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GPCR Proteomics: Mass Spectrometric and Functional Analysis of Histamine H_1 Receptor after Baculovirus-Driven and *in Vitro* Cell Free Expression

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The human histamine H_1 Receptor (hH_1R) belongs to the family of G-protein coupled receptors (GPCRs), an attractive and proven class of drug targets in a wide range of therapeutic areas. However, due to the low amount of available purified protein and the hydrophobic nature of GPCRs, limited structural information is available on ligand–receptor interaction especially for the transmembrane (TM) domain regions where the majority of ligand–receptor interactions occur. During the last decades, proteomic techniques have increasingly become an important tool to reveal detailed information on the individual GPCR class, including post-translational modifications and characterizations of GPCRs binding pocket. Herein, we report the successful functional production and mass spectrometric characterization of the hH_1R, after baculovirus-driven and *in vitro* cell-free expression. Using only MALDI-ToF, sequence coverage of more than 80%, including five hydrophobic TM domains was achieved. Moreover, we have identified an asparagine residue in the hH_1R protein that is subject to N-linked glycosylation. This information would be valuable for drug discovery efforts by allowing us to further study H_1R−ligand interactions using histaminergic ligands that covalently bind the hH_1R, and eventually revealing binding sites of hH_1R and other GPCRs.

Keywords: Mass spectrometry • G-protein coupled receptor • histamine H_1 receptor • cell-free expression • membrane protein purification

Introduction

G-protein coupled receptors (GPCRs) represent the largest family of transmembrane (TM) signaling proteins and play key roles in (patho) physiology by binding a chemically diverse array of extracellular signaling molecules. Consequently, GPCRs are major drug targets, with approximately 50% of all currently marketed drugs affecting function of these membrane receptors.[1] Despite the large number of GPCRs and their pharmacotherapeutic relevance, the structural characterization by biophysical techniques such as NMR or X-ray crystallography studies still remains a challenge, mainly due to difficulties for their successful protein production and purification. Up to now, the only known GPCR crystal structures are those of the naturally abundant rhodopsin, which is responsible for our dim-light vision.[2–4]

So far, structure-based drug design for GPCRs is mainly derived from computational structure prediction based on homology modeling combined with mutagenesis experiments.[5–8] During the past decade, mass spectrometry (MS) analysis has gained interest for elucidating protein–protein and ligand−protein interactions.[9–13] The use of selective ligands to covalently bind a receptor of interest, followed by MS analysis, could in principle precisely reveal amino acid residues that are part of the respective ligand–receptor binding sites. Moreover, MS techniques are well-suited for the analysis of post-translational modifications but not frequently applied in the analysis of GPCRs. To apply MS techniques to GPCRs, improved methods for membrane protein production and proteomics analysis are needed to overcome the major hurdles for these important integral membrane proteins, for example, low

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expression levels and, more importantly, their highly hydrophobic nature at the TM domains, which form the main ligand binding pocket for most small molecules.[1]

We are particularly interested in the therapeutically relevant human histamine H_1 receptor (hH_1R), a prototypic member of the family A subfamily of GPCRs, and have studied it here as a model system to address several aspects of GPCR production, purification, and detection with the emphasis on mass spectrometric analysis of transmembrane regions and post-translational modifications. Milligrams of hH_1R protein were produced using a baculovirus/insect cells expression system and a relatively new *in vitro* cell-free expression system, which proves to be an alternative method for rapidly producing GPCRs. The hH_1R protein was subsequently solubilized and purified using immobilized metal affinity chromatography (IMAC),[14] reconstituted into artificial membranes, and functionally active as measured by radioligand binding experiments. Following SDS-PAGE and tryptic digestion, hH_1R fragments were analyzed by MALDI-ToF and/or LC-ion trap MS. Using these techniques, we were able to directly determine most of the primary amino acid composition of the hH_1R protein covering five of the TM domain regions from both sources of protein production. Furthermore, we were able to identify an amino acid residue in the hH_1R protein that is subject to glycosylation upon expression in insect cells.

Experimental Procedures

Reagents and Materials. Standard laboratory chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) if not otherwise specified. Insect-Xpress insect medium, penicillin, and Streptomycin were purchased from Bio Whittaker. Ni-NTA Agarose resin, NuPAGE Novex Bis-Tris Gel, and SimplyBlue Safestain were purchased from Invitrogen.

***In Vitro* Cell-Free hH_1R Protein Synthesis.** The C-terminally His_6 hH_1R cDNA was amplified by PCR using Pfu DNA polymerase (Fermentas) and directly subcloned into the pET102/D-TOPO (Invitrogen), according to manufacturer's protocol. In a 1 mL reaction mixture of the RTS 500 ProteoMaster *Escherichia coli* HY (Roche), 20 μg of plasmid DNA was incubated at 25 °C for 24 h. The RTS reaction mixture was collected, and aliquot amounts were analyzed by gel electrophoresis and Western blotting.

Baculovirus Expression System. *Spodoptera frugiperda* ovarian cells (*Sf9*) were cultured as monolayers at 27 °C in tissue culture flasks in a serum-free insect medium supplemented with 50 units/mL penicillin and 50 mg/mL streptomycin. Under these conditions, the cell doubling time was typically 20–24 h. Cells were infected with pBac-H_1His_{10} at a multiplicity of infection (MOI) of 0.1. Infected *Sf*9 cells were maintained in a serum-free insect medium supplemented with 5 μM leupeptin, 50 units/mL penicillin, and 50 mg/mL streptomycin and harvested at 5 days post infection (dpi).

