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Functional studies of membrane-bound and purified human Hedgehog receptor Patched expressed in yeast

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ABSTRACT

The Sonic Hedgehog (Shh) signalling pathway plays an important role both in embryonic development and in adult stem cell function. Inappropriate regulation of this pathway is often due to dysfunction between two membrane receptors Patched (Ptc) and Smoothened (Smo), which lead to birth defects, cancer or neurodegenerative diseases. However, little is known about Ptc, the receptor of the Shh protein, and the way Ptc regulates Smo, the receptor responsible for the transduction of the signal. To develop structure–function studies of these receptors, we expressed human Ptc (hPtc) in the yeast *Saccharomyces cerevisiae*. We demonstrated that hPtc expressed in a yeast membrane fraction is able to interact with its purified ligand Shh, indicating that hPtc is produced in yeast in its native conformational state. Using Surface Plasmon Resonance technology, we showed that fluorinated surfactants preserve the ability of hPtc to interact with its ligand after purification. This is the first report on the heterologous expression and the purification of a native and stable conformation of the human receptor Ptc. This work will allow the scale-up of hPtc production enabling its biochemical characterization, allowing the development of new therapeutic approaches against diseases induced by Shh signalling dysfunction.

1. Introduction

Hedgehog (Hh) proteins constitute a family of secreted signalling molecules that govern patterning and morphogenesis in most multicellular organisms [1]. In adult animals, the Hh pathway regulates tissue stem cells required for organ repair and maintenance [2]. Sonic Hedgehog (Shh), the most studied of the three vertebrate homologs of *Drosophila* Hedgehog, is a secreted protein that acts on target cells to increase transcription of several genes, including members of the *Wnt* and transforming growth factor β families, and of its own receptor Patched (Ptc). Cellular responses to the secreted morphogen Shh are mediated by two integral membrane proteins, Ptc and Smoothened (Smo), which were first identified by genetic screens in *Drosophila* [3,4]. In the absence of Shh, Ptc represses the G protein coupled receptor Smo, which is a positive regulator of the pathway. Once the Shh protein binds to Ptc, the inhibition of Smo is released and this allows transduction of the Hh signal [5]. Smo activation then triggers a series of intracellular events that results in the expression of Hh target genes through the Ci/Gli family of transcription factors [6]. Dysregulation of the pathway causes birth defects and human cancer [2]. A recent paper suggests that primary cilia sense Shh and transduce the signal through Ptc regulation of Smo accumulation within cilia, and play a critical role in development, carcinogenesis, and stem cell function [7]. Despite the importance of Hh signalling in mammals, there are gaps in our understanding of the pathway, such as the mechanisms allowing Ptc to regulate Smo activation.

Purification of Ptc and resolution of its structure are essential to understand how this protein functions and to identify Ptc ligands involved in Smo regulation. However, Ptc, like the majority of medically important mammalian membrane proteins, is present in tissues at very low concentrations, making overexpression in

Abbreviations: Hh, Hedgehog; Shh, Sonic Hedgehog; hSmo, human Smoothened; hPtc, human Patched; AOX1, alcohol oxidase 1; PMSF, phenylmethylsulfonyl fluoride; CBD, calmodulin binding domain; HA, hemagglutinin A; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; DDM, *n*-dodecyl-β-D-maltoside; Ni-NTA, Ni^{2+}-nitrilotriacetate; FSs, fluorinated surfactants; F_6-TAC, $C_6F_{13}C_2H_4$-S-poly-*Tris*-(hydroxymethyl)aminomethane; F_8-TAC, $C_8F_{17}C_2H_4$-S-poly-*Tris*-(hydroxymethyl)aminomethane; SPR, surface plasmon resonance

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heterologous systems a prerequisite for structural studies [8,9]. In an earlier paper, we showed that the human receptor Patched (hPtc) could be functionally expressed in *Drosophila* Schneider 2 cells [10]. We show in the present paper that hPtc can be expressed at the membrane fraction of the yeast *Saccharomyces cerevisiae* and purified in a native and stable conformation. To demonstrate that, we expressed and purified the fully active amino-terminal signalling domain of the secreted protein Shh (N-Shh) and used it to measure the ability of hPtc to interact with its ligand N-Shh both in the yeast membrane fraction and after purification in solution with different surfactants.

2. Materials and methods

2.1. Construction of expression vectors

For *S. cerevisiae*, we used the YEpGAL vector containing the GAL1-10 promotor and the YEpPMA vector containing the plasma membrane proton ATPase (PMA) promotor, generously given by Al-Shawi et al. [11]. The Multitag Affinity Purification (MAP) sequence encoding (i) a factor Xa, a TEV and thrombin cleavage sites to eliminate the MAP sequence; (ii) a calmodulin binding domain (CBD), a streptavidin tag and an hexahistidine tag for affinity chromatography; and (iii) an hemagglutinin peptide (HA) for anti-HA Western blot analysis [12], was inserted into the BamHI and XhoI restriction sites of the YEpGAL or YEpPMA polylinker, to yield YEpGAL-MAP, YEpPMA-MAP vectors. PCR with the Proofstart polymerase (Qiagen) was carried out on the hPtc cDNA to introduce at the 5′ end two restriction sites (SpeI) and a sequence of six adenosines, and at the 3′ end, a NheI restriction site, using the following primers: 5′-ACT AGT TCT AGA GAG CTC CCG CGG AAA AAA TCG GCT GGT AAC GCC GCC GAG CCC, and 5′-GGT ACC TCT AGA TCA GCT AGC GTT GGA GCT TCC CCG GGG CCT CTC. The PCR product was subcloned in the pCR™ 2.1 plasmid (Invitrogen) and sequenced. hPtc cDNA was then digested by SpeI and NheI and subcloned in MAP NheI sites, giving, YEpPMA-hPtc-MAP and YEpGAL-hPtc- MAP (Fig. 1).

2.2. Yeast strains, media, culture and transformation

S. cerevisiae strain K699 (Mata, ura3, and leu 2–3) generously given by R. Arkowitz was transformed with YEpGAL-hPtc-MAP, YEpPMA-hPtc-MAP, YEpPMA-MAP or YEpGAL-MAP by the lithium acetate procedure [13] and spread out on plates containing minimal medium and an amino acid mixture lacking leucine. Clones were precultured at 30 °C until 3 OD_{600} on minimal medium (MM) (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 for YEpPMA vectors or with 2% galactose for YEpGAL vectors. This preculture was then diluted to 0.1 OD_{600} in complete medium (yeast extract, bactopeptone, adenine), 2% D-glucose (or 2% galactose for YEpGAL vectors) and grown at 20 °C under 200 rpm shaking until 7 OD_{600} and centrifuged for 10 min at 2000 *g* and 4 °C.

2.3. Membrane preparation

All steps were performed at 4 °C. Yeast cells were washed in cold water, resuspended in 50 mM Tris–HCl (pH 7.4), 300 mM NaCl, 2.5 mM EDTA, 1 mM PMSF and 4 mM benzamidine, and broken by vortexing 15 min with glass beads (425–600 μm, Sigma). Unbroken yeasts and beads were removed by centrifugation for 5 min at 2000 *g*. Membranes were collected by centrifugation of the supernatant for 1 h at 100,000 *g*. The pellet was washed twice in the same buffer without EDTA and resuspended finally at 5 mg/mL in a solubilization buffer containing 50 mM Tris–HCl (pH 7.4), 300 mM NaCl, 10% glycerol, 1 mM PMSF and 4 mM benzamidine.

