP peptide was eluted with PBS buffer supplemented with 1 mM PMSF, 4 mM

Benzamidine and 5 mM EGTA. Purified MAP peptide was used as a standard to quantify hSmo-MAP.

2.6. Protein quantification

The proteins were quantified using the Bio-Rad protein assay.

2.7. SDS-PAGE and western-blotting

Protein samples were separated on SDS-PAGE (acrylamide/bis-acrylamide: 8% for hSmo-MAP or hSmoG435R-MAP and 15% for MAP peptide). For western-blot analysis, gels were transferred to nitrocellulose using standard techniques. Blots were first incubated for 1 h at room temperature in the blocking buffer (20 mM Tris–HCl (pH 7.4), 450 mM NaCl, 0.1% Tween-20 supplemented with 4% non-fat milk) and probed overnight at 4 °C with monoclonal mouse anti-HA antibodies (laboratory-made, dilution 1:20). The blots were then washed three times in the blocking buffer. Secondary polyclonal goat anti-mouse immunoglobulins coupled to horseradish peroxidase (Dako) (dilution 1:5000) were then applied for 2 h at 4 °C. Revelation was carried out using ECL kit (Amersham Biosciences), a CCD camera and the Las 3000 system® (Fuji).

2.8. Native gel

Protein samples were separated on native gel under non-denaturing conditions in which acrylamide gels, running, loading and transfer buffers lacked SDS. The gels were then transferred to nitrocellulose membrane and probed with anti-HA antibodies.

2.9. Fluorescent ligand binding measurements

100 µg of membrane preparation were incubated in PBS buffer and increasing concentrations of Bodipy-cyclopamine (Bo-CPN) (between 0 and 25 nM) 4 h at RT, centrifuged for 1 h at 25,000 *g* and re-suspended in 1 mL of PBS buffer. The fluorescence measurements were realized on a spectrofluorimeter SAFAS FLX-Xenius. The fluorescence variations $\Delta F/F$ were calculated for each concentration of Bo-CPN and analyzed using Origin software. The Bo-CPN standard curve was realized by measuring the fluorescence variations before centrifugation.

2.10. Surface plasmon resonance (SPR) experiments

SPR experiments were performed on a Biacore 3000 (Biacore/GE Healthcare Uppsala, Sweden) with a CM5 sensor chip from Biacore/GE Healthcare. A stock solution of N-(2-Aminoethyl) cyclopamine in DMSO (5 mg/mL) was prepared and diluted 1:10 in sodium acetate buffer pH 5.5 for covalent amine-coupling to the CM5 sensor chip. Flow cell 2 (Fc2) was activated with 200 mM *N*-ethyl-*N*-(3-dimethylaminopropyl)-carbodiimide (EDC) and 50 mM *N*-hydroxysuccinimide (NHS), and 100 µL (50 µg) of N-(2-Aminoethyl) cyclopamine were injected at 10 µL/min. The surface was deactivated with ethanolamine. Flow cell 1 (Fc1) was activated and deactivated, and used as a reference flow cell. The surfaces were then washed with three pulses of 50 mM NaOH (15 µL at 5 µL/min).

Experiments on membrane preparations were carried out at 25 °C. 50 µL of membrane preparation from hSmo-expressing yeasts or from yeasts that do not express hSmo, were diluted in HBS-N running buffer (10 mM HEPES (pH 7.4) and 150 mM NaCl, GE Healthcare) at 100 µg/mL and injected over both the reference (Fc1) and the CPN-coupled (Fc2) flow cells at a flow rate of 5 µL/min. The difference between sensorgrams recorded on Fc2 and Fc1 was reported. The sensor chip surface was regenerated twice with 10 µL of 50 mM NaOH, 0.05% SDS at 20 µL/min.

Experiments on purified hSmo were performed at 10 °C. Purification buffer containing 2 mM of surfactant was used as a running buffer, otherwise the specific buffer was stated. 40 µL (80 ng) of purified hSmo were injected on both the reference (Fc1) and the CPN-coupled (Fc2) flow cells at a flow rate of 10 µL/min. The difference between the sensorgrams recorded on Fc2 and Fc1 was reported. The sensor chip surface was regenerated twice with 10 µL of 50 mM NaOH, 0.05% SDS at 20 µL/min.

To determine the stability of hSmo in surfactants over time, purified hSmo fractions were stored at 4 °C and binding was measured 10, 20 and 30 days after purification. The percentages of remaining binding compared to initial binding measured 1 day after purification were calculated from at least two independent experiments.

For thermostability assays, sensorgrams were recorded after incubating purified Smo for 30 min at temperatures ranging from 4 to 70 °C prior to injection on the reference (Fc1) and the CPN-coupled (Fc2) flow cells at a flow rate of 10 µL/min. The percentage of remaining binding after incubation was determined with respect to its own unheated control. In order to determine the inflection point of the melting curves, which was assumed to equal the melting temperature (T_m), a Boltzmann sigmoid equation was fitted to the raw data with the Origin software.

To determine the kinetic constants of the CPN-Smo binding, Fc2, 3 and 4 of a new CM5 sensor chip were coupled with different amounts of N-(2-Aminoethyl) cyclopamine, Fc1 being the reference flow cell. Increasing concentrations of purified Smo were injected onto the four flow cells and the differences between the sensorgrams recorded on each CPN-coupled flow cell and Fc1 were reported and analyzed using BiaEva software.

2.11. Surface Immunofluorescent labeling of Drosophila Schneider 2 cells

S2 cells were cultured and transiently transfected with 0.5 µg of pAc-hSmo-MAP or pAc-hSmo-G435R-MAP as described in [9]. Three days after transfection, the cells were treated or not with Hh-conditioned medium, and cell surface labeling was performed as described in [9] by incubating cells on ice for 1 h with a rabbit polyclonal antibody raised against amino acids 488–787 of human Smo (1:100; N-300; Santa Cruz Biotechnology) in S2 medium containing 10% of fetal bovine serum. After dilution and centrifugation, the cells were incubated on ice for 30 min with a rhodamine fluorescent secondary antibody (Alexa 568 goat anti-rabbit IgG (1:500; Molecular Probes)) in S2 medium containing 10% of fetal bovine serum. After dilution and centrifugation, the cells were re-suspended in phosphate-buffered saline plus 1% formaldehyde. Rhodamine fluorescence was analyzed by fluorescence microscopy and image acquisition was performed using a confocal system (Zeiss LSM510 Meta) with an objective Plan Apochromat ×63/1.4 oil differential interference contrast. Cell fluorescence was analyzed using Image J software.

