

# Phenylalanine 169 in the Second Extracellular Loop of the Human Histamine H<sub>4</sub> Receptor Is Responsible for the Difference in Agonist Binding between Human and Mouse H<sub>4</sub> Receptors<sup>[S]</sup>

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## ABSTRACT

Using the natural variation in histamine H<sub>4</sub> receptor protein sequence, we tried to identify amino acids involved in the binding of H<sub>4</sub> receptor agonists. To this end, we constructed a variety of chimeric human-mouse H<sub>4</sub> receptor proteins to localize the domain responsible for the observed pharmacological differences between human and mouse H<sub>4</sub> receptors in the binding of H<sub>4</sub> receptor agonists, such as histamine, clozapine, and VUF 8430 [S-(2-guanidylethyl)-isothioureia]. After identification of a domain between the top of transmembrane domain 4 and the top of transmembrane domain 5 as being responsible for the differences in agonist affinity between human and

mouse H<sub>4</sub>Rs, detailed site-directed mutagenesis studies were performed. These studies identified Phe<sup>169</sup> in the second extracellular loop as the single amino acid responsible for the differences in agonist affinity between the human and mouse H<sub>4</sub>Rs. Phe<sup>169</sup> is part of a Phe-Phe motif, which is also present in the recently crystallized  $\beta_2$ -adrenergic receptor. These results point to an important role of the second extracellular loop in the agonist binding to the H<sub>4</sub> receptor and provide a molecular explanation for the species difference between human and mouse H<sub>4</sub> receptors.

Histamine is an important chemical mediator that exerts various (patho)physiological effects via a family of four histamine receptors, which in the last decade have been shown to belong to the large multi-gene family of membrane-bound G-protein coupled receptors (GPCR) (Hough, 2001). Like many ligands acting at GPCRs, histamine receptor antagonists have been shown previously to have important therapeutic use or promise. Histamine H<sub>1</sub> receptor (H<sub>1</sub>R) antagonists are widely used to control allergic conditions, whereas histamine H<sub>2</sub> receptor (H<sub>2</sub>R) antagonists have been widely

used to treat gastric ulcers (Parsons and Ganellin, 2006). The histamine H<sub>3</sub> receptor (H<sub>3</sub>R) has also attracted considerable attention. Currently, several potent H<sub>3</sub>R antagonists are tested in phase I or phase II clinical trials for diseases of the central nervous system, such as Alzheimer's disease, attention-deficit hyperactivity disorder, obesity, and schizophrenia (Celanire et al., 2005).

The histamine H<sub>4</sub> receptor (H<sub>4</sub>R) is the latest member of the histamine receptor family and was first reported in 2000 as a direct consequence of the efforts to elucidate the human genome sequence (Oda et al., 2000). The H<sub>4</sub>R is mainly expressed in a variety of cells of the immune system, and activation of the H<sub>4</sub>R results in chemotaxis of eosinophils, mast cells, and monocyte-derived dendritic cells, and modulation of chemical mediator production (Hofstra et al., 2003; Ling et al., 2004; Gutzmer et al., 2005; Dunford et al., 2006). Current experimental evidence suggests that the H<sub>4</sub>R is a

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**ABBREVIATIONS:** GPCR, G-protein coupled receptor;  $\beta_2$ AR,  $\beta_2$ -adrenergic receptor; EL2, second extracellular loop; H<sub>1</sub>R, histamine H<sub>1</sub> receptor; H<sub>2</sub>R, histamine H<sub>2</sub> receptor; H<sub>3</sub>R, histamine H<sub>3</sub> receptor; H<sub>4</sub>R, histamine H<sub>4</sub> receptor; VUF 8430, S-(2-guanidylethyl)-isothioureia; TM, transmembrane; JNJ 7777120, 1-[(5-chloro-1H-indol-2-yl)carbonyl]-4-methylpiperazine; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; NFAT, nuclear factor of activated T cells; IL, interleukin.

potential drug target for inflammatory diseases, such as allergic asthma, rheumatoid arthritis, and pruritis (de Esch et al., 2005; Thurmond et al., 2008).