Protein Purification and Reconstitution of the hH_1R Protein. The RTS reaction mixture containing hH_1R protein obtained from *in vitro* cell-free protein synthesis was solubilized in Bis-Tris propane buffer (20 mM Bis-Tris propane, 1 M NaCl, 1 mM Histidine, 5 mM β-mercaptoethanol, and 5 μM Leupeptin, pH 7.8), containing 2% (w/v) *n*-dodecyl-β-D-maltoside (DDM), under gentle rotation at 4 °C for 4 h. The supernatant was separated by ultracentrifugation at 80 000*g* for 20 min at 4 °C and incubated with 2 mL Ni-NTA Agarose resin, pre-equilibrated with Bis-Tris propane buffer. After overnight incubation at 4 °C, Ni-NTA agarose resin was settled by gravity, and the unbound lysate was aspirated. The resin was washed with Bis-Tris propane buffer, containing 0.2% (w/v) DDM, and imidazole (concentration 20 and 100 mM). The bound hH_1R proteins were eluted with Bis-Tris propane buffer, containing 0.2% (w/v) DDM and 200 mM imidazole. Purified hH_1R protein was added to asolectin liposomes, prepared according to a protocol based on Hale and co-workers,[15] at a molar ratio of protein/lipid of 1/300. The mixture was incubated on ice for 30 min. Subsequently, prewashed SM-2 Bio-Beads (Bio-Rad) were added in 10-fold excess to the detergent, and the mixture was incubated overnight at 4 °C on a shaker. Proteoliposomes were collected by centrifugation at 40 000*g* for 1 h at 4 °C and resuspended in H_1R binding buffer (50 mM Na_2/K Phosphate buffer, pH 7.4). The purity and identity of the reconstituted hH_1R protein was analyzed by gel electrophoresis, followed by protein staining or Western blotting. For hH_1R protein obtained from baculovirus expression system, the hH_1R *Sf9* cell membrane was prepared based on the protocol according to Ratnala and co-workers,[14] and subsequently solubilized, purified, and reconstituted in a similar manner as described above.

Gel Electrophoresis and Western Blotting. Protein samples were mixed with SDS-PAGE sample buffer (2% (w/v) sodium dodecyl sulfate, 0.04 M dithiothreitol, and 0.015% bromophenol blue in 0.5 M Tris-HCl, final pH 6.8) to a final volume of 25–50 μL. Samples were run on a NuPAGE Novex Bis-Tris Gel at 200 V constant, according to the manufacturer's protocol. For immunodetection, proteins were blotted onto a nitrocellulose membrane (1 h at 100 V) in ice-cold blot buffer (25 mM Tris and 0.2 M glycine in 20% methanol). Blots were subsequently immunoassayed for the presence of His-tagged receptor, using anti-His antibody (1:2000 dilution) (Qiagen). Protein staining was performed using Coomassie G-250 stain (SimplyBlue Safestain).

Radioligand Binding Assays. The collected proteoliposomes were resuspended and homogenized in Bis-Tris propane buffer. Proteoliposomes (1 μg protein/mL) were incubated with 15 nM [^{3}H] mepyramine (specific activity 30 Ci/mmol) (Perkin-Elmer) in the presence of unlabeled ligands (10^{-3}–10^{-10}) in a final volume of 200 μL of H_1R binding buffer (50 mM Na_2/K Phosphate buffer, pH 7.4) for 30 min at 25 °C. Nonspecific binding was determined by incubation with 1 μM of the selective H_1R antagonist mianserin. Nonbound radioactivity was removed by filtration through Whatman GF/C filters that had been treated with 0.3% polyethyleneimine. Filters were washed 3–4 times with ice-cold H_1R binding buffer, and radioactivity retained on the filters was measured by liquid scintillation counting. Binding data was evaluated by a non-linear, least-squares, curve-fitting procedure using GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

In-Gel Tryptic Digestion and Deglycosylation. Protein bands were excised from a NuPAGE Novex Bis-Tris gel, cut into small pieces, and washed with 25 mM NH_4HCO_3 followed by dehydration with 100% acetonitrile (ACN) for 10 min. For reduction and alkylation, dried gel particles were first incubated with 10 mM dithiothreitol for 30 min at 56 °C. Following dehydration with ACN, gel plugs were subsequently incubated in 55 mM iodoacetamide for 20 min at room temperature. After washing with 25 mM NH_4HCO_3 and dehydration with 100% ACN, the gel particles were completely dried in a centrifugal vacuum concentrator (Eppendorf, Hamburg, Germany). These dried gel plugs were either processed for standard in-gel tryptic digestion or tryptic digestion using RapiGest™ SF (Waters).