2.4. hPtc solubilization

Membrane preparations from *S. cerevisiae* expressing hPtc (5 mg/mL) were solubilized with different detergents in the solubilization buffer at 4 °C during 20 min under gentle agitation and then centrifuged at 100,000 *g* for 1 h to discard the insoluble fraction. The detergents tested were the non-ionic detergents DDM (*n*-dodecyl-β-D-maltoside, CMC = 0.15 mM), Nonidet NP40 (a nonyl phenoxypolyethoxylethanol, CMC = 0.059 mM) and Triton X-100 (CMC = 0.2–0.9 mM), the zwitterionic detergent CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate, CMC = 6 mM), and the anionic detergent SDS (Sodium dodecyl sulfate, CMC = 7–10 mM) (CMC values obtained from Sigma Aldrich catalogue). Samples were run on SDS-PAGE for western blotting, and revealed with the anti-HA antibody to compare the solubilization conditions.

2.5. hPtc purification

The membrane protein fraction (5 mg/mL) was solubilized in solubilization buffer, in the presence of 1% (20 mM) of DDM (Calbiochem), 20 min at 4 °C under gentle agitation, and then centrifuged at 100,000 *g* for 1 h to discard the insoluble fraction. All purification steps were carried out at 4 °C. One-half of the supernatant was supplemented with 2 mM $CaCl_2$, the other half with 10 mM imidazole, and batch loaded during 3 h onto, respectively, 1 volume of calmodulin Sepharose (Amersham), or 1 volume of Ni-NTA agarose (Qiagen) pre-equilibrated. The resins were centrifuged for 1 min at 400 *g*, resuspended in 10 volumes of washing buffer (solubilization buffer plus 2 mM DDM, and 2 mM $CaCl_2$ or 10 mM imidazole) and centrifuged. This washing step was repeated three times. The resins were then incubated for 10 min with 1 volume of elution buffer (solubilization buffer plus 2 mM DDM and 4 mM EGTA for calmodulin sepharose, 50–500 mM imidazole for Ni-NTA agarose). The eluates were collected after centrifugation for 1 min at 400 *g*. The elution step was also repeated three times. To test the stability of hPtc in fluorinated surfactants, DDM was exchanged with $C_6F_{13}C_2H_4$-S-poly-*Tris*-(hydroxymethyl)aminomethane (F_6-TAC) (CMC = 0.3 mM), or a slightly longer version, $C_8F_{17}C_2H_4$-S-poly-*Tris*-(hydroxymethyl)aminomethane (F_8-TAC) (CMC = 0.03 mM) [14,15] on calmodulin column during the washing step by replacing DDM

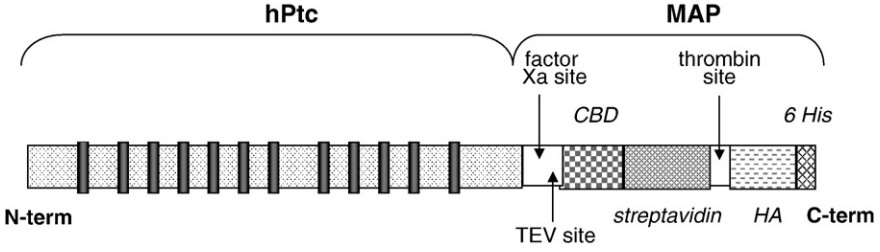

Fig. 1. MAP sequence fused at hPtc C-terminal end. Map sequence is composed of 165 amino acids: three affinity purification tags (a calmodulin binding domain (CBD), a streptavidin tag and a hexahistidine tag), the hemagglutin A epitope , and cleavage sites for factor Xa, TEV and thrombin.

with 2 mM F_6-TAC or F_8-TAC in the washing buffer. hPtc was eluted from the column with elution buffer containing 2 mM F_6-TAC or F_8-TAC in place of DDM.

2.6. Protein quantification

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

2.7. Gel electrophoresis and western blotting

Protein samples were separated on 8% SDS-PAGE and transferred to nitrocellulose membranes (Amersham) using standard techniques. Membranes were first blocked 1 h at room temperature in blocking buffer (20 mM Tris–HCl (pH 7.5); 450 mM NaCl; 0.1% Tween-20, 5% non-fat milk), probed overnight at 4 °C with monoclonal mouse anti-HA (laboratory made, dilution 1:20), polyclonal rabbit anti-Shh (laboratory made, dilution 1:1000) or polyclonal rabbit anti-Ptc (laboratory made, dilution 1:1000) antibodies, and then washed thrice in the blocking buffer. Secondary polyclonal goat anti-mouse immunoglobulins (dilution 1:5000) or polyclonal anti-rabbit immunoglobulins (dilution 1:2000) coupled to horseradish peroxidase (Dako) 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. Expression of N-Shh protein

The active amino-terminal part of the murine Shh protein (N-Shh corresponding to amino acids 25 to 198) was cloned into the XhoI site of the mammalian pABWN expression vector (pHH015 plasmid). The expression is under control of CMV enhancer/chicken β-actin promoter. This construct was transfected into mouse fibroblast L-cells (LTK-P2 cells). Cells stably expressing the construct were selected by addition of 800 μg/mL G418 (Gibco/BRL) in the culture medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% Na-pyruvate, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (all from Biowhittaker)). The expression of N-Shh protein was assayed in the culture medium using western blot experiment. The cell population expressing the higher level of N-Shh was adapted for growth in suspension by sequential dilution in different serum free culture media. Best results have been obtained by progressive adaptation to the ProCHO4-CDM medium (Cambrex). Cells were then grown in suspension in this medium, using either plastic Erlenmeyer flasks in regulated Minitron (INFORS) or CellSpin (Integra Biosciences) in regulated incubator (8% CO_2 atmosphere in both cases).

2.9. Purification of N-Shh— protein

Purification was routinely started from 5 L conditioned culture medium. The pH of the conditioned medium was adjusted to 7.4 by adding Tris–HCl buffer (50 mM final concentration). DTT (0.5 mM) and PMSF (0.5 mM) were also added at that step. The conditioned medium was clarified either by centrifugation at 42,000 *g* for 3 h at 4 °C or by filtration using a Stericup 0.22 μm GP Express Plus (Millipore). The conductivity of the clarified fraction was adjusted to 14 mS by addition of NaCl before loading on 25 mL SP Sepharose Fast Flow column (XK 26/20 column, both from GE HealthCare). The column was equilibrated with buffer A (25 mM Tris, pH 7.4, 50 mM NaCl and 0.5 mM DTT). Elution was carried out with a linear gradient of buffer B (buffer A containing 1 M NaCl) to 600 mM NaCl. Fractions were analyzed for N-Shh content by SDS-PAGE followed with Coomassie blue staining (Thermo). The N-Shh protein was eluted at about 400 mM NaCl. The protein was diluted 5 times in 25 mM Tris–HCl, pH 7.4 and 0.5 mM DTT, then further purified and concentrated on a 5 mL Hitrap SP column (GE HealthCare) using the buffers described for the first chromatography. Elution was performed by applying a gradient to 100% buffer B (i.e. 1 M NaCl) in 5 column volumes. The fractions concentrated in N-Shh protein were pooled (purified N-Shh protein concentrations ranging from 300 to 600 μg/mL) then stored at −80 °C until use.

2.10. Alkaline phosphatase (ALP) activity

C3H10T1/2 cells were seeded at 6000 cells/well in 96-well plates containing alpha-MEM supplemented with 10% fetal bovine serum, 1% Na-pyruvate, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (all from Biowhittaker). The day after, the medium was changed and FBS concentration decreased to 2%. At the same time 200 ng/mL BMP2 was added with purified N-Shh protein in the presence or absence of anti-Shh antibody 5E1 (Developmental Studies Hybridoma Bank). The culture was continued for 48 h then cells were lysed using 75 μL lysis buffer ($MgCl_2$ 0.02%, NP40 0.2%) for 1 h at 37 °C. ALP activity was read on 10 μL of cell extract by fluorescence measurement (Ex: 435 nm–Em: 555 nm) after addition of 100 μL substrate (AttoPhos® AP Fluorescent Substrate System Promega). The activity was standardized with the total protein amount evaluated by BCA (Pierce).