The H<sub>4</sub>R protein is a G<sub>i</sub>-protein-coupled receptor of 390 amino acids, possessing all the hallmarks of the family A or rhodopsin-like family of GPCRs (de Esch et al., 2005). Like all aminergic receptors, the H<sub>4</sub>R possesses an aspartic acid residue in transmembrane domain (TM) 3, which has been implicated in the binding of a variety of aminergic ligands to their respective GPCR proteins (Shi and Javitch, 2002). Moreover, in TM5, a glutamic acid residue is conserved with the related H<sub>3</sub>R protein, and this amino acid is most likely responsible for the relative high affinity of both receptors for their agonist histamine. Mutagenesis and computational studies indeed confirm the involvement of those two amino acids (Shin et al., 2002; Uveges et al., 2002; Jongejan et al., 2008).

It has been reported that histamine and clozapine have a lower affinity for the mouse H<sub>4</sub>R in comparison with the human H<sub>4</sub>R (Liu et al., 2001). The existence of such pharmacological differences is not surprising given that the homology between the protein sequences of human and mouse H<sub>4</sub>R is relatively low (67%) for species orthologs. However, the previously identified aspartic acid and glutamic acid residues in TM3 and 5, respectively, are conserved in both species (Liu et al., 2001), indicating that additional residues are involved in the agonist binding to the H<sub>4</sub>R protein. By using the natural variation in H<sub>4</sub>R protein sequence, we tried to identify additional amino acids involved in agonist binding to the H<sub>4</sub>R. We constructed a variety of chimeric human-mouse H<sub>4</sub>R proteins to localize domains responsible for the observed pharmacological differences. After identification of a domain between the top of TM4 and the top of TM5 as being responsible for the observed differences in agonist affinity between human and mouse H<sub>4</sub>R, our chimeric approach was followed by site-directed mutagenesis to identify amino acid residues involved in the agonist binding to the H<sub>4</sub>R.

## Materials and Methods

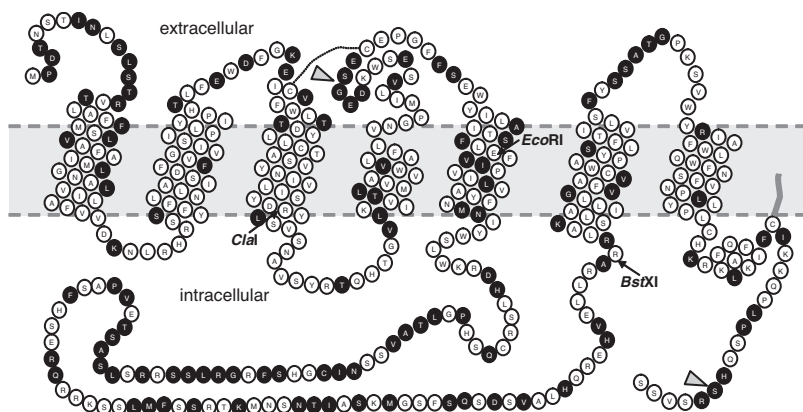
**Materials.** Dulbecco's modified Eagle medium (DMEM), penicillin, and streptomycin were purchased from Invitrogen Life Technologies (Merelbeke, Belgium). Fetal bovine serum was from Integro BV (The Netherlands), and cell culture plastic wares were obtained from Greiner Bio-one (Wommel, Belgium). Tris base was from AppliChem (Darmstadt, Germany), whereas linear 25-kDa polyethyleneimine (PEI) was from Polysciences (Warrington, PA). Histamine dihydrochloride, clozapine, and 750-kDa PEI were purchased from Sigma-

Aldrich (St. Louis, MO), whereas VUF 8430 (Lim et al., 2006) and JNJ 7777120 (Thurmond et al., 2004) were synthesized at the Department of Medicinal Chemistry (Vrije Universiteit, Amsterdam, The Netherlands). [<sup>3</sup>H]Histamine (18.10 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Oligonucleotides primers for PCR were synthesized by Isogen Bioscience (Maarsse, The Netherlands). Endonuclease restriction enzymes, T<sub>4</sub> DNA ligase, and *Pfu* DNA polymerase were obtained from MBI Fermentas (St. Leon-Rot, Germany).