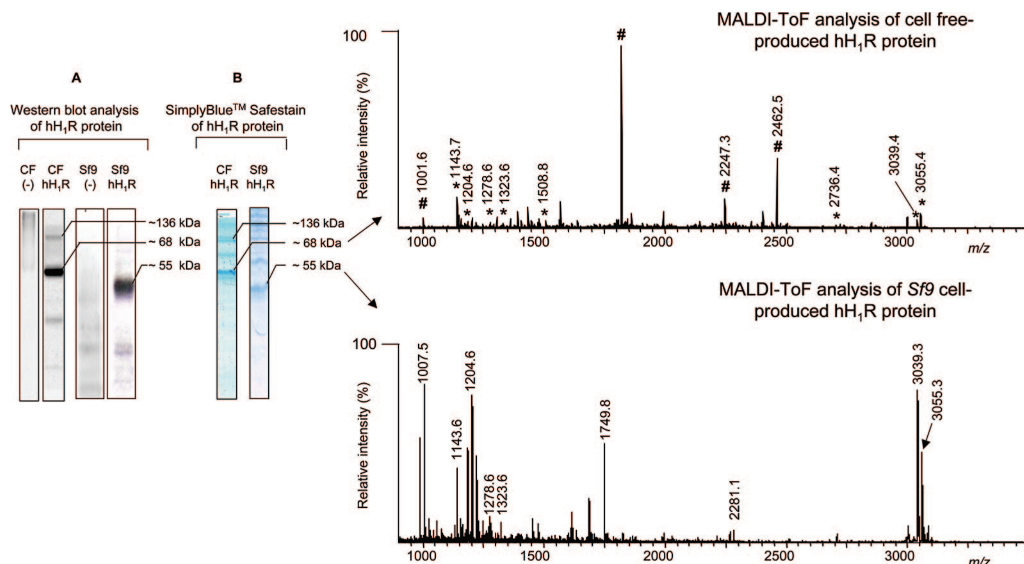

Figure 1. hH_1R protein produced via *in vitro* cell-free and baculovirus/insect *Sf*9 cells expression system. Purified hH_1R protein samples and controls from *in vitro* cell-free and baculovirus/insect *Sf*9 expression systems were detected by Western blot analysis using anti-His antibody and SimplyBlue Safestain. The controls were obtained from RTS cell free reaction without hH_1R cDNA and noninfected *Sf*9 cells. The protein bands at 68 kDa (cell free; CF) and 55 kDa (*Sf*9 cells) were excised from polyacrylamide gel, trypsin-digested, and subjected to MALDI-ToF MS analysis. *, hH_1R tryptic peptides; #, C-terminal tag derived tryptic peptides.

For standard tryptic digestion, the dried gel particles were reswollen for 45 min on ice by addition of 10–15 μL of a trypsin solution (12.5 ng/μL in 25 mM NH_4HCO_3). Tryptic digestion was subsequently performed overnight at 37 °C. For in-gel digestion using RapiGest™ SF, dried gel particles were reswollen in a 1% RapiGest™ SF solution (in 25 mM NH_4HCO_3) followed by 10 min incubation at 37 °C. Excess solution was removed, and the gel particles were dried in a centrifugal vacuum concentrator. Dried gel particles were subsequently reswollen on ice and incubated with trypsin as described above. Following tryptic digestion, the overlaying digestion-solution was collected. In case of digestion in RapiGest™ SF, 0.5% trifluoroacetic acid (TFA) (final concentration) was added to the digestion solution followed by incubation for 40 min at 37 °C. The sample was centrifuged at 20 000*g*, for 10 min. Finally, the resulting supernatant was collected, and the pellet was extracted two times with 7.5 μL of 70% isopropanol. For subsequent deglycosylation, the supernatant was adjusted to pH 8 using ammonium bicarbonate and incubated with 5 Units of PNGase F (Roche Diagnostics, Mannheim, Germany) overnight at 37 °C.

MALDI-ToF MS. MALDI-ToF(-ToF) analyses were performed on an Ultraflex II mass spectrometer (Bruker Daltonics, Bremen, Germany) using dihydroxybenzoic acid (5 mg/mL in 50%ACN/0.1% TFA) as a matrix. The mass spectrometer was used in the reflector ion mode. For fragment ion analysis in the tandem time-of-flight (ToF/ToF) mode, precursors were selected in a timed ion gate. Fragment ions generated by laser-induced decomposition of the precursor were further accelerated in the LIFT cell, and their masses were analyzed after the ion reflector passage.

Nano-LC-Iontrap MS. The samples containing the digested hH_1R in 70% isopropanol were concentrated to approximately 1–2 μL in a centrifugal vacuum concentrator. Subsequently, 7 μL of 70% ACN/0.1%TFA was added, and the sample was sonified for 10 min. Next, 0.1% TFA was added until a concentration of 20% ACN/0.1% TFA was reached. This was then shortly sonified (2 min) just prior to injection onto the

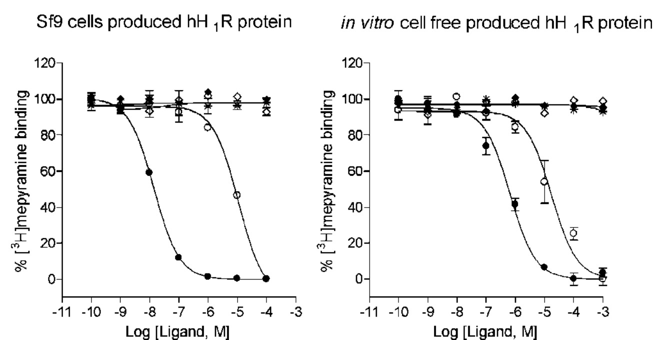

Figure 2. Pharmacological characterization of *Sf*9 cells expressed hH_1R protein and reconstituted hH_1R protein produced via *in vitro* cell-free expression. The pharmacology of the hH_1R protein was determined by competition binding assays using mepyramine (•), histamine (○), ranitidine (♦), thioperamide (◇), and JNJ 7777120 (×) to displace [^{3}H] mepyramine binding.

nanoLC system (Ultimate, Dionex-LC Packings, The Netherlands). The sample was separated on a C_{18} pepmap100 column (75 μm i.d., 15 cm long; Dionex-LC packings, Amsterdam, The Netherlands) coupled with an electrospray ionization (ESI)-iontrap mass spectrometer (HCTultra, Bruker Daltonics, Bremen). The gradient profile started with 100% solvent A (20% ACN/0.1% formic acid) for 10 min. This was followed by a 20 min linear gradient from 20% solvent B (95% ACN/0.1% formic acid) up to 60% solvent B. The system remained at 60% solvent B for 25 min, followed by a second linear gradient up to 90% solvent B in 10 min. After 30 min at 90% solvent B, the gradient was changed to 0% solvent B. The flow rate was maintained at 200 nL/min during the entire run. Peptides were detected in the positive ion mode. Upon detection of target *m*/*z* values, parent ion selection and MS/MS analysis was performed manually. Helium was used as a collision gas for the collision-induced dissociation (CID).