2.11. Gli responsive element activity

GliC53-19 is a stable clone of C3H10T1/2 cells transfected with reporter construct 5xGli responsive elements (RE) cloned in 5′ of a minimal collagenase promoter, followed by luciferase coding gene. GliC53-19 cells were seeded at 15,000 cells per well in 96-well plates and allowed to grow overnight at 37 °C and 5% CO_2 in alpha-MEM supplemented with 10% FBS, 1% Na-pyruvate, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. The following day, cells were supplemented with N-Shh at different concentrations and allowed to grow for 48 h. Cells were then lysed in 75 μL of 1× passive lysis buffer (Promega) and luciferase activity was measured in the cell lysate by luminescent assay following the manufacturer's procedure (Promega).

2.12. N-Shh western blotting

Samples were separated either on 10–20% Tris-Glycine or 4–12% NuPAGE Bis-Tris gel following the manufacturer's procedure (Invitrogen). Proteins were then transferred for 16 h at 45 mA on nitrocellulose membrane (Invitrogen). MagicMark XP Western Protein Standards (Invitrogen) were used as molecular weight markers. Membranes were incubated for 60 min in saturation buffer (PBS containing 0.5% Tween-20 (Fluka) and 5% skim milk) at room temperature, and then incubated with a polyclonal rabbit anti-Shh antibody (N-19 Santa Cruz Biotechnology, dilution 1:500) in the same buffer for 1 h. After 3 washes for 15 min in PBS, 0.2% Tween 20, 0.35 M NaCl, membranes were incubated with a Horseradish peroxidase coupled polyclonal anti-rabbit immunoglobulin (Sigma, dilution 1:8000 in the saturation buffer) for 1 h. After 3 washes for 15 min in PBS, 0.2% Tween 20, 0.35 M NaCl, blots were revealed using ECL (Amersham) and Molecular Imager FX (Bio-Rad).

2.13. N-Shh /hPtc binding measurements

2.13.1. Using immunodectection

100 μg of *S. cerevisiae* membranes expressing hPtc diluted in PBS were incubated with different concentrations of N-Shh for 1 h at 25 °C under gentle shaking. The membranes were centrifuged for 1 h at 20,000 *g* at 4 °C. The pellets were then washed twice in 100 μL of PBS and finally resuspended in 60 μL of PBS. 30 μL of these samples were run on SDS-PAGE for western blotting together with increasing concentrations of free N-Shh. The western blots were treated with laboratory made polyclonal rabbit anti-Shh antibodies (dilution 1:1000 [16]). The N-Shh signal intensities on western blots were

estimated by densitometry using Image J software. The intensities of the free N-Shh signals were used to make an N-Shh standard curve. The intensities of the N-Shh signals associated to hPtc-containing membrane samples were reported on this standard curve to estimate the quantities of N-Shh specifically bound to hPtc in the membrane fractions. This experiment has been repeated with membrane preparations from three different hPtc-expressing yeast cultures.

2.13.2. Using Surface Plasmon Resonance (SPR) experiments

SPR experiments were performed on a Biacore 3000 (Biacore/GE Healthcare, UMR IBSV, INRA Sophia Antipolis) with CM5 sensor chip from Biacore/GE Healthcare. 50 μg/mL of purified N-Shh was prepared in 10 mM Na-acetate pH5 and injected over the NHS/EDC— activated flow-cell (Fc) 2 of the Biacore CM5 sensor chip as described in the Biacore manual to achieve an immobilization level of 5000 RU. Fc1 served as an 'uncoated' reference flow-cell.

With membrane preparations, experiments were carried out at 20 °C. 40 μL of membrane preparation from hPtc-expressing yeast or from yeast that do not express hPtc, diluted in HBS-N running buffer (GE Healthcare) at 100 μg/mL were injected over both Fc1 and Fc2 at a flow rate of 10 μL/min, and the difference between sensorgrams recorded on Fc2 and Fc1 was reported. 10 μL of 4 M NaCl was injected to regenerate the surface at 10 μL/min. Membranes from hPtc-expressing yeast were mixed with purified N-Shh at four concentrations from 0.5 to 20 nM, and the resulting solutions were injected over reference (Fc1) and N-Shh (Fc2) surfaces.

With purified samples, experiments were carried out at 10 °C. 40 μL of hPtc purified in DDM, F_6-TAC or F_8-TAC were injected over both Fc1 (reference) and Fc2 (coupled to N-Shh) of the CM5 sensor chip at 10 μL/min. Specific running buffer is used for each sample: solubilization buffer plus 2 mM DDM, 2 mM C_6F-TAC or 2 mM C_8F-TAC. The difference between sensorgrams recorded on Fc2 and Fc1 is reported. 10 μL of 4 M NaCl was injected at 10 μL/min to regenerate the surface. For inhibition assay hPtc purified in DDM, F_6-TAC or F_8-TAC was incubated with free purified N-Shh at concentrations from 0.5 to 20 nM and injected over reference (Fc1) and N-Shh (Fc2) surfaces.

3. Results

3.1. hPtc is expressed in S. cerevisiae membranes

After modification of the sequence by PCR to introduce restriction sites and a sequence of six adenosines just upstream of the ATG initiation codon in order to optimize the initiation of translation and the protein expression [17], the hPtc cDNA was subcloned in the expression vectors at the 5′-end of the MAP sequence. We therefore expected to express hPtc with the MAP sequence at its C-terminal extremity at a theoretical molecular weight of 180 kDa, allowing detection of hPtc by western blot using antibodies directed against the hemagglutinin tag, and purification of hPtc using three affinity resins (calmodulin, streptavidin, and Ni-NTA), as already described for the other receptor of the Hh pathway hSmo [12] (Fig. 1). *S. cerevisiae* was then transformed with YEpGAL-hPtc-MAP and YEpPMA-hPtc-MAP plasmids, allowing an inducible expression of hPtc by galactose or a constitutive expression of hPtc respectively. Clones that integrated one of the plasmids were able to grow on plates containing minimum medium without leucine and were screened for expression of hPtc in the membrane fraction by western blotting using antibodies directed against the HA peptide present in the MAP sequence or against hPtc (Fig. 2A). The same specific signal corresponding to hPtc-MAP is observed around 180 kDa on western blots using both antibodies. The intensities of this signal in different clones and culture conditions were compared. We first observed that the expression of hPtc was comparable using both plasmids (data not shown). Therefore, all further experiments were performed with the *S. cerevisiae* YEpPMA-hPtc-MAP strain. Then, the best expressing clone was grown at 30 °C

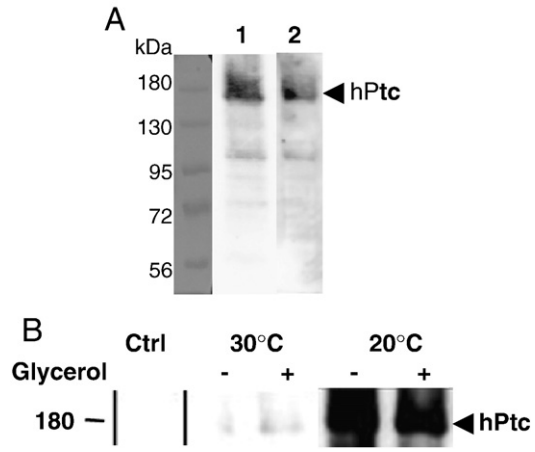

Fig. 2. hPtc expression in *S. cerevisiae* membranes. (A) Western blot with 40 μg of a membrane protein preparation from hPtc-expressing *S. cerevisiae* blotted with anti-HA (1:20) (lane 1) or laboratory made anti-Ptc (1:1000) (lane 2) antibody. (B) Effect of temperature and glycerol on the expression of hPtc at *S. cerevisiae* membrane fraction. The western blot was probed with an anti-HA antibody. The control lane is loaded with 40 μg of a membrane protein preparation of wild-type *S. cerevisiae* grown at 20 °C without glycerol. In the other lanes, 40 μg of membrane preparation from hPtc-expressing *S. cerevisiae* were loaded. Yeasts were grown in the indicated conditions.