3. Results

3.1. Expression, optimization and large-scale production of human Smoothened

Human Smoothened was expressed in the yeast *S. cerevisiae* with a multitag affinity purification sequence of 165 amino acids fused at its C-terminus (hSmo-MAP, Fig. 1A) allowing detection of hSmo by western-blotting using antibodies directed against the hemagglutinin (HA) tag, and purification of hSmo using three affinity resins (calmodulin, streptavidin, Ni–NTA), as already described in [8]. Expression of hSmo-MAP was improved and subsequently scaled up. We evaluated the impact of aeration on the hSmo-MAP expression level by growing the yeast cells with different medium/air volume ratios of 1/10, 1/7, 1/5 and 1/4 in a 2-L flask. Yeasts were grown until the OD_{600} was 7 and plasma membranes were prepared. Expression of hSmo-MAP in each culture condition was checked by western-blot

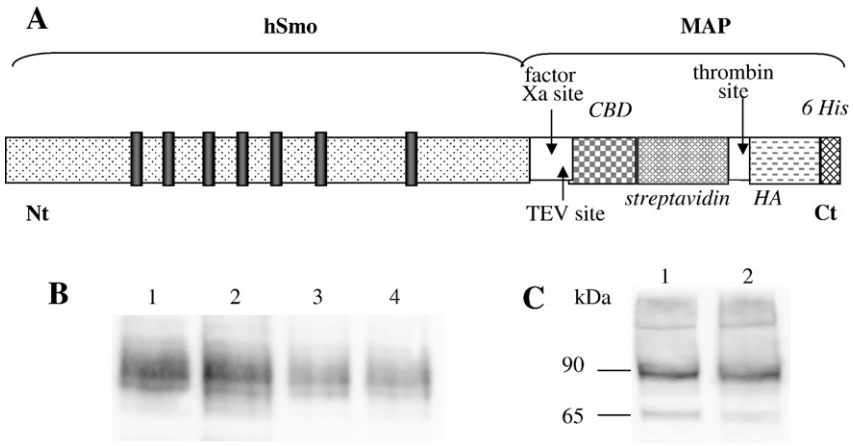


Fig. 1. Expression of human Smoothened in *S. cerevisiae*. A. MAP sequence fused at hSmo C-terminus. The MAP sequence is composed of 165 amino acids: three affinity purification tags (a calmodulin binding domain (CBD), a streptavidin tag and a hexa-histidine tag), the hemagglutin A epitope, and cleavage sites for factor Xa, TEV and thrombin. B. Effect of the yeast culture oxygenation on hSmo-MAP expression level. Western-blot with plasma membrane preparations from *S. cerevisiae* expressing hSmo-MAP cultured with indicated medium/air ratio (v/v): 1/10 (lane 1), 1/7 (lane 2), 1/5 (lane 3) and 1/4 (lane 4) (respectively 200 mL, 300 mL, 400 mL and 500 mL of YEP medium in a 2-L flask). Samples were run on SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-HA antibodies. C. Large-scale production of hSmo-MAP in a 40-L fermentor. Plasma membranes were prepared from hSmo-MAP-expressing yeast grown in 2-L flasks (lane 1) or in 40-L fermentors (lane 2). 40 μg of membranes were loaded on SDS-PAGE, western-blotted and probed with anti-HA antibodies.

analysis (Fig. 1B) which showed a major band around 90 kDa close to the predicted size of hSmo-MAP (100 kDa) and a weaker band around 65 kDa. The highest expression level of hSmo-MAP was obtained in the higher oxygenation conditions (medium/air volume ratios of 1/10 and 1/7). We used the ratio 1/7 for yeast cultures grown in flasks. This experiment allowed air/volume adaptation in the 40-L fermentor for hSmo-MAP scale-up production. Western-blot analysis (Fig. 1C) shows that the quantity of hSmo-MAP expressed at the yeast plasma membrane was comparable in 2-L flask and 40-L fermentor cultures.

To quantify the amounts of hSmo-MAP expressed on the yeast plasma membrane, the production of the MAP tag alone was necessary. Therefore we carried out the expression of the MAP peptide in *E. coli* and the peptide was then purified using calmodulin affinity resin. Purified MAP peptide was used as a standard to quantify hSmo-MAP on western-blots with anti-HA antibodies (Fig. 2). The expression level of hSmo-MAP was estimated at about 50 pmol/mg of membrane protein, which in mass represents 150 μg of hSmo-MAP per liter of yeast culture. However, due to the fact that the transfer of the hydrophobic protein hSmo-MAP was surely less efficient than that of the soluble peptide MAP, this value could have been underestimated.

3.2. Antagonist-binding properties of Smoothened expressed on the yeast plasma membrane

In order to find out if hSmo-MAP was expressed in *S. cerevisiae* in a native conformation, its ability to interact with its antagonist cyclopamine (CPN) was measured.

Binding experiments using a fluorescent derivative of the cyclopamine, the Bodipy-cyclopamine (Bo-CPN) (Fig. 3A) were carried out on membrane prepared from yeast expressing hSmo-MAP and control yeast. The fluorescence variations associated with hSmo-MAP-containing membranes as a function of the concentration of Bo-CPN allowed the calculation of apparent K_D values between 1.7 nM and 5.7 nM, and of the number of active hSmo-MAP per mg of membrane proteins between 20 and 50 pmol depending on membrane preparation (Fig. 3A).

Biacore surface plasmon resonance (SPR) technology forms the basis of a rapid and reliable automated biosensor system and allows real-time detection of binding without labeling [14]. One of the binding partners is immobilized on the sensor chip surface and the other is passed over it in solution. Binding causes a change in the refractive index at the biosensor surface which is transformed into a

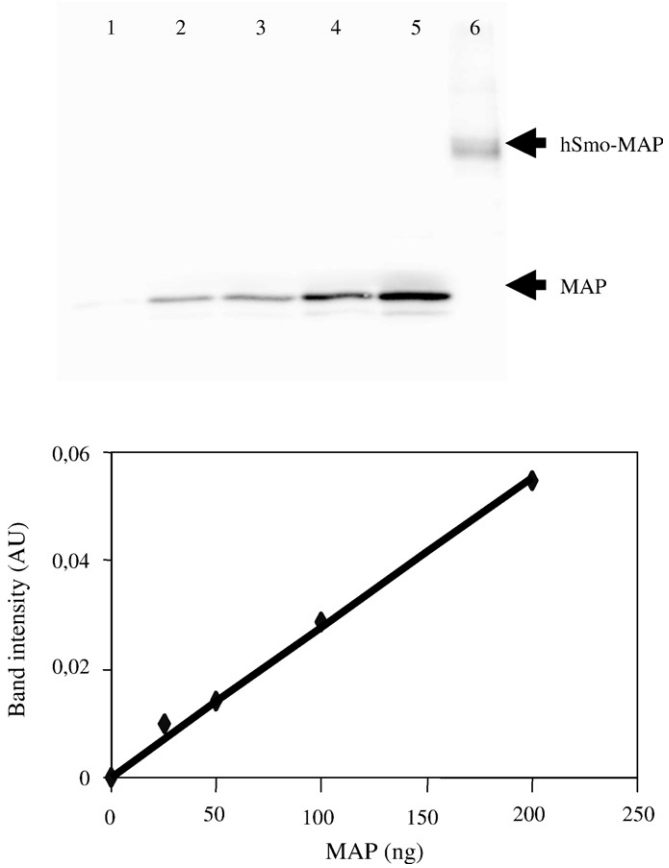


Fig. 2. Quantification of human Smoothened expression using the MAP peptide as a standard. MAP was expressed in *E. coli* and purified on calmodulin resin. Different quantities of purified MAP peptide (lane 1: 10 ng, lane 2: 25 ng, lane 3: 50 ng, lane 4: 100 ng, lane 5: 200 ng) and 5 μg of membrane fraction from yeast expressing hSmo-MAP (lane 6) were loaded on SDS-PAGE, transferred to a membrane and probed with anti-HA antibodies. The intensities of the bands corresponding to the MAP peptide were estimated by densitometry using the ImageJ software and used to draw the MAP standard curve. The intensity of hSmo-MAP band was reported on this standard curve to estimate the quantity of hSmo-MAP produced.