For the analysis of glycopeptides, samples were injected onto the same system as described above (equilibrated with 100% buffer A, 0.4 ACN/0.1% formic acid) but eluted using a linear

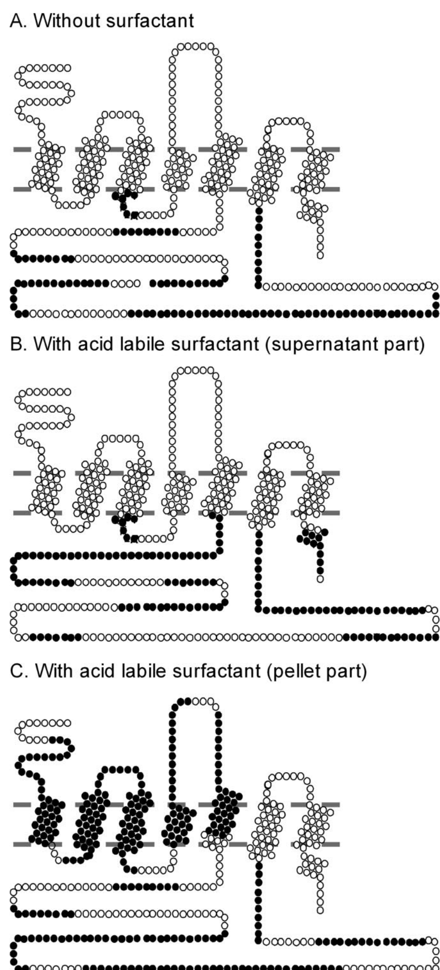

Figure 3. Schematic representation of sequence coverage obtained by MALDI-ToF-MS analysis after tryptic digestion of the hH_1R protein using different digestion conditions. The filled circles in the typical snake plot representation of the hH_1R protein indicate amino acids covered by MALDI-ToF analysis, after in-gel tryptic digestion, (A) without surfactant, (B) with acid labile surfactant (supernatant part), and (C) with acid labile surfactant (pellet part).

gradient of 0% solvent B (95% ACN/0.1% formic acid) up to 60% solvent B in 60 min. In this case, MS/MS analysis was performed in a data-dependent manner using the five most intense peaks in an MS spectrum.

Results and Discussion

Expression, Purification, and Reconstitution of hH_1R. For the expression of hH_1R, we used two different approaches, a classical baculovirus/insect cell expression system,[14] and a relatively new *in vitro* cell-free expression system.[16–23] Generally, the baculovirus/insect cell expression system generates correct folding of recombinant protein, as well as disulfide bond formation, and allows post-translational modifications.[3] However, due to its laborious nature, we also employed an *in vitro* cell-free expression system as another approach to rapidly produce milligrams of hH_1R protein. There are several reports on the expression of membrane proteins using cell-free protein expression system.[16–23] However, most of these expressed proteins are small multidrug transporter membrane proteins. So far, only a very limited number of reports showed the successful use of cell-free expression on large membrane proteins, such as GPCRs.[18,19,21]

Using the baculovirus/insect cell expression system, *Sf9* cells were infected with baculovirus encoding C-terminal His_6-tagged hH_1R.[14] An *N*-dodecyl-*β*-D-maltoside cell membrane extract containing hH_1R protein was applied to Ni-NTA resin. After extensive washing, hH_1R protein was eluted with buffer containing 200 mM imidazole. The purified hH_1R protein was further reconstituted into asolectin liposomes. Over a large number of experiments, we obtained functional expression levels of hH_1R in *Sf9* cells in the range of 15–25 pmol/mg protein as estimated from radioligand binding assay. After purification and reconstitution process, approximately 0.9–1.5 mg of purified and functional receptor per liter of culture could be achieved. The purified protein was analyzed by SDS-PAGE followed by Western blot analysis (Anti-His antibody) or SimplyBlue Safestain. Both techniques detected a broadband with an apparent molecular weight of 55 kDa (Figure 1, A,B) corresponding to the molecular weight of hH_1R. In general, glycosylated proteins appear as broad bands in SDS-PAGE, and therefore, the *Sf*9 cell-produced hH_1R could be partially glycosylated (see below).

For *in vitro* cell-free expression, the hH_1R cDNA was inserted as a thioredoxin-fusion construct into the plasmid, pET102/D-TOPO. Previously, thioredoxin-fusion has been identified as a critical factor to obtain elevated expression levels for the β_2 adrenergic receptor, another prototypical GPCR family member.[21] The hH_1R-thioredoxin fusion protein was synthesized under the control of a T7 promoter and generally resulted in the production of 3–5 mg of hH_1R protein/mL of reaction within 20–24 h. Following IMAC purification, hH_1R protein was reconstituted into asolectin liposomes. In general, we obtained approximately 0.2–0.7 mg of purified and functional hH_1R using this system. The purified hH_1R protein was subsequently analyzed by Western blot analysis or SimplyBlue Safestain. The hH_1R protein, produced via the *in vitro* cell-free expression system appeared as a sharp band with an apparent molecular weight of 68 kDa (Figure 1, A,B). The increase in molecular weight is the result of the additional N- and C-terminal tags in the hH_1R-pET102/D-TOPO construct.