**DNA Constructs and Site-Directed Mutagenesis.** The human H<sub>4</sub>R cDNA cloned in pcDNA3.1 was purchased from cDNA Resource Center (Guthrie Research Institute, Sayre, PA) and subcloned into a mammalian expression vector, pcDEF3 (a gift from Dr. J. Langer), at BamHI and XbaI sites (Goldman et al., 1996). Mouse H<sub>4</sub>R cDNA obtained from Johnson & Johnson Pharmaceutical Research & Development, LLC (La Jolla, CA) was originally cloned in pcNeo (Liu et al., 2001) and subcloned into pcDEF3. The constructs were amplified in *Escherichia coli* JM109 (Promega, Leiden, The Netherlands). The human and mouse H<sub>4</sub>R both have a set of three unique conserved restriction sites that can be used to swap the cDNAs of these two receptors with each other (the sites are indicated in Fig. 1). ClaI was used to construct chimeras 1 and 2, EcoRI was used for chimeras 3 and 4, and BstXI was used for chimeras 5 and 6. Chimera 7 was constructed by swapping the C-terminal part of chimera 2 with that of mouse H<sub>4</sub>R at the EcoRI site, and chimera 8 was created by exchanging the C-terminal part of chimera 1 with that of human H<sub>4</sub>R at the EcoRI site. A complementary pair of oligonucleotide primers was used to introduce a BamHI restriction site around the codon encoding for residue 141 by PCR. This restriction site was used to create chimera 9, by swapping the N-terminal part of chimera 1 and the C-terminal part of the human H<sub>4</sub>R, and chimera 10, by combining the N-terminal fragment of the human H<sub>4</sub>R and the C-terminal of chimera 4. Moreover, introduction of the BamHI site also resulted in the V141I mutation in the human H<sub>4</sub>R.

Site-directed mutagenesis, including the multiple residue alteration in the second extracellular loop (EL2), was performed with a fusion PCR method by introducing the mutation in oligonucleotide primers. To allow detection of expressed mutant receptor proteins, all mutant receptors were tagged with an N-terminal FLAG peptide (DYKDDDDK). This epitope tagging does not affect H<sub>4</sub>R ligand binding (Shin et al., 2002). The identity of mutant cDNAs was verified by sequence analysis at ServiceXS (Leiden, The Netherlands).

**Cell Culture and Transfection.** HEK 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum, 50 IU/ml penicillin, and 50 μg/ml streptomycin in a humidified 5% CO<sub>2</sub> atmosphere at 37°C. For transfection, approximately 4 million cells were seeded in a 10-cm dish and cultured overnight. For transfection of each dish of cells, a transfection mixture was prepared in 0.5 ml of serum-free DMEM containing 5 μg of receptor plasmid and 25 μl of 1 mg/ml 25-kDa linear PEI. The mixture was incubated for 5 to 10 min at room temperature before it was added to the HEK 293T cell



**Fig. 1.** Snake plot of the human H<sub>4</sub>R protein. The residues of the human H<sub>4</sub>R identical with those of the mouse H<sub>4</sub>R are depicted in open circles. Differences between the two species homologs are indicated by the filled circles. The triangle pointing to the EL2 indicates the location of insertion of two residues in the mouse H<sub>4</sub>R, whereas the triangle pointing to the cytoplasmic tail shows the location of a deletion in the mouse H<sub>4</sub>R. The three arrows indicate the location of the used endonuclease restriction sites in the corresponding cDNA.

monolayer loaded with 5 ml of fresh cell culture medium. Two days after transfection, the cells were detached from the plastic surface by adding 5 ml/dish of phosphate-buffered saline containing 1 mM EDTA. Transfected cells were collected as pellets by centrifugation for 3 min at 200g and stored at  $-20^{\circ}\text{C}$  until use.