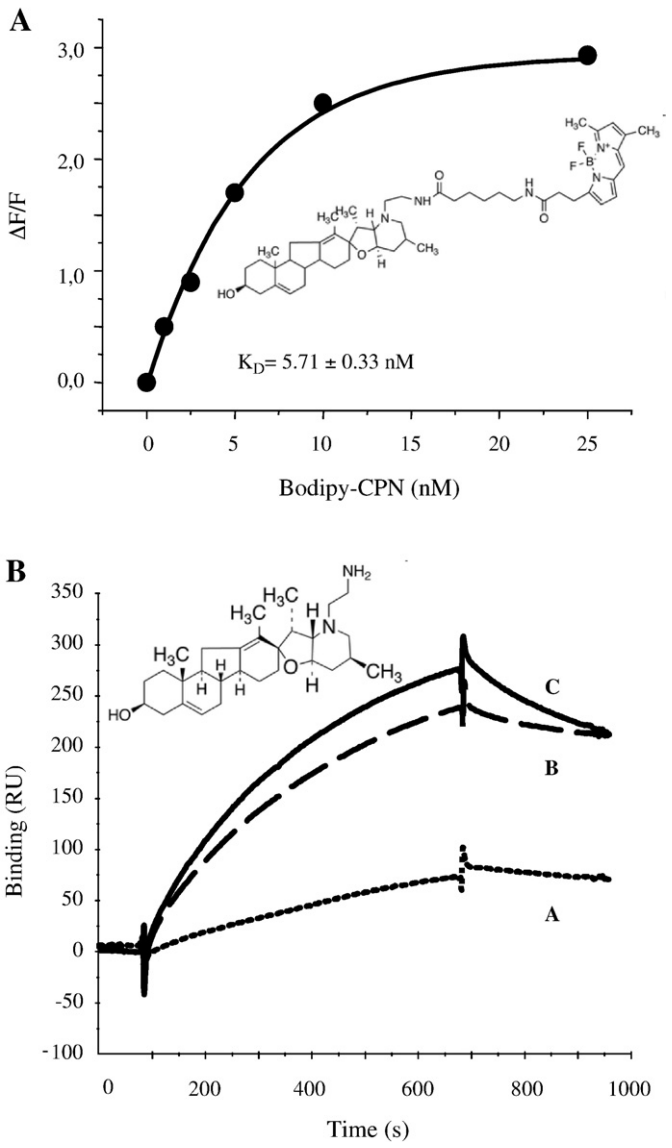


Fig. 3. Smoothened expressed on the yeast plasma membrane bound to its antagonist, cyclopamine. A. Fluorescent-cyclopamine-binding assay. 100 µg of membrane preparation from yeast expressing hSmo-MAP were incubated with increasing concentrations of Bodipy-cyclopamine (Bo-CPN, structure shown), centrifuged, re-resuspended in PBS, and fluorescence emission was recorded at 520 nm (ex. 490 nm). Fluorescence variations ($\Delta F/F$) were plotted as a function of the Bo-CPN concentration. The apparent K_D and the number of active hSmo-MAP were calculated using Origin software. B. Surface plasmon resonance experiments. An amine-derivative of the cyclopamine (N-(2-Aminoethyl) cyclopamine, structure shown) was covalently coupled to the flow cell Fc2 of a Biacore CM5 sensor chip. The sensorgrams presented are the difference between signals recorded on the CPN-coupled flow cell Fc2 and on the reference flow cell. 50 µL of membrane fraction (100 µg/mL) from control yeasts that do not express hSmo (sensorgram A), from 2 L flask-cultured yeast expressing hSmo-MAP (sensorgram B), and from fermentor-cultured yeast expressing hSmo-MAP (sensorgram C), were injected over both Fc1 and Fc2.

measurable signal by a light-sensitive detector and expressed as resonance units (RU) on the recorded sensorgram. An amine-derivative of CPN, N-(2-aminoethyl) cyclopamine (Fig. 3B) was covalently immobilized by amine-coupling on the flow cell 2 (Fc2) of a CM5 sensor chip producing a change of the refractive index of the Fc2 surface of 3600 RU. Membrane preparations from yeast that do or do not express hSmo-MAP were injected on both the reference flow cell (Fc1), and the measure flow cell (Fc2) coupled to CPN (Fig. 3B). Membrane preparations from yeasts that do not express hSmo-MAP presented a binding level of 64 RU corresponding to non-specific binding (Fig. 3B sensorgram A). This non-specific binding was expected due to the high hydrophobicity of the CPN. The signal recorded with membrane preparations from yeast expressing hSmo-MAP reached 211 RU (Fig. 3B sensorgram B), indicating that hSmo present in the yeast membrane interacted with the CPN. Membrane fractions obtained from yeast expressing hSmo-MAP cultured in the 40-L fermentor exhibited a comparable binding level (214 RU) (Fig. 3B sensorgram C).

Therefore, hSmo-MAP expressed on the yeast plasma membrane is able to interact with its antagonist, meaning that we succeeded in large-scale production of human Smo in yeast in a native conformation.

3.3. Antagonist-binding properties of purified Smoothened

S. cerevisiae expressing hSmo-MAP was grown using a medium/air volume ratio of 1/7, at 18 °C until the OD_{600} was 7. Then the plasma membrane fraction was prepared and the hSmo-MAP receptor was solubilized with DDM, which is one of the most efficient detergents for hSmo solubilization [8]. Around 60% of hSmo-MAP was solubilized. The hSmo-MAP was fully purified using a calmodulin column followed by an Ni–NTA column. The silver stained SDS-PAGE presented in Fig. 4 clearly showed the presence of two bands between 90 and 95 kDa, which were also visible on some western-blots. These two bands could correspond to non-glycosylated and weakly glycosylated forms of hSmo-MAP, respectively.

In order to compare the stability of hSmo-MAP in DDM and in the fluorinated surfactants (FSs) C_6F_{13}TAC, or C_8F_{17}TAC [12,13], DDM was exchanged with FSs on the calmodulin resin during the washing steps. The resin was incubated with 5 volumes of buffer containing 2 mM of C_6F_{13}TAC or C_8F_{17}TAC, slowly centrifuged and the supernatant removed. This step was repeated 3 times before elution. hSmo-MAP was eluted from the column by using a buffer containing 2 mM of surfactant (DDM, C_6F_{13}TAC or C_8F_{17}TAC). This extensive wash using batched resin is one of the most efficient techniques for exchanging detergents. Theoretical DDM concentration after exchange was 10 times bellow its CMC, suggesting that very few DDM molecules were present in the hSmo/FS micelles. The western-blot analysis (Fig. 5A) showed a band around 90 kDa of comparable intensity for hSmo-MAP purified in DDM, C_6F_{13}TAC or C_8F_{17}TAC, suggesting that FSs maintained hSmo in a soluble state as well as DDM.