hH_1R protein bands were subjected to standard trypsin digestion followed by MALDI-ToF MS. Next to hH_1R-specific peptides (Figure 1, right upper part, indicated with an asterisk (*)), the highest signals from the *in vitro* cell-free produced hH_1R covered the C-terminal tag of the protein (Figure 1, right upper part, indicated with a pound sign (#)). Moreover, this spectrum contained peptides derived from the N-terminal tag (thioredoxin, data not shown). Because of the absence of these particular tags in the *Sf*9 produced hH_1R, we observed a clearer hH_1R fingerprint in this tryptic digest (Figure 1, right lower part). The total sequence coverage obtained with the standard tryptic digestion was relatively low and covered only peptides corresponding to intracellular domains (representative sequence coverage is shown in Figure 3A). We also observed an additional band at approximately 136 kDa in Western blot analysis of hH_1R obtained using the *in vitro* cell-free expression system (Figure 1, A,B, lane: cell free). Following tryptic digestion and MALDI-ToF MS, this band was also identified as hH_1R and, thus, probably represents a dimeric form of hH_1R (result not shown). To certify that this finding was not solely a result of smearing, we also further identified the protein bands surrounding this putative hH_1R dimer (Figure 1, A,B, lane: cell free). The protein band just below the putative hH_1R dimer was identified as pyruvate dehydrogenase complex component E1 (GI_75208426, 78 kDa) from *E. coli*, while the protein band

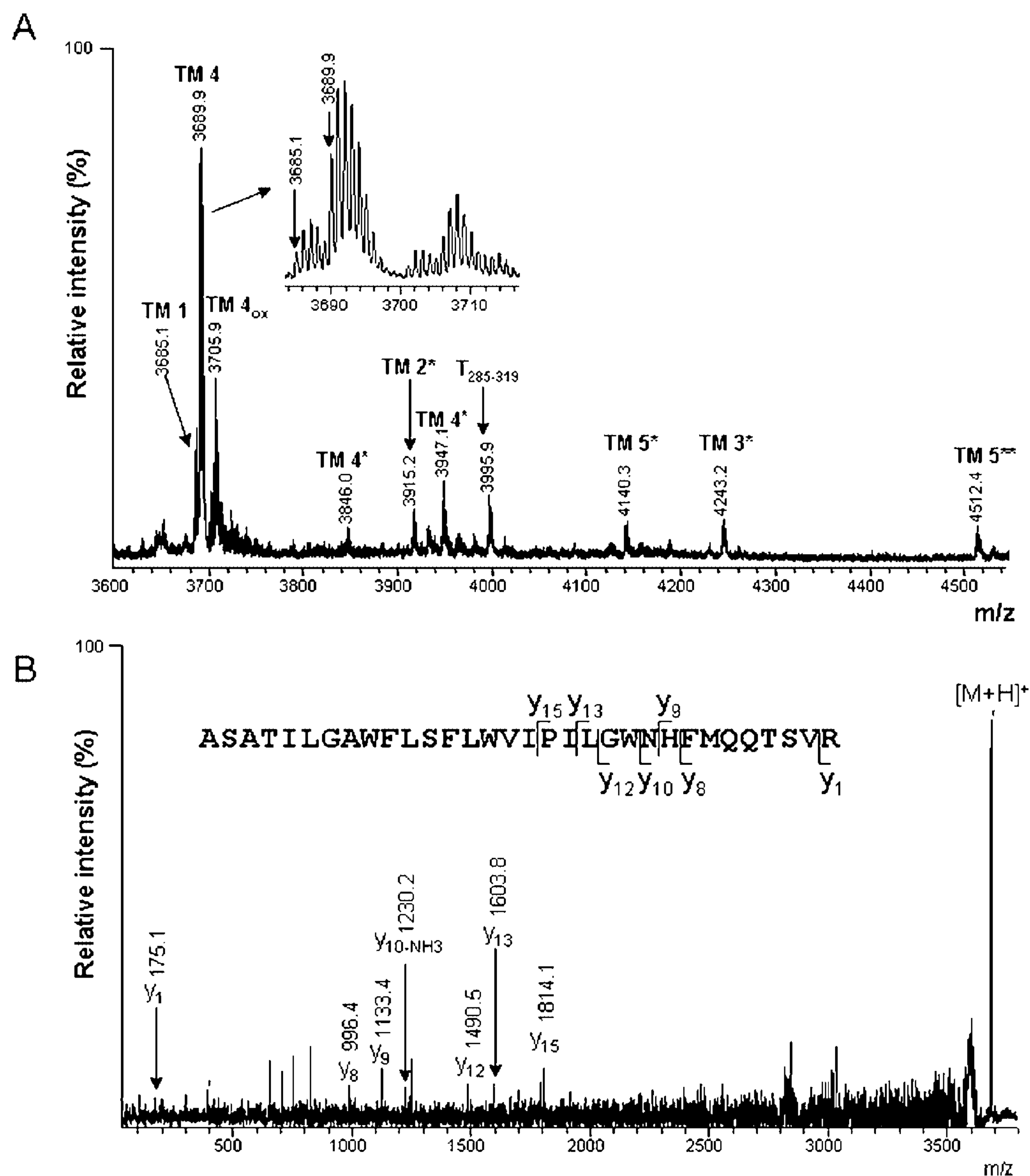

Figure 4. MALDI-ToF-MS analysis of tryptic peptides covering TM regions of hH_1R protein. The hydrophobic fraction obtained after tryptic digestion of hH_1R protein using Rapigest^{TM} SF was analyzed using MALDI-ToF. (A) Tryptic peptides covering TM 1–5 were resolved in the range of *m/z* 3600–4300. An asterisk (*) indicates a peptide with one and two asterisks (**) a peptide with two missed cleavage site(s); (B) MALDI-ToF-ToF of *m/z* 3689.9 corresponding to TM 4 of hH_1R.