or 20 °C, with or without 10% glycerol which as been shown to increase human P-glycoprotein expression [11]. Fig. 2B shows that the signal corresponding to hPtc-MAP is strongly increased when yeasts are grown at 20 °C. The presence of glycerol does not increase the intensity of the signal, meaning that its addition is not relevant for a better expression of our protein. At 20 °C, the expression in the membrane protein fraction reached a maximum at 7 $OD_{600\ nm}$ over which, at 9 $OD_{600\ nm}$ for instance, it decreases.

3.2. hPtc can be purified using the MAP strategy

The best hPtc-expressing clone of *S. cerevisiae* was grown in the best conditions established, at 20 °C until the $OD_{600\ nm}$ was 7 and then the membrane fraction was prepared. In order to purify hPtc, the membrane fraction was solubilized with different detergents (Fig. 3A). hPtc solubilization was equally efficient in DDM, NP40 or Triton X100, but was more efficient in DDM than in CHAPS. Since DDM is one of the most efficient detergents for hSmo solubilization [12] and one of the most frequently used non-ionic detergents for purification and crystallization of membrane proteins, it was chosen for hPtc purification. Therefore all purification steps have been performed in the presence of DDM. Different times of solubilization (from 20 min to 180 min) and concentrations of detergent (from 2 to 20 mM) were tested. The best solubilization of hPtc was obtained after 20 min at 4 °C in 20 mM DDM. Same amounts of solubilized hPtc have been incubated in batches with Ni-NTA and calmodulin resins in appropriate buffers and a western blot analysis was used to ascertain binding to the resins (Fig. 3B). Half of the solubilized fraction was loaded onto the Ni-NTA resin in the presence of 10 mM imidazole and the resin was washed in the same buffer. Then we applied an imidazole gradient from 50 mM to 300 mM (Fig. 3B, lanes 2–6). No hPtc was detected in the flow through (lane 1) and hPtc was eluted with 100 or 150 mM imidazole as confirmed by the strong signal obtained in lanes 3 and 4 respectively. The other part of the solubilized fraction was loaded on calmodulin resin with 2 mM $CaCl_2$. After incubation, we noticed that a certain amount is lost in the flow through (lane 7). The resin was then washed in the same buffer and hPtc was eluted three times with 4 mM of EGTA (lanes 8–10). A band at 180 kDa corresponding to the predicted size of hPtc appears strongly in the first elution (lane 8) and a weaker signal is observed in later elutions (lanes 9 and 10). Purification on Ni-NTA is only partial and hPtc

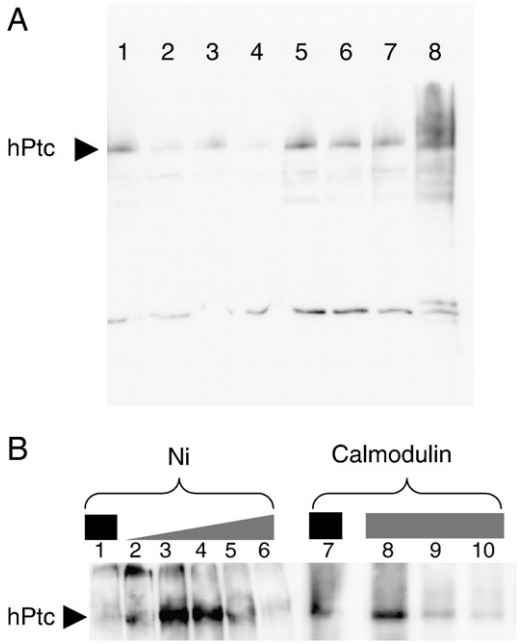

Fig. 3. Solubilization and purification of hPtc expressed in *S. cerevisiae*. (A) A membrane preparation from *S. cerevisiae* expressing hPtc (5 mg/mL) was solubilized with either 1% NP40 (lane 1), a mixture of 2% DDM and 1% CHAPS (lane 2), 2% CHAPS (lane 3), 1% CHAPS (lane 4), 2% DDM (lane 5), 3% DDM (lane 6), 2% Triton × 100 (lane 7) or 1% SDS (lane 8). Samples were run on SDS-PAGE, transferred to a western blot and probed with the anti-HA antibody. (B) Purification of hPtc on Ni-NTA and calmodulin resins: the grey triangle corresponds to the elution in 5 steps from 50 to 250 mM imidazole from the Ni-NTA column (lanes 2–6), the grey rectangle corresponds to the elution with 4 mM EGTA from the calmodulin column (lanes 8–9), the flow through of each resin was loaded (black squares, lanes 1 and 7). Samples were run on SDS-PAGE, transferred to a western blot and probed with the anti-HA antibody.

represents around 30% of the purified fraction. The yield of purification of hPtc is higher after calmodulin column than after Ni-NTA, however, purification on calmodulin resin allows to recover only 1% of the fraction of hPtc initially present in the membrane preparation. Both resins, Ni-NTA and calmodulin, can be used to purify hPtc. However, Ni-NTA seems to be more efficient as a first step of purification.

3.3. Production of pure and active N-Shh

The active amino-terminal part of the murine Sonic Hedgehog protein (N-Shh), corresponding to amino acids 25 to 198 (89% identity with human N-Shh) was expressed under the control of the CMV enhancer/chicken β-actin promoter. This construct was transfected into mouse fibroblast L-cells and stably expressing populations were obtained after antibiotic selection. In order to facilitate further production scale-up, the best expressing cells were adapted to growth in suspension. Various serum free medium were tested and the best results were obtained with ProCHO4-CDM. Using this medium, cells were growing well (about one division/24 h) and the N-Shh expression level was as good as previously measured for the parental adherent cells, about 2 to 4 mg/L in conditioned medium.

A standard purification procedure was then designed using 5 L of conditioned medium as starting material. Clarification of this medium was very efficient using either centrifugation or filtration, both without loss of N-Shh. The first ion exchange chromatographic step allowed the recovery of about 95% of the N-Shh protein. The large majority of contaminant proteins were eliminated by a column wash containing 200 mM NaCl. The protein was eluted at about 450 mM NaCl. Since the purity of the N-Shh protein obtained after this purification step was already good, a second ion exchange chromatographic step using the same resin was performed to mainly concentrate the protein. Purity analysis of the final product using Coomassie blue staining after SDS-PAGE showed that the protein was more than 90% pure with an occasional minor contamination by a protein of very high molecular mass (Fig. 4A).