**[ $^3\text{H}$ ]Histamine Binding.** For radioligand binding studies, pellets of transfected cells were homogenized in  $\text{H}_4\text{R}$  binding buffer (100 mM Tris-HCl, pH 7.4). Saturation binding assay was performed using different concentrations of [ $^3\text{H}$ ]histamine (18.10 Ci/mmol), whereas nonspecific binding was determined by incubation in the presence of 3 to 10  $\mu\text{M}$  JNJ 7777120 in a total assay volume of 200  $\mu\text{l}$ . For displacement studies, cell homogenates were typically incubated with  $10^{-4}$  to  $10^{-11}$  M ligands (stock concentration was 10 mM in dimethyl sulfoxide) in the presence of approximately 7 or 20 nM [ $^3\text{H}$ ]histamine for the human  $\text{H}_4\text{R}$ -like or mouse  $\text{H}_4\text{R}$ -like receptors, respectively, in a total volume of 200  $\mu\text{l}$ . The reaction mixtures were incubated for 1 h at room temperature ( $22^{\circ}\text{C}$ ) and harvested on 96-well glass fiber C plates that were pretreated with 0.3% 750-kDa PEI. Binding data were analyzed using Prism 4.0 (GraphPad Software Inc., San Diego, CA).

**NFAT-Luciferase Reporter Gene Assay.** Approximately 4 million resuspended HEK 293T cells were transfected with a mixture containing 5  $\mu\text{g}$  of receptor plasmid, 2.5  $\mu\text{g}$  of a pcDNA3.1-based plasmid construct that bears both an NFAT-luciferase reporter gene and a copy of  $\text{G}\alpha_{\text{q}15}$  gene (Conklin et al., 1993), and 35  $\mu\text{l}$  of 1 mg/ml 25-kDa linear PEI. The transfected cells were immediately exposed to ligands in a white 96-well plate and incubated for 24 h. The luciferase activity was measured with a Victor<sup>2</sup> microplate reader (PerkinElmer Life and Analytical Sciences).

**Homology Modeling.** The human  $\text{H}_4\text{R}$  was modeled based on the crystal structure of  $\beta_2$ -adrenergic receptor (Protein Data Bank code 2RH1) (Cherezov et al., 2007), which lacks the N-terminal tail and contains  $\text{T}_4$  ligase structure in the third intracellular loop (IL). The latter was removed in the model template. Sequence alignment, homology modeling, and energy minimization were all performed using default settings in Molecular Operating Environment, version 2007.09 (Chemical Computing Group, Inc., Montreal, QC, Canada). A large part of IL3 of the  $\text{H}_4\text{R}$  was removed to fit the length of the IL3 of the template. Alignment constraints were applied to avoid gaps in TM domains between Thr<sup>146</sup> and Gly<sup>128</sup>, Leu<sup>167</sup>, and Pro<sup>149</sup> of  $\beta_2$ -adrenergic receptor and  $\text{H}_4\text{R}$ , respectively. In EL2, constraints were put between Cys<sup>191</sup> and Cys<sup>164</sup>, Phe<sup>193</sup>, and Phe<sup>168</sup>, as well as Tyr<sup>174</sup> and Ser<sup>156</sup> of the  $\beta_2$ -adrenergic receptor and  $\text{H}_4\text{R}$ , respectively. This alignment was used to run homology modeling and resulted in models with a preserved disulfide bridge.