Table 1. Summary of Transmembrane Spanning Tryptic Peptides from hH_1R Identified by MALDI-ToF MS[a]

region	peptide sequence	aa	theoretical mass $(M + H)^+$	measured mass $(M + H)^+$	missed cleavages	GRAVY index[b]
TM1	TTMASPQLMPLVVVLSTICLVTVGLNLLVLYAVR	20–53	3685.09	3685.09	0	1.571
TM2	KLHTVGNLYIVSLSVADLIVGAVVMPMNILYLLMSK	57–92	3915.19	3915.16	1	1.250
TM3	WSLGRPLCLFWLSMDYVASTASIFSVFILCIDRYR	93–127	4243.13	4243.24	1	0.806
TM4	TRASATILGAWFLSFLWVIP ILGWNHFMQQTSVR	142–175	3947.09	3947.12	1	0.603
	ASATILGAWFLSFLWVIPILGWNHFMQQTSVR	144–175	3689.95	3689.96	0	0.803
	ASATILGAWFLSFLWVIPILGWNHFMQQTSVRR	144–176	3846.05	3846.04	1	0.642
TM5	EDKCETDFYDVTWFKVMTAI INFYLPTLLMLWFYAK	177–212	4512.20	4512.36	2	0.303
	CETDFYDVTWFKVMTAIINFYLPTLLMLWFYAK	180–212	4140.04	4140.26	1	0.661
TM6	QLGFIMAAFILCWIPYFIFFMVIAFCK	416–442	3346.71	Not found	0[c]	1.989
TM7	NCCNEHLHMFTIWLGYINSTLNPLIYPLCNENFK	443–476	4225.98	Not found	0[c]	−0.003

[a] aa, amino acid; TM, transmembrane. [b] Grand average of hydropathicity, calculated at www.expasy.ch. [c] Tryptic peptides containing 1 or 2 missed cleavages were also not identified.

just above the putative hH_1R dimer was identified as DNA-directed RNA polymerase beta subunit (GI_26250759, 155 kDa) and RNA polymerase beta subunit (GI_12518912, 150 kDa) from *E. coli*, respectively. These findings provide evidence that the hH_1R protein is present most likely as both a monomer and dimer form when produced via *in vitro* cell-free expression

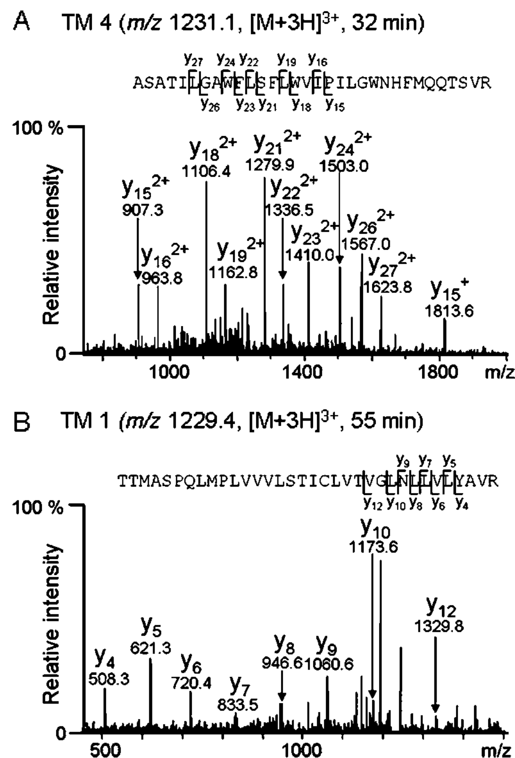

Figure 5. LC-ion trap MS analysis of tryptic peptides covering TM regions of *in vitro* cell-free produced hH_1R protein. The hydrophobic fraction obtained after tryptic digestion of hH_1R protein using Rapigest^{TM} SF was analyzed by LC-Liontrap MS. Shown are the MS/MS spectra of *m*/*z* 1231.1 corresponding to TM 4 (A) and at *m*/*z* 1229.4 corresponding to TM 1 (B).

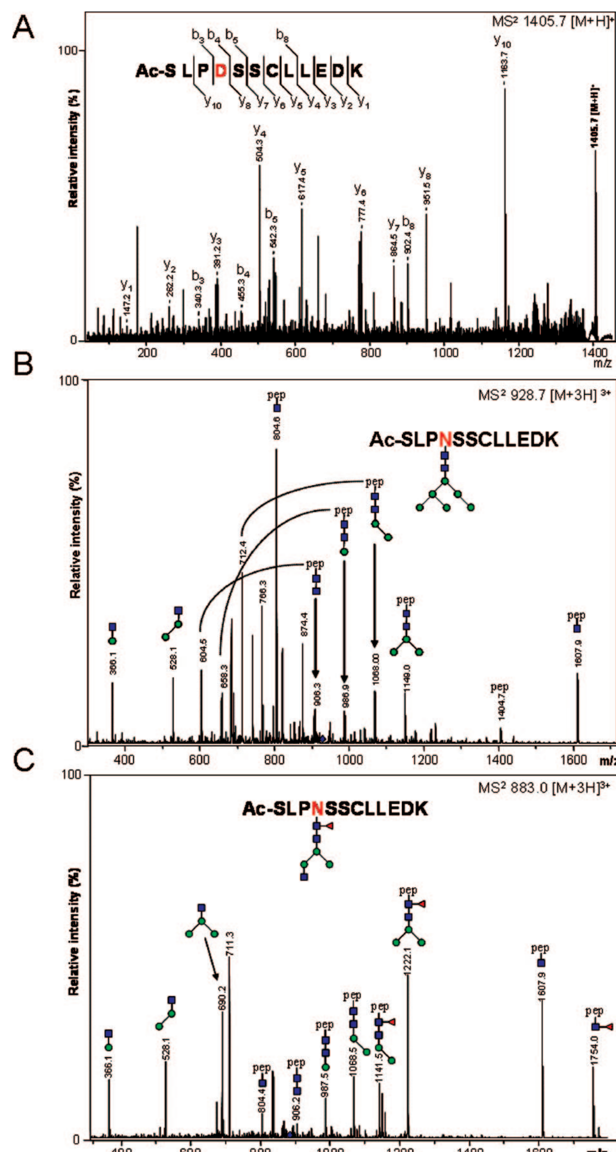

Figure 6. Identification of hH_1R glycosylation. Tryptic peptides from *Sf*9 cell produced hH_1R were deglycosilated using PNGaseF and analysed by MALDI-ToF MS. A peptide at *m*/*z* 1405.7 was selected for MALDI-ToF-ToF and identified as the N-terminal tryptic peptide from hH1R containing an Asp residue at position 4 which corresponds to the Asn-5 residue in full-length hH_1R after PNGase F treatment (A). Glycopeptides containing Asn-5 were further analysed by LC-iontrap MS/MS (B and C). Red triangle, fucose; blue square, *N*-acetylglucosamine; green circle, mannose.

system, as confirmed by Western blot and subsequent MS analysis after in-gel tryptic digestion.