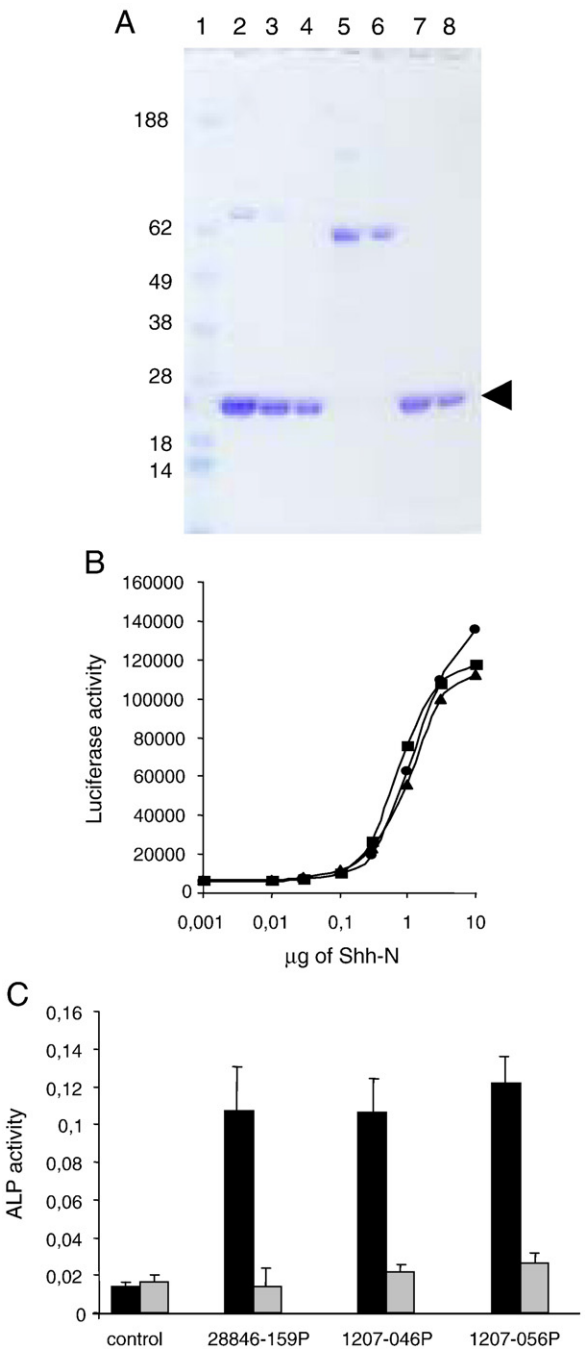

Fig. 4. N-Shh purification and activity. (A) Purity analysis. N-Shh was expressed in mouse fibroblast L-cells and purified as described. Samples were run on 4–12% NuPage Bis-Tris gels in MES buffer and stained with Coomassie blue. Lane 1: 2.5 µL SeeBlue molecular weight markers (Invitrogen); lane 2 to 4: batch 1207-056P (10, 5 and 2.5 µL respectively); lanes 5–6: BSA (500 and 250 ng respectively); lanes 7–8 : batch 28846-159P, 10 and 5 µL respectively. N-Shh is indicated by an arrow. (B) Induction of the Gli transduction pathway. 3 Different purified N-Shh batches were tested for their ability to induce Gli responsive element in GliC53-19 cells. (●): 1207-046P; (▲): 28846-159P; (■): 1207-056P. (C) Induction of alkaline phosphatase (ALP) activity is reversed by an anti-Shh 5E1 antibody. Three different purified N-Shh batches (28846-159P, 1207-046P and 1207-056P) were tested for their ability to synergize with BMP2 in ALP activity induction in C3H10T1/2 cells. All samples contain 200 ng/mL BMP2. Control: treatment without N-Shh. Incubation without or with 5E1 antibody at 10 µg/mL (dark and grey boxes respectively).

As expected for a biologically active protein, our purified N-Shh protein was very active at inducing the Gli transduction pathway. Using a cell line stably transfected with the luciferase gene under the control of 5 Gli responsive elements, we observed a clear correlation between an increase in the amount of N-Shh added to the cells and an increase in luciferase activity (Fig. 4B). The same results were obtained with different N-Shh preparations. Moreover, these different preparations of our N-Shh protein showed exactly the same specific Alkaline Phosphatase (ALP) activity in C3H10T1/2 cells, an activity which was clearly inhibited by co-incubation with the commercially available inhibiting anti-Shh antibody 5E1 (Fig. 4C).

3.4. Human Ptc expressed in yeast interacts with purified Shh in membrane preparations

In order to know if human Ptc is expressed in *S. cerevisiae* in a native conformation, its ability to interact with purified N-Shh was measured using two different techniques, either immunodetection or SPR.

3.4.1. Using immunodetection

After incubation with different concentrations of pure N-Shh followed by three washes, the membrane protein preparations from three different hPtc-expressing yeast cultures present a signal corresponding to N-Shh on western blots treated with anti-Shh antibodies (see Fig. 5A as an example). This observation indicates that hPtc expressed in *S. cerevisiae* interacted with its ligand N-Shh. In order to estimate the quantity of N-Shh bound to hPtc, the N-Shh signal intensities associated with hPtc-containing membranes were measured using Image J software and compared with an N-Shh standard curve established with the signal intensities corresponding to increasing concentrations of pure N-Shh in three independent experiments (see Fig. 5A as an example). Knowing that N-Shh interacts with Ptc in a 1:1 molar ratio [18], the N-Shh signal obtained in the experiment presented in Fig. 5A allowed us to estimate that this membrane preparation contains around 75 pmol of hPtc able to interact with N-Shh per mg of membrane proteins. The two other membrane preparations analyzed for N-Shh interaction presented around 50 pmol of functional hPtc per mg of membrane proteins. For each liter of yeast giving around 20 mg of membrane proteins, we estimate that between 200 and 250 μg of functional hPtc are produced per liter of *S. cerevisiae*.

3.4.2. Using SPR technology

The Biacore 3000 Surface Plasmon Resonance (SPR) biosensor allows the direct real-time detection of binding without chemical labelling [19]. One of the binding partners is immobilized on the