One day after purification, the ability of purified hSmo-MAP to bind the CPN was investigated. A quantity of hSmo-MAP purified in DDM, C_6F_{13}TAC or C_8F_{17}TAC was injected onto both the reference (Fc1) and the CPN-coupled (Fc2) flow cells of the Biacore sensor chip, and the difference between sensorgrams recorded on Fc2 and Fc1 was reported (Fig. 5B). These results indicate that hSmo-MAP was able to interact with its antagonist after purification in DDM, but also in FSs. The lower values of RU obtained with hSmo-MAP in FSs solution could be due to a difference in refractive index between DDM and FSs, and/

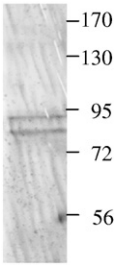
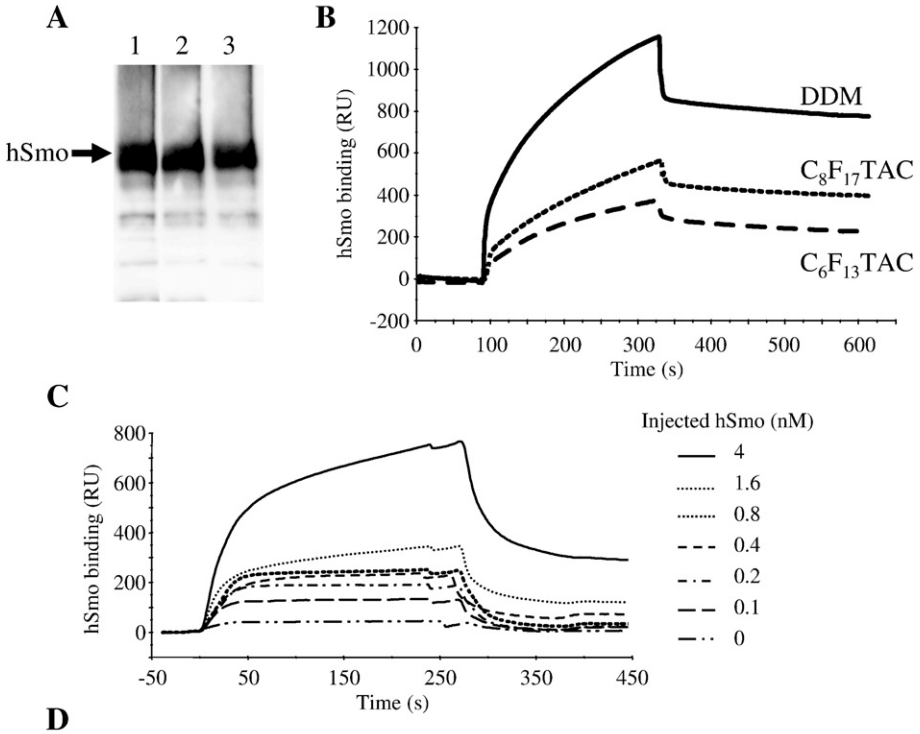


Fig. 4. Silver stained SDS-PAGE of purified hSmo-MAP. hSmo-MAP was purified on calmoduline resin followed by Ni–NTA resin. Purified hSmo-MAP was loaded on an 8% SDS-PAGE and silver stained.

or to the difference of hydrophilicity of the surfactant polymer heads. The fact that the specific refractive index increments (dñ/dc) of the surfactants used to purify hSmo were different (0.14 for DDM and 0.08 for FSs) and that these surfactants may have an effect on the binding response recorded by SPR, made it difficult to compare between the signals recorded in the presence of the different surfactants. However, in order to clarify this point, different amounts of cyclopamine were immobilized on Fc2 (11825 RU), Fc3 (8345 RU) and Fc4 (2971 RU) of a new CM5 sensor chip, the Fc1 being the reference flow cell. Using buffer containing 2 mM DDM as running buffer, we observed that the injection of buffer containing 2 mM of C_8F_{17}TAC had a different effect depending on the amount of CPN-coupled: it was positive in the absence of CPN (on Fc1) and negative in the presence of CPN, the negative response being proportional to the amount of coupled-CPN (Fig. S1A). This result suggests that each surfactant interacts with the CPN coupled on the flow cells and has a different effect on the SPR response. To further consider surfactant effects a second experiment was carried out in which hSmo purified in DDM, C_6F_{13}TAC, C_8F_{17}TAC was diluted ten times in the DDM-containing running buffer and injected on Fc1 and Fc2. The sensorgrams resulting from the difference between signals recorded on Fc2 and Fc1 were very different, suggesting that the surfactant surrounding hSmo had an effect on the CPN and the SPR response. However, after dissociation time, recorded sensorgrams showed similar RU values suggesting a recovery of DDM signals when FS-purified Smo was diluted in DDM-containing buffer (Fig. S1B).

As a direct comparison of binding responses of hSmo purified in the different surfactants was not possible, kinetic constants of hSmo/CPN binding were determined. Increasing concentrations of hSmo purified in DDM or FSs were injected on the flow cells coupled to different quantities of CPN and the sensorgrams recorded from the difference between CPN-coupled Fc and the reference Fc1 were superposed and analyzed using BiaEva software (Fig. 5C and D). The affinity constants of hSmo purified in DDM or C_8F_{17}TAC for its antagonist were of the same order (5.8 nM and 2 nM respectively). We also calculated from the R_{max} values that the same amounts of hSmo-MAP purified in DDM or in C_8F_{17}TAC (0.9 nmol) interacted with the CPN coupled to the sensor chip per mg of protein injected. These values, which represent about 10% of the injected samples, were calculated considering that the totality of the injected sample could interact with the cyclopamine coupled to the sensor chip. However, since the experiments were carried out with a continuous flow, it was impossible to know the volume of sample that was really in contact with the cyclopamine. Unfortunately, the technique used did not permit calculation of the number of active receptors, but just allowed us to compare the amount of hSmo that interacted with cyclopamine



D

	K_D (M)	K_A (M^{-1})	k_a ($M^{-1}.s^{-1}$)	k_d (s^{-1})	Bound Smo (nmole/mg)
hSmo in DDM	$5{,}8.10^{-9}$	$1{,}7.10^{8}$	$7{,}3.10^{5}$	$4{,}3.10^{-3}$	0.9
hSmo in C_8F_{17}TAC	$2{,}0.10^{-9}$	$4{,}9.10^{8}$	$8{,}0.10^{6}$	$1{,}6.10^{-2}$	0.9

Fig. 5. DDM and fluorinated surfactants preserved Smoothened integrity after purification. A. hSmo-MAP purified in DDM (lane 1), C_8F_{17}TAC (lane 2) or C_6F_{13}TAC (lane 3) was loaded on SDS-PAGE and western-blotted with anti-HA antibodies. B. SPR measurements showing that purified hSmo-MAP-bound to its antagonist, cyclopamine. 40 µL of purified hSmo-MAP in DDM, in C_8F_{17}TAC or in C_6F_{13}TAC were injected over both Fc1 (reference flow cell) and Fc2 (on which CPN was immobilized by amine-coupling). The SPR responses reported in resonance units (RU) are the differences between sensorgrams recorded on Fc2 and Fc1, and correspond to the specific binding of hSmo-MAP on immobilized CPN. C. SPR responses of increasing concentrations of hSmo-MAP purified in DDM. 50 µL of increasing concentrations of hSmo-MAP purified in DDM were injected over both reference Fc1 and CPN-coupled Fc2. The differences between sensorgrams recorded on Fc2 and Fc1 at each hSmo-MAP concentration are presented. D. CPN-binding kinetic constants for hSmo-MAP purified in DDM and in C_8F_{17}TAC. Sensorgrams recorded at increasing concentrations of hSmo-MAP purified in DDM and in C_8F_{17}TAC were analyzed using BiaEva software and the calculated kinetic constants were reported.

in each injected sample. Taking into account this point and the fact that the samples injected onto the sensor chip were purified on the calmodulin column and only gave a purity yield of about 60 to 70%, the amount of active hSmo after purification in DDM and in C_8F_{17}TAC should be clearly higher than 10%.