Pharmacological Properties of Purified and Reconstituted hH_1R Protein. The ability of the purified and reconstituted hH_1R protein to interact with H_1R ligands was assessed by radioligand binding studies using the inverse agonist [^{3}H]mepyramine as H_1R selective radioligand and competition with histamine and selective H_1, H_2, H_3, and H_4 receptor antagonists. The selective H_2R antagonist ranitidine, the H_3R/H_4R selective inverse agonist thioperamide, and the H_4R selective antagonist JNJ 7777120 all failed to displace [^{3}H]mepyramine binding to purified hH_1R proteins (Figure 2). Yet, the binding of [^{3}H]mepyramine to baculovirus-produced hH_1R protein was displaced by the endogenous hH_1R agonist (histamine) and by unlabeled mepyramine with pK_i values of 4.3 ± 0.2 (mean ± SEM, $n = 3$) and 8.1 ± 0.1 (mean ± SEM, $n = 3$), respectively (Figure 2). These values are in good agreement with results reported previously,[14] and in mammalian COS-7 cells expressing hH_1R.[24] Similar values were observed for the cell-free produced hH_1R (pK_i values of 4.6 ± 0.3 (mean ± SEM, $n = 3$) and 6.7 ± 0.2 (mean ± SEM, $n = 3$), respectively) (Figure 2). The reduction in binding affinity observed for the potent H_1R inverse agonist mepyramine might be explained by the absence of post-translational modifications, such as glycosylation and palmitoylation in the cell-free expression system.[25–28] Moreover, also the interaction with G proteins has previously been reported to be important for high-affinity binding of mepyramine.[27] At this moment, we cannot distinguish between the various options, and future experiments should address this issue. Notwithstanding this, the cell-free system allows rapid production of hH_1R, which upon reconstitution is functionally active in our binding assays and therefore is a very attractive alternative for the more laborious baculovirus-driven expression.

Mass Spectrometry Analysis of hH_1R Transmembrane (TM) Regions. As described above, the total sequence coverage of the hH_1R, obtained from standard in-gel tryptic digestion was relatively low, and only peptides corresponding to intracellular domains were identified (Figure 3A). The lack of MS signals for the TM peptides following the standard tryptic digestion most probably arises from a combination of inefficient digestion and poor recovery of the very hydrophobic fragments in combination with inefficient mass spectrometric analysis. Several studies have addressed the difficulties in

proteomic analysis of highly hydrophobic transmembrane proteins, especially with respect to MS/MS analysis of GPCR transmembrane spanning peptides.[29] MALDI-ToF MS has given variable results for the detection of transmembrane regions.[30–32] Considering sample preparation, several alternative digestion methods (e.g., CNBr, Chymotrypsin, and Pepsin), in-solution versus in-gel digestion, and the use of detergents (e.g., octyl-β-glucopyranoside) have been applied.[13,30–34] The use of detergents often results in reduced proteolytic activity and compatibility problems with downstream mass spectrometric techniques.[35–37] Recently, a mass spectrometry compatible acid-labile surfactant (ALS) was developed and was proven successful to improve sequence coverage for trypsin digestion.[38–40] We are particularly interested in the development of methods for in-gel digestion of GPCRs in combination with MALDI-ToF MS because it can be applied to SDS-PAGE separated, and therefore more complex mixtures, and it is favorable for high-throughput screening. The *in silico* tryptic digest of hH_1R shows large hydrophobic peptides corresponding to the transmembrane domains, and as shown above, we were confronted with difficulties in recovering these peptides. Therefore, we performed in-gel digestions of hH_1R using the acid-labile surfactant. Upon acidification and centrifugation of the hH_1R digest, we analyzed the supernatant and the pellet fraction separately by MALDI-ToF-MS. In the pellet fraction, we expected to find the hydrophobic peptides corresponding to the transmembrane domains. Indeed, by using an extraction with 70% isopropanol, we could clearly identify *m/z* values corresponding to tryptic peptides from TM 1–5 in this fraction. (Figure 4A). This was true for both the *Sf*9 cell and cell-free produced hH_1R. The tryptic peptide fragments corresponding to the transmembrane domains are shown in Table 1. The highest intensity was observed for the tryptic peptide of TM 4 (aa 144–175) at *m/z* 3689.9. Because a tryptic peptide with a similar *m/z* value, 3689.7 (aa 327–360, 1 missed cleavage), is present within the *in silico* tryptic digest of the hH_1R, we additionally performed MALDI-ToF-ToF analysis of this ion to confirm its identity. Although the fragmentation of this peptide was not very efficient, the MS/MS data clearly showed that the observed *m/z* (3689.9) value corresponds to the tryptic peptide of TM 4 (Figure 4B). The reason we did not detect the tryptic peptides corresponding to TM 6 and 7 in our MS analysis remains unclear, but this could be due to either sample preparation (e.g., improper digestion, poor solubility) or mass spectrometric detection (poor ionization). The use of other enzymes such as pepsin for protein digestion and in-gel tryptic digestions in the presence of solvents such as methanol, isopropanol, or acetonitrile, also did not result in the identification of these peptides. We do not expect specific modifications that have been unrecognized to be responsible for the failure to detect these peptides, because they were not observed in the digest of both the *Sf*9 as well as in the cell-free produced hH_1R.