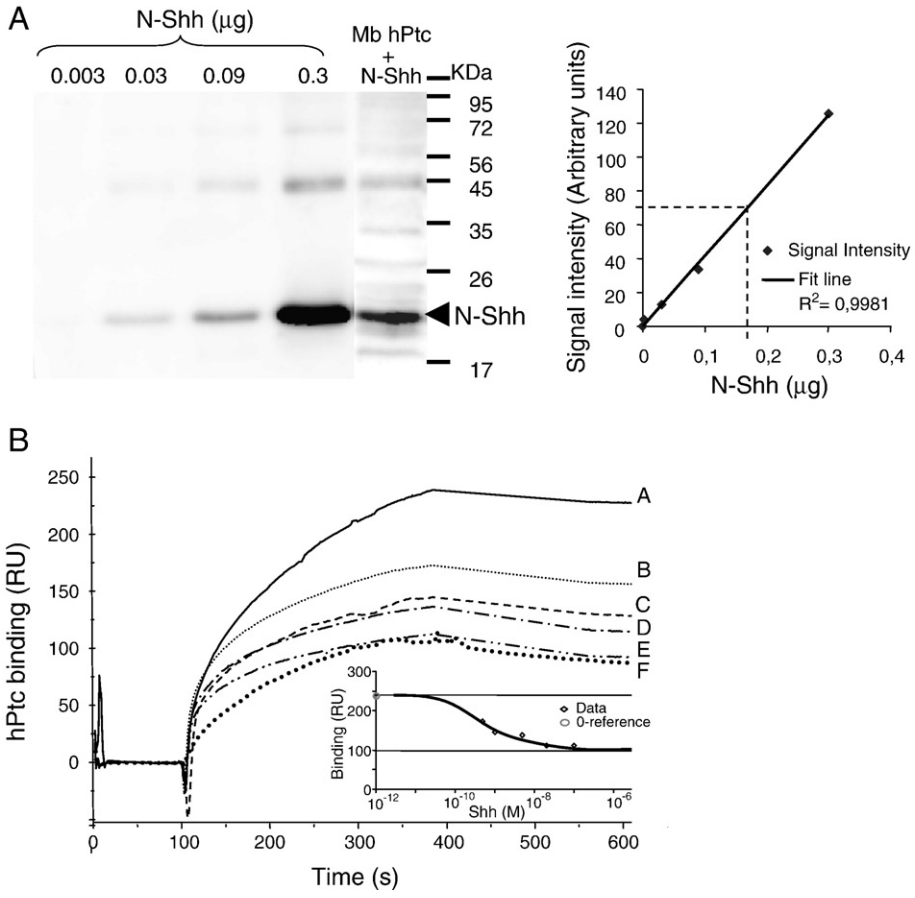

Fig. 5. hPtc expressed in the yeast interacts with purified N-Shh. (A) Immunodetection measurements. Lanes 1 to 4: 0.003, 0.03, 0.09 and 0.3 μg of pure N-Shh. Lanes 5: hPtc-containing membrane preparation incubated with 15 nM of N-Shh. Samples were run on SDS-PAGE, transferred on western blot and probed with laboratory made anti-Shh. The corresponding western blot is presented at the left of the figure. The intensities of the free N-Shh signals were estimated by densitometry using the Image J software, and used to draw the N-Shh standard curve presented in the right part of the figure. The intensity of the N-Shh signal associated with hPtc-containing membranes was reported on this standard curve to estimate the quantity of N-Shh bound to hPtc. (B) Surface Plasmon Resonance measurements. The sensorgrams presented are the difference between signal recorded on the N-Shh-coupling channel Fc2 (on which purified N-Shh was immobilized by amine coupling) and the reference channel Fc1 (activated with amine coupling reagents) of a Biacore CM5 sensorchip. Sensorgram A: 100 μL of hPtc-containing membrane preparation (100 μg/mL) have been injected both over Fc1 and Fc2 producing a specific signal of 240 resonance units (RU). Sensorgrams B, C, D, and E: inhibition assay in solution: binding responses of hPtc-containing membrane preparation (100 μg/mL) mixed with 0.5, 1, 5 and 20 nM of purified N-Shh injected both over Fc1 and Fc2 (B: 173 RU, C: 145 RU, D: 137 RU, E: 112 RU). Sensorgram F: 100 μL of control membrane preparation from yeasts that do not express hPtc (100 μg/mL) have been injected both over Fc1 and Fc2 producing a signal corresponding to non-specific binding. Window: binding response against N-Shh concentration for IC_{50} determination.

sensor chip surface and the other passed over it in solution. Binding causes a change in the refractive index at the biosensor surface which is transformed into a measurable signal by light-sensitive detector and expressed as resonance units (RU) on the recorded sensorgram. Purified N-Shh was immobilized by covalent coupling to a Biacore CM5 sensor chip (channel Fc2) producing a signal of 5000 RU. A membrane preparation containing hPtc have been injected on both the reference channel (Fc1) treated with coupling reagents but without N-Shh and the measure channel (Fc2) coupled to N-Shh. The difference between the sensorgrams recorded on Fc2 and Fc1 shows a signal increase of 240 RU on Fc2 (Fig. 5B, sensorgram A). An inhibition assay in solution was then carried out with purified N-Shh added at increasing concentrations to solutions of membrane preparation containing hPtc before injection on N-Shh coated sensor chip. The superposition of the sensorgrams shows that the binding of hPtc-containing membranes decreases with increasing concentration of added N-Shh (Fig. 5B, sensorgrams B, C, D, and E). Therefore, N-Shh in solution bound to hPtc contained in yeast membranes and inhibits binding of the latter to the N-Shh surface. The sensorgram F recorded with membrane preparation from yeasts that don't express hPtc reports the non-specific binding on N-Shh. The binding signal obtained when hPtc-containing membranes were incubated with 20 nM of free N-Shh is just with the top of the non-specific one indicating that 20 nM of free N-Shh inhibits almost completely hPtc binding to N-Shh coupled to the sensor chip. Plotting the binding response against N-Shh concentration produced a binding curve from which the calculated IC_{50} is between 0.5 and 1 nM.

3.5. Fluorinated surfactants preserve human Ptc integrity in solution

1 day after purification in DDM, hPtc was injected on both the reference (Fc1) and the measure (Fc2) channels of the CM5/N-Shh sensor chip. The difference between the sensorgrams recorded on Fc2 and Fc1 gave a signal of 244 RU indicating that a certain amount of hPtc was able to interact with its ligand after purification in DDM (Fig. 6 A). The signal recorded was relatively stable in time (measures realized until 1 month after purification). Fluorinated surfactants (FSs) have been shown to be less aggressive towards certain membrane proteins than classical detergents [20–22]. FSs show 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, comprises fluorine atoms [14,15]. Because FSs are poor solvents for lipids, membranes were solubilized with DDM, and DDM was then exchanged on the calmodulin column to F_6-TAC or a slightly longer version, F_8-TAC. The capacity of hPtc to interact with its purified ligand N-Shh when purified in DDM, F_6-TAC and F_8-TAC was measured on the CM5/N-Shh Biacore sensor chip. 1 day after purification, hPtc purified in F_6-TAC or F_8-TAC bound to N-Shh produced a signal of approximately 430 RU, suggesting that a larger amount of hPtc was able to interact with N-Shh when purified in FSs in comparison to DDM (Fig. 6A). The binding signals recorded slightly decreased (10 to 40 % depending on purifications) 3 days after purification and stayed stable in time until 1 month after purification. An inhibition assay in solution was then carried out with free purified N-Shh added at increasing concentrations to solutions of hPtc purified in the fluorinated surfactants before injection on N-Shh coupled to the sensor chip. Fig. 6B shows that the binding of hPtc in solution with F_8-TAC decreases with increasing concentration of free N-Shh added. Analysis of the results indicates that free N-Shh inhibits binding of hPtc to N-Shh coupled on the sensor chip with an IC_{50} around 0.5 nM, comparable to the IC_{50} obtained with hPtc in membrane preparations. These results suggest that fluorinated surfactants are efficient for preserving hPtc integrity and stability in solution.

4. Discussion

In this study, the human receptor of the morphogen Shh (hPtc) has been expressed in the yeast *S. cerevisiae* in fusion with a multitag affinity purification sequence at its C-terminal end. Expression of hPtc at the yeast membrane fraction was tested under several promoters, clones and growth conditions. We observed that constitutive expression of hPtc had no effect on yeast growth and gave the same expression levels as the inducible system. As observed for human Smo expression in *S. cerevisiae* [12] and other integral membrane proteins, decreasing growth temperature from 30 °C to 20 °C strongly increased the expression level of hPtc. This is consistent with various other studies suggesting that folding and stability of membrane proteins are often favorably influenced by low temperatures [23]. As for hSmo, the level of hPtc at the yeast membrane fraction decreased if cells reached an $OD_{600\ nm}$ over 7 and this is probably the result of translational or post-translational events such as proteolysis or protein degradation [24]. We concluded that constitutive expression from the promoter of the plasma membrane ATPase, in rich medium at 20 °C at 7 $OD_{600\ nm}$ units were the best conditions for the expression of hPtc at *S. cerevisiae* membrane fraction.