3.4. Smoothened stability in solution

To compare the stability of purified detergent solubilized hSmo receptor, we developed two stability assays based on SPR.

Time-dependent stability was determined from SPR binding assays 10, 20 and 30 days after purification in the different surfactants with hSmo-MAP samples kept at 4 °C. Results showed that 1 month after purification, 45% of the initial hSmo still bound to the CPN in DDM, and that this value was slightly higher in FSs (55% and 50% for C_6F_{13}TAC and C_8F_{17}TAC respectively) (Fig. 6A). Our results indicated that FSs were at least as efficient as DDM in maintaining hSmo in solution and in preserving hSmo–cyclopamine binding over time after purification.

The thermostability of hSmo-MAP purified in DDM and in FSs was determined from SPR binding assays on hSmo-MAP incubated for 30 min at increasing temperatures ranging from 10 to 70 °C prior to injection on the reference (Fc1) and the CPN-coupled (Fc2) flow cells of the Biacore sensor chip. The difference between sensorgrams recorded on Fc2 and Fc1 was reported, giving the binding level for each sample. The maximal level of binding was determined on control samples kept on ice. The percentage of remaining native receptors was determined by comparing the amount of binding (in RU) after heating with its unheated control (Fig. 6B). The apparent T_m, defined as the temperature at which 50% of the binding activity remained after a 30-min incubation was calculated and reported. The hSmo-MAP in DDM showed apparent T_m values of 40.7 ± 2.3 °C. The apparent T_m in C_6F_{13}TAC was lower (33.6 ± 2.4 °C) than that in DDM, whereas the T_m was shifted to 45.1 ± 0.8 °C when hSmo-MAP was purified in C_8F_{17}TAC.

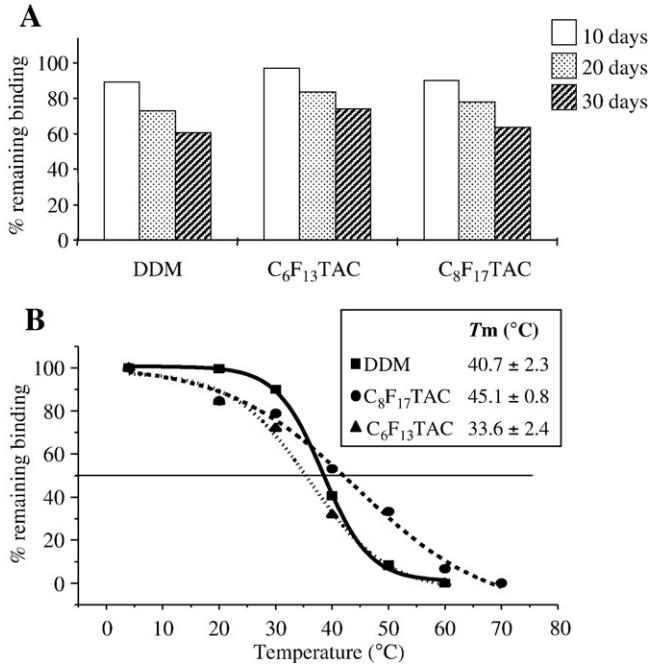


Fig. 6. Smoothened stability in solution. A. Time-dependent stability of hSmo-MAP in different surfactants. SPR responses were recorded 10, 20 and 30 days after purification, samples being kept at 4 °C. Remaining hSmo-MAP binding is represented as percentages of the initial binding recorded 1 day after purification. B. Thermostability of purified hSmo-MAP. Samples of hSmo-MAP purified in DDM, C_6F_{13}TAC, or C_8F_{17}TAC were heated for 30 min at increasing temperatures and injected on the Biacore sensor chip to determine remaining hSmo-MAP binding activity, which was normalized against the unheated control in each surfactant (100%). Data points are from duplicate measurements and are representative of at least two independent experiments. Apparent T_m values determined from the curves by nonlinear regression are indicated.

Samples of hSmo-MAP purified in DDM, C_8F_{17}TAC and C_6F_{13}TAC heated at increasing temperatures were mixed with loading buffer, loaded on SDS-PAGE and western-blotted with anti-HA antibodies (Fig. S2A). The western-blots showed that heating Smo did not induce the appearance of new bands, suggesting that the loss of antagonist-binding was not due to proteolysis. The band at 90 kDa was quantified using Image J software and the percentage of remaining Smo at 90 kDa was plotted as a function of the temperature using Origin software (Fig. S2B). For hSmo-MAP purified in DDM, the 90-kDa band began to decrease at 40 °C, the T_m value being around 60 °C. For hSmo-MAP in C_8F_{17}TAC, the 90-kDa band decreased by 20% at 20 °C, was stable until 40 °C, and then decreased as for Smo in DDM with a T_m value around 60 °C. These data suggest that the 20% decrease observed at 20 °C in the antagonist-binding activity could be due to a precipitation of Smo: it is possible that some of the Smo proteins did not get enough C_8F_{17}TAC molecules to stabilize them. This could also explain why the thermal transition was less cooperative in C_8F_{17}TAC than in DDM. For hSmo-MAP purified in C_6F_{13}TAC, the T_m value calculated from the intensity of the 90-kDa band was around 35 °C, which was in good agreement with the T_m value calculated from the antagonist-binding activity. These data suggest that the loss of antagonist-binding after heating could be due to a progressive unfolding followed by the precipitation of Smo in DDM and in C_8F_{17}TAC, and to a more rapid precipitation in C_6F_{13}TAC.

SPR and western-blot analyses clearly indicated that hSmo-MAP was at least as stable in the FS C_8F_{17}TAC as in the classical detergent DDM, and less stable in C_6F_{13}TAC. The 12 °C shift in thermostability between the two FSs could be due to the length of the fluorocarbon chain, which is longer for the C_8F_{17}TAC and would better protect membrane proteins.

3.5. Characterization of the Smoothened mutant G435R

This mutant is the result of an error introduced during the PCR carried out to modify the 5′ and 3′ extremities of hSmo cDNA for cloning into the YEpPMA-MAP expression vector. The guanine in position 1303 was substituted for adenine giving an arginine instead of a glycine in position 435 of the third intracellular loop of hSmo.