## Results

### The Human and Mouse $\text{H}_4\text{R}$ s Display Pharmacological Differences. To delineate the structural basis for the

species differences between human and rodent  $\text{H}_4\text{R}$  proteins (Liu et al., 2001), we transiently expressed human and mouse  $\text{H}_4\text{R}$  in human embryonic kidney HEK 293T cells. Upon expression, both mouse and human  $\text{H}_4\text{R}$  are well expressed but display a clearly different affinity for [ $^3\text{H}$ ]histamine as determined by saturation binding analysis (Table 1; Supplemental Fig. 1). Whereas the human  $\text{H}_4\text{R}$  binds [ $^3\text{H}$ ]histamine with a  $K_d$  value of 9 nM, the mouse homolog binds [ $^3\text{H}$ ]histamine with an almost 9-fold lower affinity of 78 nM (Table 1). Similar results were obtained by displacement of [ $^3\text{H}$ ]histamine binding with unlabeled histamine (Table 1). Furthermore, displacement studies also revealed species differences for other  $\text{H}_4\text{R}$  agonists, such as clozapine and VUF 8430 (Table 1). Both compounds have a higher affinity for the human  $\text{H}_4\text{R}$  ( $\text{p}K_i$  values of 6.35 and 7.46, respectively) in comparison with the mouse  $\text{H}_4\text{R}$  ( $\text{p}K_i$  values of 5.49 and 6.75, respectively). For the  $\text{H}_4\text{R}$  antagonist JNJ 7777120, no difference in affinity for the two receptor proteins was noticed (Table 1). The results obtained for JNJ 7777120, histamine, and clozapine corroborate previous findings (Liu et al., 2001; Thurmond et al., 2004).

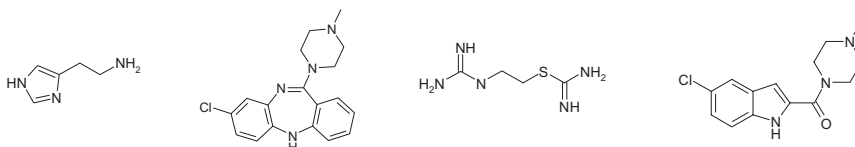
**Generation and Characterization of Human-Mouse  $\text{H}_4\text{R}$  Chimeras.** The pharmacological differences observed between human and mouse  $\text{H}_4\text{R}$ s are not surprising in view of the relative low homology between the human and mouse  $\text{H}_4\text{R}$  protein sequences (67%). As can be seen in the snakeplot in Fig. 1, the residues that differ between the two species variants are distributed throughout the entire receptor protein. TM domains TM2, TM3, and TM7 and the first and second intracellular loops are relatively conserved, but in the other regions, substantial differences are observed, including the insertion/deletion of nonconserved stretches in the EL2 (DEGSE<sup>159–163</sup> in the human  $\text{H}_4\text{R}$  and NSTNTKD<sup>159–165</sup> in the mouse  $\text{H}_4\text{R}$ ) and the C-terminal tail (HSR<sup>384–386</sup> in the human  $\text{H}_4\text{R}$  and NQ<sup>386–387</sup> in the mouse  $\text{H}_4\text{R}$ ) (Fig. 1).

In view of the relatively large sequence variation, we adopted a chimeric receptor strategy to determine structural features responsible for the differences between the human and mouse  $\text{H}_4\text{R}$  in the binding of the  $\text{H}_4\text{R}$  agonists histamine, clozapine, and VUF 8430. Unique endonuclease restriction sites in the cDNAs, encoding the human and mouse  $\text{H}_4\text{R}$ s (Fig. 1), allowed us to conveniently swap receptor domains at the bottom of TM3 (using ClaI), at the middle of TM5 (using EcoRI), and at the bottom of TM6 (using BstXI). In chimeras 1, 3, and 5, we gradually increased the extent of human  $\text{H}_4\text{R}$

TABLE 1

Pharmacological analysis of human and mouse  $\text{H}_4\text{R}$  proteins, using [ $^3\text{H}$ ]histamine saturation analysis and displacement studies. Equilibrium dissociation constants ( $K_d$ ) and  $B_{\text{max}}$  values for [ $^3\text{H}$ ]histamine (picomole per milligram protein) and affinity ( $\text{p}K_i$ ) of  $\text{H}_4\text{R}$  agonists for the human and mouse  $\text{H}_4\text{R}$ s are presented as average  $\pm$  S.E.M. of results of at least three independent experiments.