A way to evaluate the conformational state of hPtc expressed in *S. cerevisiae* is to measure its ability to interact with its natural ligand N-Shh. N-Shh derives from the Shh precursor through an autoprocessing

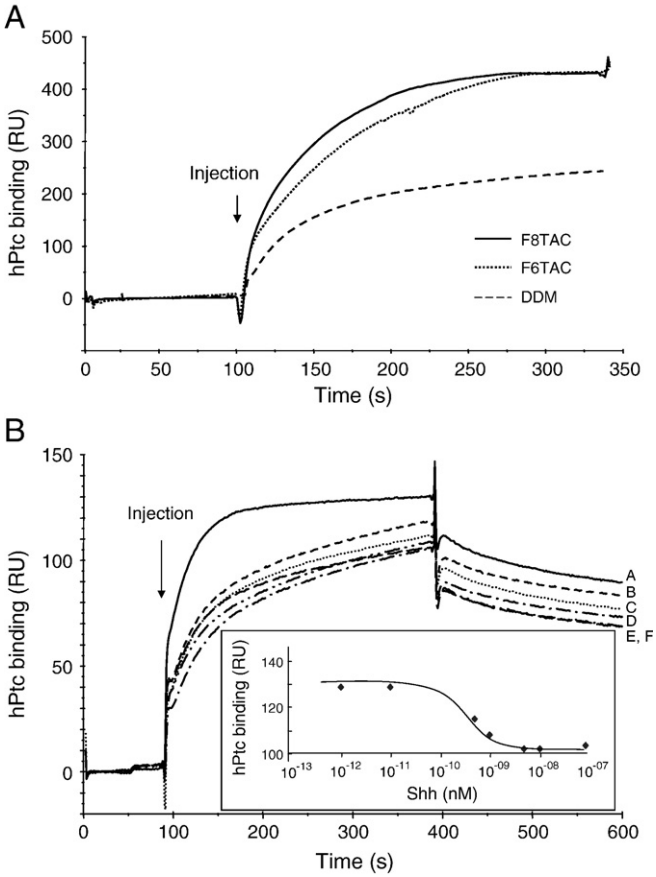

Fig. 6. hPtc purified in fluorinated surfactants interacts with N-Shh. (A) hPtc purified in DDM, F_6-TAC or F_8-TAC was injected over both the N-Shh-coupling channel Fc2 (on which purified N-Shh was immobilized by amine coupling) and the reference channel Fc1 (activated with amine coupling reagents) of the Biacore CM5 sensor chip. The Biacore SPR responses reported in resonance units (RU) are the differences between sensorgrams recorded on Fc2 and Fc1, and correspond to the specific binding of hPtc on immobilized N-Shh. (B) Inhibition assay in solution: binding response of hPtc purified in F_8-TAC mixed with 0 (sensorgram A), 0.5 nM (sensorgram B), 1 nM (sensorgram C), 5 nM (sensorgram D), 10 nM (sensorgram E) and 20 nM (sensorgram F) of free N-Shh. Window: binding response against free N-Shh concentration for IC_{50} determination.

reaction mediated by the carboxyl-terminal domain and is the part of the Shh protein active in signalling as a ligand for the receptor Ptc [18]. In order to measure the interaction between hPtc and its ligand, the N-terminal part of murine Shh was expressed in fibroblast L-cells and purified. Our results show that purified recombinant N-Shh is fully active and able to induce the Hh pathway in mouse pluripotent stem cells. Experiments carried out on membrane preparations using two different techniques, immunodetection and Surface Plasmon Resonance, clearly demonstrate that human Ptc expressed in *S. cerevisiae* is able to interact with purified N-Shh. Knowing that N-Shh interacts with Ptc in a 1:1 molar ratio [18], we estimated from immunodetection experiments that our membrane preparations contain 50 to 75 pmol of hPtc per mg of membrane proteins, meaning that between 200 and 250 μg of functional hPtc are produced per liter of *S. cerevisiae*. This is not a high expression level but should be sufficient to allow purification of hPtc for biochemical and structural studies. Using Biacore SPR technology, we observed that N-Shh in solution bound to hPtc contained in membranes, therefore inhibiting the binding of hPtc to the N-Shh-coupled surface. An IC50 value of 0.5 to 1 nM was obtained, which is in close agreement with the K_D value of 1.2 nM reported using labelled recombinant human N-Shh on oocytes injected with chick ptc mRNA [25]. This is also the same order of magnitude of the concentration of N-Shh required for biological effect *in vivo* [26], suggesting that hPtc is expressed in a native conformation in *S. cerevisiae*.

After solubilization of hPtc from the membrane with the non-ionic detergent DDM, we showed that hPtc could be purified using affinity tags present in the MAP sequence at its C-terminal end. However, Biacore SPR experiments showed that only a fraction of hPtc purified in this detergent was able to interact with its ligand N-Shh. Several studies have reported that fluorinated surfactants are less aggressive towards certain membrane proteins than classical detergents [20–22]. Presumably because they do not compete effectively with the protein–protein and protein–lipid interactions that stabilize the native fold of membrane proteins, fluorinated surfactants can keep membrane proteins water-soluble under particularly mild conditions. In the present study, we show using Biacore SPR technology that when DDM is exchanged with fluorinated surfactants during purification, the fraction of hPtc able to interact with its ligand N-Shh is higher than in DDM, and stable in time after purification. Moreover, the IC_{50} determined for N-Shh binding with hPtc in solution with the fluorinated surfactants is comparable to the IC_{50} determined with hPtc in a membrane environment. This result suggests that the fraction of hPtc able to interact with its ligand in solution with the fluorinated surfactants is in the same conformation than in the membrane. This is the first study reporting conditions in which the human receptor Ptc is in a native and stable conformational state able to interact with its natural ligand N-Shh in solution after purification.

These results encourage us to work on the purification procedure scale-up to develop crystallization trials and structural characterization of hPtc. Yeast is the simplest eukaryotic cell that performs many of the post-translational modifications seen in higher eukaryotic cells. Moreover, it is easy to grow in large volumes in a short period of time [27]. *S. cerevisiae* has been successfully used to functionally express and purify various mammalian membrane proteins such as the recombinant rabbit SERCA1a Ca^{2+}-ATPase, allowing the determination of the first structure of a mammalian membrane protein derived from a heterologous expression system [28]. This structure was shown to be the same as the one obtained from native protein purified from muscle [29]. This result demonstrates that a recombinant membrane protein produced in a heterologous expression system can adopt the same structure as the native one. Among ten atomic structures of mammalian membrane proteins derived from heterologous expression solved since 2005, five were derived from proteins expressed in yeast [28,30–33]. This is very encouraging, and although the process of producing, purifying and crystallizing these integral membrane proteins is challenging, the relevance for pharmacological research justifies these efforts. Mutations that lead to constitutive Hh signalling are found in Gorlin's syndrome, a familial cancer syndrome characterized by neoplasms that include basal cell carcinomas and medulloblastomas [34,35]. Inappropriate activation of the pathway has been implicated in the pathogenesis of sporadic human cancers of the skin, brain, lung, prostate, pancreas and gastrointestinal tract [2,36]. The first and often genetically damaged step in the Hh pathway is the interaction between the two membrane receptors, Ptc and Smo. Purification of Ptc will allow biochemical and structural studies which will shed light on the mechanism of the regulation of Smo activity by Ptc, and thus provide new strategies for cancer treatment.