Yeasts expressing hSmo-MAP or hSmoG435R-MAP were grown in the same conditions, membrane proteins were prepared and loaded on SDS-PAGE for western-blotting with anti-HA antibodies (Fig. 7A). The first observation was that, in the line where membranes prepared from yeast expressing hSmoG435R-MAP were loaded, anti-HA antibodies did not detect a band at 90 kDa as for hSmo-MAP but only the band at 65 kDa. The second observation was that the densitometric measure of the 65-kDa band corresponding to hSmoG435R-MAP was 2.5 times that of the 90-kDa corresponding to hSmo-MAP. Using the purified MAP peptide as a standard, we could estimate that 120 pmol of hSmoG435R-MAP was produced per mg of membrane proteins, which corresponded to 360 μg/L of yeast culture. The western-blot presented in Fig. 7A also shows that the 90-kDa band disappeared after heating at 95 °C in the loading buffer whereas the 65-kDa band was not affected by heating. This suggests that the 90-kDa protein and the 65-kDa protein have differences in their folding. Membrane preparations from yeast expressing hSmo-MAP and yeast expressing hSmo-G435R-MAP were loaded on a native gel and the western-blot revealed with anti-HA antibodies showed that both proteins migrated identically on this native gel, with a stronger signal for hSmoG435R-MAP than for hSmo-MAP (Fig. 7B). Moreover, we carried out digestions with increasing amounts of trypsine on purified hSmo-MAP and hSmoG435R-MAP. The western-blot analysis showed that trypsine did not enhance the 65-kDa band for hSmo-MAP but produced only one C-terminal fragment around 35 kDa both with

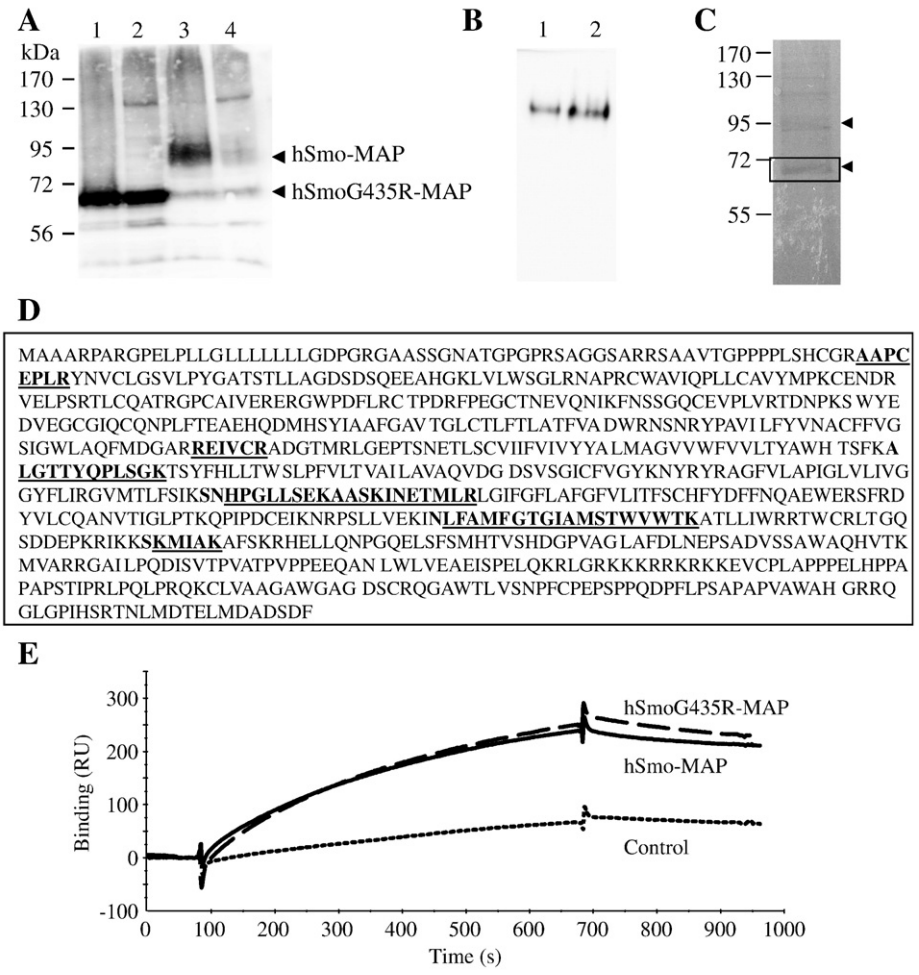


Fig. 7. Expression of the G435R-mutant Smoothened. A. Plasma membranes prepared from yeasts expressing hSmoG435R-MAP (lanes 1 and 2) or hSmo-MAP (lanes 3 and 4). 80 μg of membranes were heated for 2 min at 95 °C in loading buffer (lanes 2 and 4) or not (lanes 1 and 3) and loaded on SDS-PAGE, western-blotted and probed with anti-HA antibodies. Arrows indicate the 90-kDa and the 65-kDa bands. B. Membranes prepared from yeasts expressing hSmo-MAP (lane 1) or hSmoG435R-MAP (lane 2) were loaded on a native gel, western-blotted and probed with anti-HA antibodies. C. Coomassie blue stained gel of purified hSmoG435R-MAP. hSmoG435R-MAP was purified in DDM on Ni/NTA and streptavidin columns successively, loaded on a SDS-PAGE and stained by Coomassie blue. The 65-kDa band indicated by an arrow was cut for mass spectrometry analysis. D. Amino-acid sequence of human Smoothened (*Homo sapiens*) (from NP 005622; NCBI, http://www.ncbi.nlm.nih.gov.gate1.inist.fr). Peptides detected by mass spectrometry from the cut 65-kDa band are in bold and underlined. They occur in the N-terminal domain, the first extracellular loop, the 2nd intracellular loop, the 3rd intracellular loop, the 3rd extracellular loop, the 7th TMS and the C-terminal domain of the human receptor Smoothened. E. CPN-binding of membrane-bound G435R-mutant Smoothened measured by SPR. 50 μL of membranes (100 μg/mL) prepared from control yeasts that do not express hSmo, from yeasts expressing hSmo-MAP, and from yeasts expressing hSmoG435R-MAP were injected over both the reference flow cell Fc1 and the CPN-coupled flow cell Fc2 of a CM5 sensor chip. The sensorgrams presented show the difference between signals recorded on Fc2 and Fc1.

wild-type and mutant Smo (Fig. S3). This result suggests that the 65-kDa band observed on hSmo-MAP western-blot does not correspond to a proteolysed form of Smo. Taken together, these observations suggested that the mutant receptor hSmo-G435R-MAP could have a more stable conformation making it less denatured by SDS which could explain the faster running on SDS-PAGE.

However, in order to verify that the special migration of hSmoG435R-MAP was not due to a truncated protein, hSmoG435R-MAP was purified in DDM on an Ni/NTA column followed by a streptavidin column. The 65-kDa band observed on the Coomassie blue stained SDS-PAGE (Fig. 7C) was cut and analyzed by MALDI-TOF–TOF mass spectrometry. Several peptides were identified between the amino acids number 66 and 575 (Fig. 7D). Moreover, the 65-kDa band being detected by anti-HA antibodies on western-blots indicated that the MAP sequence was present at the C-terminal extremity of mutant Smo. The 65 N-terminus residues could have been truncated resulting in 8 kDa deletion but this cannot explain the running at 65 kDa of the protein. Therefore, the data suggested that the 65-kDa band would correspond to the full-length MAP-tagged hSmoG435R protein and would result from atypical running behavior.

3.5.1. Antagonist-binding properties of the Smoothened mutant G435R

We tested the ability of membrane-bound hSmoG435R-MAP to bind to the Smo antagonist cyclopamine by SPR studies. Membrane preparations from yeast either expressing hSmo-MAP or hSmoG435R-MAP, or from a control yeast strain that did not express hSmo, were injected onto the reference flow cell (Fc1) and the measure flow cell (Fc2) coupled to the CPN. We observed that membranes prepared from yeast expressing hSmoG435R-MAP gave a comparable binding response to membranes prepared from yeast expressing hSmo-MAP (Fig. 7E), indicating that the mutation G435R did not prevent antagonist-binding. However, we expected a higher binding activity with hSmoG435R-MAP-bound membranes since western-blotting suggested that the mutant receptor was more strongly expressed than the wild-type.