Receptor	$K_d$	$B_{\text{max}}$	$\text{p}K_i$			
			Histamine	Clozapine	VUF 8430	JNJ 7777120
	<i>nM</i>					
h $\text{H}_4\text{R}$	9.0 $\pm$ 0.1	2.71 $\pm$ 1.00	7.89 $\pm$ 0.05	6.35 $\pm$ 0.03	7.46 $\pm$ 0.02	8.31 $\pm$ 0.08
m $\text{H}_4\text{R}$	77.5 $\pm$ 11	1.48 $\pm$ 0.24	7.09 $\pm$ 0.04	5.49 $\pm$ 0.07	6.75 $\pm$ 0.10	8.40 $\pm$ 0.04





protein sequence, whereas in chimeras 2, 4, and 6, reciprocal changes were introduced (Table 2). All six chimeric H<sub>4</sub>R proteins were expressed upon transient expression in HEK 293T cells and bound histamine with moderate to high affinity. Exchanging the first part of the mouse H<sub>4</sub>R protein (up to Arg<sup>112</sup> at the bottom of TM3) with the related human protein sequence as in chimera 1 resulted in a chimeric protein with moderate affinity for histamine (Fig. 2,  $pK_i = 7.20$ ) and a pharmacological profile similar to the mouse H<sub>4</sub>R (Table 2). Chimera 2, in which the first part of the human H<sub>4</sub>R protein is replaced by the mouse protein sequence (Met<sup>1</sup>-Arg<sup>112</sup>), retained a high affinity for histamine (Fig. 2,  $pK_i = 7.84$ ), similar to value obtained for the human H<sub>4</sub>R (Table 2). The same trend was observed for the other H<sub>4</sub>R agonists, clozapine and VUF 8430 (Table 2), indicating that the TM domains 1 to 3 are not involved in the differential binding of the H<sub>4</sub>R agonists.

When a larger part of the human H<sub>4</sub>R sequence was used to replace the mouse sequence, as in chimera 3 (Met<sup>1</sup>-Glu<sup>182</sup> in the middle of TM5) and chimera 5 (Met<sup>1</sup>-Ser<sup>304</sup> in the bottom of TM6), the affinity of histamine increased to  $pK_i$  values of 7.98 (chimera 3) and 8.00 (chimera 5). These values are not significantly different from the value obtained for the binding of histamine to the human H<sub>4</sub>R (Fig. 2; Table 2). The reciprocal counterparts, chimeras 4 and 6, showed opposite results. Both chimeric receptors lost affinity for histamine upon the introduction of larger stretches of the mouse H<sub>4</sub>R sequence (Fig. 2; Table 2). The change in affinity of chimeras 3, 4, 5, and 6 is not only observed for histamine, similar changes in affinity were found for clozapine and VUF 8430 (Table 2).