We also analyzed the hydrophobic peptides by LC−MS/MS. The additional power of this technique was most apparent for the MS/MS analysis of TM1 (*m/z* 3685.1) because proper selection of this peptide in our MALDI-ToF-ToF analysis was hampered by the presence of the more intense peak at *m/z* 3689.9 (TM 4). With LC−MS, the peptides corresponding to these two transmembrane domains were nicely separated, and their nature was confirmed by MS/MS analysis (Figure 5).

As expected, the supernatant of the acidified and centrifuged hH_1R digest contained only peptides covering intracellular domains (Figure 3B). In combination with the coverage obtained for the hydrophobic peptides (Figure 3C), a very high total sequence coverage (~80%) of the hH_1R was obtained. Importantly for our purposes, this was obtained by analyzing only two spots with MALDI-ToF MS (pellet and supernatant fraction from ALS digestion).

Analysis of hH_1R Glycosylation. Many GPCRs are glycosylated *in vivo*, and glycosylation has been shown to be implicated in proper GPCR expression, signaling, and trafficking.[25,26,41,42] The analysis of GPCR glycosylation have relied mainly on in-direct methods such as apparent molecular weight shifts after Western blotting of tunicamycin-treated cells or PNGase- treated protein extracts.[41,43] Moreover, site-directed mutagenesis of asparagines within N-glycosylation consensus sites have yielded valuable information, not only about the exact localization of the glycan moiety, but also on the functional consequences.[44,45]

Because of the common difficulties in proteomic analysis of GPCRs described above, studies applying mass spectrometric techniques for the direct assessment of the glycosylation sites in GPCRs are restricted to rhodopsin,[46–50] but MS/MS analysis of glycopeptides is often lacking. As described above, the hH_1R protein obtained from the baculovirus/insect cell expression system appeared as a smear (Figure 1A,B) possibly reflecting hH_1R glycosylation. The presence of post-translational modifications is one of the advantages of using this system in comparison with the cell-free expression.

Within the amino terminal domain, hH_1R contains 2 sequences (N^5SS and $N^{18}KT$) that fit with the consensus sequence for N-glycosylation (Asn-Xaa-Ser/Thr, Xaa being any amino acid except proline). It is hitherto unknown whether these sites are actually glycosylated. To address this question, we first analyzed a tryptic digest of hH_1R before and after treatment with PNGase F, using MALDI-ToF MS. PNGase F cleaves asparagine N-linked oligosaccharides at the β-aspartylglycosylamine linkage, converting asparagine into aspartic acid. Within the PNGaseF-treated sample, we identified a peptide at *m/z* 1405.7 which was absent in the untreated sample (data not shown). MALDI-ToF-ToF analysis revealed that it corresponds to the acetylated N-terminal tryptic peptide of hH_1R containing an aspartic acid at position 5. This aspartic acid corresponds to the converted asparagine within the consensus sequence Asn-Ser-Ser after PNGase F release, identifying this Asn^5 as a glycan attachment site. In these experiments we did not observe glycosylation at Asn^{18}. Although this probably reflects site-specific glycosylation at Asn^5, we cannot formally exclude the possibility of protein glycosylation at Asn^{18} as well. On the contrary, but not surprisingly, Asn^5 was not fully occupied since we also identified the nonglycosylated counterpart of this peptide (data not shown).

To analyze the Asn^5 glycosylation in more detail, we specifically searched for glycopeptides within our LC-iontrap MS data through the analysis of the glycopeptide marker ion at *m/z* 366 ($hexose_1HexNAc_1$[51]) in our MS/MS spectra. Two major groups of glycopeptides corresponding to different glycan structures attached to the Asn^5-containing tryptic peptide were observed. The first contained a heterogeneous population of high mannose structures (exemplified in Figure 6B). The other major group consists of N-glycans with terminal GlcNAc-residues with or without fucosyl residues linked to the *N,N'*-diacetyl-chitobiose core (exemplified in Figure 6C). These glycan structures were predicted based on what is known from glycosylation in insects.[52] Since glycosylation in insect cells does not resemble

mammalian type of glycosylation, future MS analysis of hH_1R protein purified from insect *Sf9* cells, stably expressing mammalian glycosyltransferases (Mimic^{TM} *Sf*9 insect cells), or purified from mammalian cells overexpressing the hH_1R should be performed to reveal the mammalian glycosylation pattern of the hH_1R protein.

Conclusion

In this study, we employed the therapeutically relevant hH_1R as a prototypical rhodopsin-like GPCR protein for the study of several aspects in protein production, purification, reconstitution, and the MS analysis of its transmembrane regions and Asn-linked glycosylation. We have shown that substantial quantities of hH_1R protein can be readily obtained using either baculovirus/insect cells as well as by using an *in vitro* cell-free expression system. The protein obtained can subsequently be purified by a simple and cost-effective affinity chromatography (IMAC). The hH_1R protein, purified from both expression systems, was successfully reconstituted into artificial membranes and found to retain its functionality as confirmed by radioligand binding experiments. The purified hH_1R protein was analyzed by Western blot analysis and ligand binding, as well as by MALDI-ToF and LC−MS/MS analysis. By employing an acid labile surfactant in our tryptic digestion, we were able to obtain very high sequence coverage, including five out of the seven transmembrane domains, only using MALDI-ToF MS. Furthermore, we succeeded for the first time in identifying a single amino acid residue (Asn^5) in the H_1R that is glycosylated upon expression in insect cells. Taken together, we have demonstrated successful functional expression in combination with comprehensive proteomic characterization of a prototype GPCR which will be important for future drug discovery and development.

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