3.5.2. Thermostability of the Smoothened mutant G435R in solution

We also studied the thermostability of hSmoG435R-MAP after purification in different surfactants, as was done for the wild-type receptor (Fig. 8). T_m values obtained for hSmoG435R-MAP in DDM were relatively close to that of hSmo-MAP (38 ± 1.3 °C and 40.7 ± 2.3 °C respectively, Table 1). In C_8F_{17}TAC, however, we obtained T_m

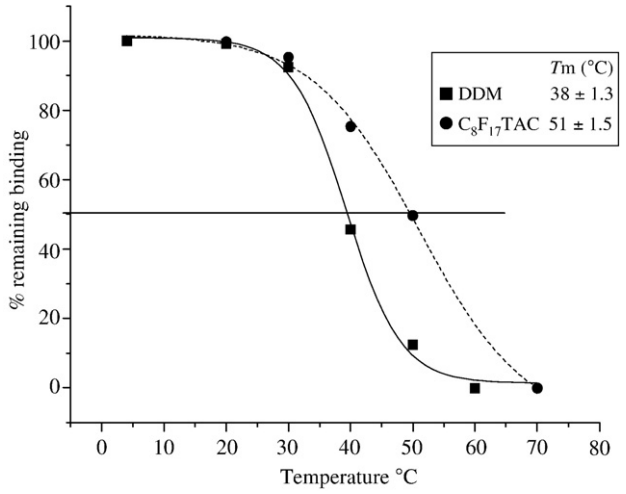


Fig. 8. Thermostability of the purified G435R-mutant Smoothened. Samples of hSmoG435R-MAP purified in DDM or C_8F_{17}TAC were heated for 30 min at increasing temperatures and injected on the Biacore sensor chip to determine remaining binding activity which was normalized against the unheated control in each surfactant (100%). Data points are from duplicate measurements and are representative of at least two independent experiments. Apparent T_m values were determined from the curves by nonlinear regression.

values of about 51 °C for hSmoG435R-MAP, which was about 6 °C higher than T_m values obtained with hSmo-MAP in the same surfactant (Table 1).

3.5.3. Activation of the Smoothened mutant G435R by Hedgehog

To find out if the mutation G435R had an effect on the localization of hSmo in response to Hedgehog, we performed surface immunofluorescence labeling on *Drosophila* Schneider 2 (S2) cells transiently transfected with pAc-hSmo-MAP or pAc-hSmoG435R-MAP. The cells were incubated with a rhodamine fluorescent polyclonal antibody raised against the 300 last amino acids of hSmo that recognized the 3rd extracellular loop of hSmo, and analyzed by confocal fluorescent microscopy. In agreement with our previously published results [9], hSmo-MAP expressed in S2 cells was present on the plasma membrane in the absence of Hh, and treatment of cells with Hh enriched hSmo-MAP at the cell surface. Cell surface immunofluorescent labeling on hSmoG435R-MAP gave comparable results (Fig. 9A). The amount of hSmoG435R-MAP present on the plasma membrane before Hh treatment was slightly lower than that for the wild-type, and incubation of the cells with Hh increased the amount of hSmoG435R-MAP on the plasma membrane 1.8 times instead of 1.5 times for the wild-type (Fig. 9B).

4. Discussion

This study reports the large-scale expression, the purification, and the stabilization of the human GPCR Smoothened.

Table 1
Tm values obtained for Smoothened wild type and G435R purified in DDM and C_8F_{17}TAC. hSmo-MAP and hSmoG435R-MAP purified in DDM or C_8F_{17}TAC were heated 30 min at increasing temperatures and injected on the Biacore sensor chip to determine remaining CPN binding activity which was normalized against the unheated control in each surfactant (100%). Apparent *Tm* values were determined from thermostability curves by nonlinear regression using Origin software.

	Tm (°C)	
	hSmo-MAP	hSmoG435R-MAP
DDM	40.7 ± 2.3	38 ± 1.3
C_8F_{17}TAC	45.1 ± 0.8	51 ± 1.5

hSmo was expressed in the yeast *S. cerevisiae* with a multitag affinity purification sequence of 165 amino acids fused at its C-terminus. We showed in a previous study that hSmo-MAP expressed in *Drosophila* Schneider 2 cells was inhibited by Patched in the absence of Hh, stabilized at the surface of *Drosophila* cells in response to Hh, and bound its antagonist cyclopamine as wild-type Smo [9], meaning that the presence of the MAP sequence at the C-terminal extremity of hSmo did not affect its activity. We estimated the amount of MAP-tagged hSmo expressed on the plasma membrane of the yeast *S. cerevisiae* to be around 150 μg of hSmo per L of yeast culture, 20 to 50 pmol/mg of membrane proteins (meaning 50 to 100% of expressed hSmo) being able to bind cyclopamine. The latter is a steroidal alkaloid extracted from the lily corn plant *Veratrum californicum* that binds to the heptahelical bundle of mammalian Smoothened, inhibiting the Hedgehog pathway [15], and does not recognize *Drosophila* Smoothened. The expression level obtained is not very high, but the 40-L fermentor production should be sufficient to perform biochemical and structural studies.

Surface plasmon resonance experiments were developed to measure the interaction of hSmo with its antagonist, the cyclopamine, covalently coupled to the sensor chip flow cells. The binding of membrane-bound hSmo-MAP to the cyclopamine indicated that hSmo-MAP was expressed in a native conformational state in *S. cerevisiae* grown in 2-L flasks as well as in a 40-L fermentor. After solubilization of hSmo-MAP from the membrane with the non-ionic detergent DDM, we showed that hSmo-MAP could be purified in DDM but also in fluorinated surfactants using the affinity tags present in the MAP. Solubilization of membrane proteins often results in destabilization, unfolding and subsequent aggregation. To investigate whether purified hSmo-MAP kept its integrity and antagonist-binding ability in surfactant solution, we performed SPR experiments and checked time-dependent stability and thermostability. Biacore SPR experiments showed that DDM preserved the ability of hSmo-MAP to interact with its antagonist in solution at 4 °C for at least 1 month after purification. Thermostability assays allowed the melting temperature (T_m) of hSmo-MAP in DDM to be estimated at about 40 °C, indicating that hSmo is a relatively stable GPCR.

Because fluorinated surfactants (FSs) have been shown to be less aggressive towards certain membrane proteins than classical detergents [16,17], and because we have recently shown that these FSs preserved the integrity and stability of human Patched, the other membrane protein of the Hedgehog pathway [18], we studied the ability of FSs to maintain hSmo-MAP integrity in solution. Our results showed that the amount of hSmo-MAP obtained after purification was comparable in DDM and in FSs. However, the cyclopamine-binding signals observed by SPR were lower in FSs than in DDM. It has to be recalled that FSs possess the same general structure as classical detergents, *i.e.* a hydrophilic head group and a hydrophobic tail, but the latter, rather than being a hydrogenated aliphatic chain, is a fluorocarbon chain [12,13]. The polymer head of FSs is much more hydrophilic than that of DDM. This could induce a repulsion effect between hSmo/FS micelles and the highly hydrophobic cyclopamine bound to the flow cell surface, which could be compared to the brush effect observed with the stealth liposomes [19]. This property could explain the lower binding of hSmo-MAP with the cyclopamine in the presence of FSs. Kinetic analysis allowed us to calculate that affinity for cyclopamine was of the same order in DDM or in C_8F_{17}TAC and comparable to the values obtained for Smo in membrane preparations, and that the same amount of active Smo was present in DDM or in C_8F_{17}TAC solution. The amount of remaining binding activity 1 month after purification in FSs suggested that FSs were at least as efficient as the classical detergent DDM in maintaining hSmo-MAP in solution and in preserving hSmo–cyclopamine binding. It is noticeable that the apparent T_m of hSmo-MAP in C_8F_{17}TAC was 4 to 5 °C higher than that of hSmo-MAP in DDM. This shift in T_m could be important for crystallization, as reported for the β1 adrenergic receptor [20] and the adenosine receptor [21].