The pharmacological properties of the chimeric human-

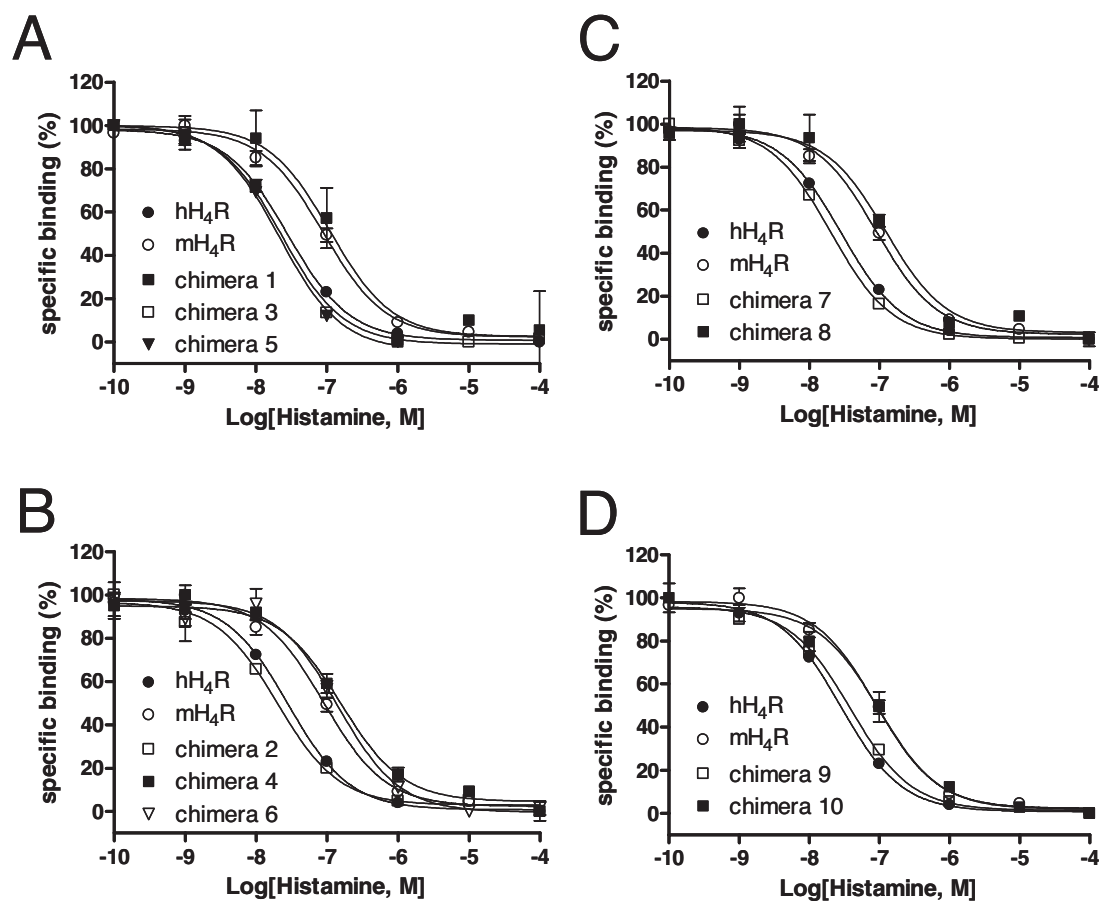
mouse H<sub>4</sub>R proteins 1 to 6 altogether suggest that the domain determining the differences in binding of the tested H<sub>4</sub> agonists is located between Arg<sup>112</sup> at the bottom of TM3 and Glu<sup>182</sup> in the middle of TM5. To investigate this hypothesis, we constructed chimeras 7 and 8 in which just the middle parts of the receptor proteins (bottom TM3 up to the middle of TM5 using the ClaI and EcoRI DNA restriction sites; Fig. 1) were exchanged. As can be seen in Table 3 and Fig. 2, the affinity of all three agonists is dependent on the nature of the middle part of the H<sub>4</sub>R protein sequence. Chimera 7, which possesses a small part of the human protein sequence (Arg<sup>112</sup> up to Glu<sup>182</sup>) in a large mouse H<sub>4</sub>R backbone, shows a human H<sub>4</sub>R pharmacology with respect to the three tested agonists (Table 3). The findings with the reciprocal chimera 8 are completely in line with these results (Table 3; Fig. 2). Introduction of the mouse protein sequence between the bottom of TM3 and the top of TM5 (Arg<sup>112</sup> up to Glu<sup>184</sup>) in the human H<sub>4</sub>R backbone results in a chimeric protein with mouse H<sub>4</sub>R pharmacology.

To further refine our search to the H<sub>4</sub>R domain responsible for the human-mouse species difference, we created chimera 9 and chimera 10, which both have only small amino acid stretches exchanged within TM domains 4 and 5 (Table 3). Chimera 9 displays similar affinity for histamine (Fig. 2D), clozapine, and VUF 8430 as found for the human H<sub>4</sub>R (Table 3), whereas the binding properties of chimera 10 closely resemble the binding profile of the mouse H<sub>4</sub>R (Fig. 2; Table 3). These data clearly pinpoint to the receptor domain between the extracellular half of TM4 and extracellular half of TM5 (Val<sup>141</sup> up to Glu<sup>182</sup>) as a structural determinant for high affinity agonist binding to the H<sub>4</sub>R.