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References

[1] P.W. Ingham, A.P. McMahon, Hedgehog signaling in animal development: paradigms and principles, Genes Dev. 15 (2001) 3059–3087.
[2] P.A. Beachy, S.S. Karhadkar, D.M. Berman, Tissue repair and stem cell renewal in carcinogenesis, Nature 432 (2004) 324–331.
[3] J.E. Hooper, M.P. Scott, The *Drosophila* patched gene encodes a putative membrane protein required for segmental patterning, Cell 59 (1989) 751–765.
[4] D.M. Stone, M. Hynes, M. Armanini, T.A. Swanson, Q. Gu, R.L. Johnson, M.P. Scott, D. Pennica, A. Goddard, H. Phillips, M. Noll, J.E. Hooper, F. de Sauvage, A. Rosenthal, The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog, Nature 384 (1996) 129–134.
[5] H.E. Karpen, J.T. Bukowski, T. Hughes, J.P. Gratton, W.C. Sessa, M.R. Gailani, The sonic hedgehog receptor patched associates with caveolin-1 in cholesterol-rich microdomains of the plasma membrane, J. Biol. Chem. 276 (2001) 19503–19511.
[6] J. Taipale, P.A. Beachy, The Hedgehog and Wnt signalling pathways in cancer, Nature 411 (2001) 349–354.
[7] R. Rohatgi, L. Milenkovic, M.P. Scott, Patched1 regulates hedgehog signaling at the primary cilium, Science 317 (2007) 372–376.
[8] C.G. Tate, Overexpression of mammalian integral membrane proteins for structural studies, FEBS Lett. 504 (2001) 94–98.
[9] I. Mus-Veteau, Heterologous expression and purification systems for structural proteomics of mammalian membrane proteins, Compar. Funct. Genom. 3 (2002) 511–517.
[10] M. De Rivoyre, L. Ruel, M. Varjosalo, A. Loubat, M. Bidet, P. Therond, I. Mus-Veteau, Human receptors patched and smoothened partially transduce hedgehog signal when expressed in *Drosophila* cells, J. Biol. Chem. 281 (2006) 28584–28595.
[11] R.A. Figler, H. Omote, R.K. Nakamoto, M.K. Al-Shawi, Use of chemical chaperones in the yeast *Saccharomyces cerevisiae* to enhance heterologous membrane protein expression: high-yield expression and purification of human P-glycoprotein, Arch. Biochem. Biophys. 376 (2000) 34–46.
[12] M. De Rivoyre, F. Bonino, L. Ruel, M. Bidet, P. Therond, I. Mus-Veteau, Human receptor Smoothened, a mediator of Hedgehog signalling, expressed in its native conformation in yeast, FEBS Lett. 579 (2005) 1529–1533.
[13] R. Elble, A simple and efficient procedure for transformation of yeasts, Biotechniques 13 (1992) 18–20.
[14] A.A. Pavia, B. Pucci, J.G. Riess, L. Zarif, New perfluoroalkylated telomeric non ionic surfactants synthesis physicochemical and biological properties, Makromol. Chem. 193 (1992) 2505–2517.
[15] E. Chabaud, P. Barthelemy, N. Mora, J.L. Popot, B. Pucci, Stabilization of integral membrane proteins in aqueous solution using fluorinated surfactants, Biochimie 80 (1998) 515–530.
[16] E. Traiffort, K.L. Moya, H. Faure, R. Hassig, M. Ruat, High expression and anterograde axonal transport of aminoterminal sonic hedgehog in the adult hamster brain, Eur. J. Neurosci. 14 (2001) 839–850.
[17] L. Beaudet, L. Urbatsch, P. Gros, High-level expression of mouse Mdr3 P-glycoprotein in yeast *Pichia pastoris* and characterization of ATPase activity, Methods Enzymol. 292 (1998) 397–413.
[18] N. Fuse, T. Maiti, B. Wang, J.A. Porter, T.M. Hall, D.J. Leahy, P.A. Beachy, Sonic hedgehog protein signals not as a hydrolytic enzyme but as an apparent ligand for patched, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 10992–10999.

[19] S. Sen, V.P. Jaakola, P. Pirila, M. Finel, A. Goldman, Functional studies with membrane-bound and detergent-solubilized alpha2-adrenergic receptors expressed in Sf9 cells, Biochim. Biophys. Acta 1712 (2005) 62–70.
[20] C. Breyton, E. Chabaud, Y. Chaudier, B. Pucci, J.L. Popot, Hemifluorinated surfactants: a non-dissociating environment for handling membrane proteins in aqueous solutions? FEBS Lett. 564 (2004) 312–318.
[21] M. Damian, S. Perino, A. Polidori, A. Martin, L. Serre, B. Pucci, J.L. Baneres, New tensio-active molecules stabilize a human G protein-coupled receptor in solution, FEBS Lett. 581 (2007) 1944–1950.
[22] K.H. Park, C. Berrier, F. Lebaupain, B. Pucci, J.L. Popot, A. Ghazi, F. Zito, Fluorinated and hemifluorinated surfactants as alternatives to detergents for membrane protein cell-free synthesis, Biochem. J. 403 (2007) 183–187.
[23] R. Yelin, S. Schuldiner, Vesicular monoamine transporters heterologously expressed in the yeast *Saccharomyces cerevisiae* display high-affinity tetrabenazine binding, Biochim. Biophys. Acta 1510 (2001) 426–441.
[24] R.T. Niebauer, A. Wedekind, A.S. Robinson, Decreases in yeast expression yields of the human adenosine A2a receptor are a result of translational or post-translational events, Protein. Expr. Purif. 37 (2004) 134–143.
[25] V. Marigo, R.A. Davey, Y. Zuo, J.M. Cunningham, C.J. Tabin, Biochemical evidence that patched is the Hedgehog receptor, Nature 384 (1996) 176–179.
[26] H. Roelink, J.A. Porter, C. Chiang, Y. Tanabe, D.T. Chang, P.A. Beachy, T.M. Jessell, Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis, Cell 81 (1995) 445–455.
[27] V.K. Ton, R. Rao, Functional expression of heterologous proteins in yeast: insights into Ca2+ signaling and Ca2+-transporting ATPases, Am. J. Physiol. Cell. Physiol. 287 (2004) C580–589.
[28] M. Jidenko, R.C. Nielsen, T.L. Sorensen, J.V. Moller, M. le Maire, P. Nissen, C. Jaxel, Crystallization of a mammalian membrane protein overexpressed in *Saccharomyces cerevisiae*, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 11687–11691.
[29] C. Toyoshima, M. Nakasako, H. Nomura, H. Ogawa, Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 A resolution, Nature 405 (2000) 647–655.
[30] S.B. Long, E.B. Campbell, R. Mackinnon, Crystal structure of a mammalian voltage-dependent Shaker family K+ channel, Science 309 (2005) 897–903.
[31] S.B. Long, X. Tao, E.B. Campbell, R. MacKinnon, Atomic structure of a voltage-dependent K+ channel in a lipid membrane-like environment, Nature 450 (2007) 376–382.
[32] D. Martinez Molina, A. Wetterholm, A. Kohl, A.A. McCarthy, D. Niegowski, E. Ohlson, T. Hammarberg, S. Eshaghi, J.Z. Haeggstrom, P. Nordlund, Structural basis for synthesis of inflammatory mediators by human leukotriene C4 synthase, Nature 448 (2007) 613–616.
[33] H. Ago, Y. Kanaoka, D. Irikura, B.K. Lam, T. Shimamura, K.F. Austen, M. Miyano, Crystal structure of a human membrane protein involved in cysteinyl leukotriene biosynthesis, Nature 448 (2007) 609–612.
[34] H. Hahn, J. Christiansen, C. Wicking, P.G. Zaphiropoulos, A. Chidambaram, B. Gerrard, I. Vorechovsky, A.E. Bale, R. Toftgard, M. Dean, B. Wainwright, A mammalian patched homolog is expressed in target tissues of sonic hedgehog and maps to a region associated with developmental abnormalities, J. Biol. Chem. 271 (1996) 12125–12128.
[35] R.L. Johnson, A.L. Rothman, J. Xie, L.V. Goodrich, J.W. Bare, J.M. Bonifas, A.G. Quinn, R.M. Myers, D.R. Cox, E.H. Epstein Jr., M.P. Scott, Human homolog of patched, a candidate gene for the basal cell nevus syndrome, Science 272 (1996) 1668–1671.
[36] L.L. Rubin, F.J. de Sauvage, Targeting the Hedgehog pathway in cancer, Nat. Rev. Drug. Discov. 5 (2006) 1026–1033.