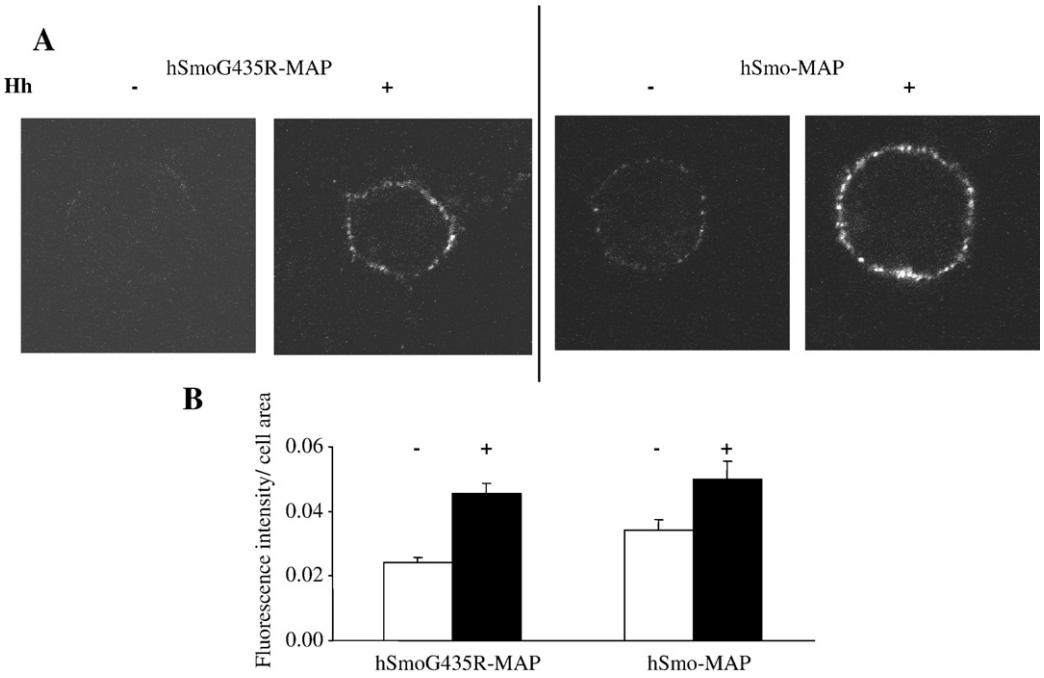


Fig. 9. Activation of the G435R-mutant Smoothened by Hedgehog in the *Drosophila* Schneider 2 cells. *Drosophila* S2 cells transfected with 0.5 μg of pAc-hSmo-MAP or pAc-hSmoG435R-MAP, treated (+) or not (−) with Hh-conditioned medium, were incubated on ice for 1 h with rabbit anti-hSmo antibody and 30 min with a rhodamine fluorescent secondary antibody. A. The rhodamine fluorescence corresponding to the hSmo amount at the cell surface was analyzed by confocal microscopy with an objective ×63. A. B. The intensity of rhodamine fluorescence of ~20 hSmo-MAP or hSmoG435R-MAP-expressing cells, normalized by cell area, was quantified using Image J software (arbitrary units; AU) and reported in the histogram.

The β1AR atomic structure was obtained with a thermostabilized mutant [22], and the use of a mutant that improves thermostabilization and/or conformational homogeneity of the receptor seems to be a good strategy for the structure determination of GPCRs [21]. One of the hSmo mutant clones generated by PCR, in which a neutral glycine residue was replaced by a positively charged arginine residue, showed a higher expression level than the wild-type hSmo in the yeast *S. cerevisiae*. Moreover, this mutation was located in the third intracellular loop of hSmo which had been shown to be very important for Smoothened activity [23]. Therefore, expecting that this mutation could improve hSmo stability, we decided to further characterize this mutant and to study its thermostability. Surprisingly, this mutant protein ran only under the 65-kDa form instead of the expected 90-kDa major form and the 65-kDa weak band obtained with the wild-type receptor on SDS-PAGE. However, mass spectrometry analysis and a running profile on native gel strongly suggested that hSmoG435R-MAP was expressed in *S. cerevisiae* along its full-length. The fact that the 65-kDa band did not disappear after heating in loading buffer, contrary to the 90-kDa band, suggested that the running behavior of hSmoG435R-MAP could be due to a more stable conformation, less denatured by SDS-containing loading buffer. It must also be mentioned that membrane proteins frequently run at lower apparent molecular weight than their theoretical value due to their high hydrophobicity [24,25]. Quantification of hSmoG435R-MAP on western-blot suggested that the level of expression of hSmoG435R-MAP was 2.5 times higher than that of hSmo-MAP; however, the cyclopamine-binding properties were comparable to those of hSmo-MAP. If the denatured state of hSmoG435R-MAP is different from that of hSmo-MAP, we cannot exclude a higher reactivity of anti-HA antibodies with the mutant protein than with the wild-type, which could explain the higher level of expression estimated. However, if G435R mutation had an effect on Smoothened conformation, surface immunofluorescent labeling showed that this mutation did not affect Smoothened enrichment on the *Drosophila* cell plasma membrane in response to Hh activation, which is in agreement with our previous data [9] and other studies [26].

Thermostability of hSmoG435R-MAP in the classical detergent dodecyl maltoside is comparable to that of hSmo-MAP with a T_m of about 40 °C. However, in the fluorinated surfactant C_8F_{17}TAC, the thermostability of the mutant is higher than that of wild-type receptor in the same surfactant, and reached 51 °C. These results showed that the FS C_8F_{17}TAC increased the thermostability of hSmoG435R-MAP by about 13 °C compared to dodecyl maltoside, and confirmed the stabilizing effect of the FS C_8F_{17}TAC on hSmo. The mutation G435R neither affected antagonist-binding nor the response of hSmo to Hh activation, but strongly stabilized the receptor in the fluorinated surfactant C_8F_{17}TAC solution. There is a synergistic effect of the mutation G435R and the FS C_8F_{17}TAC on the stability of Smoothened in solution that could be very helpful for crystallization and structural characterization [21,27].

In summary, this is the first study reporting conditions in which the human receptor Smoothened is in a native stable conformational state and interacts with its antagonist cyclopamine in solution after purification. The apparent melting temperatures obtained for hSmo in the classical detergent DDM and in the fluorinated surfactant C_8F_{17}TAC suggest that Smo is relatively stable in solution. Moreover, the results obtained with a mutant form of Smoothened presenting the replacement of a glycine by an arginine in the third intracellular loop showed that this mutation strongly stabilized the receptor in the fluorinated surfactant C_8F_{17}TAC solution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2010.02.015.

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