TABLE 2

Pharmacological analysis of chimeric human and mouse H<sub>4</sub>R proteins, using [<sup>3</sup>H]histamine saturation analysis and displacement studies. Equilibrium  $K_d$  and  $B_{max}$  values for [<sup>3</sup>H]histamine (picomole per milligram protein) and  $pK_i$  of all ligands tested at the six chimeras are presented as average  $\pm$  S.E.M. of results of at least three independent experiments.

Receptor		$K_d$	$B_{max}$	$pK_i$		
				Histamine	Clozapine	VUF 8430
		<i>nM</i>				
hH <sub>4</sub> R		9.0 $\pm$ 0.1	2.71 $\pm$ 1.00	7.89 $\pm$ 0.05	6.35 $\pm$ 0.03	7.46 $\pm$ 0.02
mH <sub>4</sub> R		77.5 $\pm$ 11	1.48 $\pm$ 0.24	7.09 $\pm$ 0.04	5.49 $\pm$ 0.07	6.75 $\pm$ 0.10
Chimera 1		80.3 $\pm$ 7.1	0.51 $\pm$ 0.15	7.20 $\pm$ 0.07	5.48 $\pm$ 0.03	6.74 $\pm$ 0.13
Chimera 2		10.1 $\pm$ 0.6	2.64 $\pm$ 0.46	7.84 $\pm$ 0.13	6.42 $\pm$ 0.10	7.52 $\pm$ 0.06
Chimera 3		10.9 $\pm$ 0.8	1.90 $\pm$ 0.27	7.98 $\pm$ 0.07	6.57 $\pm$ 0.06	7.63 $\pm$ 0.09
Chimera 4		89.3 $\pm$ 7.0	0.92 $\pm$ 0.15	7.06 $\pm$ 0.12	5.29 $\pm$ 0.09	6.51 $\pm$ 0.14
Chimera 5		9.6 $\pm$ 0.3	1.11 $\pm$ 0.25	8.00 $\pm$ 0.06	6.59 $\pm$ 0.11	7.62 $\pm$ 0.04
Chimera 6		79.1 $\pm$ 13.1	1.00 $\pm$ 0.10	7.12 $\pm$ 0.06	5.33 $\pm$ 0.09	6.66 $\pm$ 0.13



**Fig. 2.** Homologous displacement of [<sup>3</sup>H]histamine binding to chimeras 1, 3, and 5 (A), 2, 4, and 6 (B), 7 and 8 (C), and 9 and 10 (D) transiently expressed in HEK 293T cells. Data shown are representative of at least three independent experiments.

TABLE 3

Pharmacological analysis of chimeric human and mouse H<sub>4</sub>R proteins 7 to 10, using [<sup>3</sup>H]histamine saturation analysis and displacement studies. Equilibrium  $K_d$  and  $B_{max}$  values for [<sup>3</sup>H]histamine (picomole per milligram protein) and  $pK_i$  of all ligands tested at chimeras 7 to 10 are presented as average  $\pm$  S.E.M. of results of at least three independent experiments.

Receptor	$K_d$	$B_{max}$	$pK_i$		
			Histamine	Clozapine	VUF 8430
	<i>nM</i>				
hH <sub>4</sub> R	9.0 $\pm$ 0.1	2.71 $\pm$ 1.00	7.89 $\pm$ 0.05	6.35 $\pm$ 0.03	7.46 $\pm$ 0.02
mH <sub>4</sub> R	77.5 $\pm$ 11	1.48 $\pm$ 0.24	7.09 $\pm$ 0.04	5.49 $\pm$ 0.07	6.75 $\pm$ 0.10
Chimera 7	7.4 $\pm$ 0.7	1.57 $\pm$ 0.42	7.96 $\pm$ 0.08	6.57 $\pm$ 0.06	7.56 $\pm$ 0.12
Chimera 8	91.2 $\pm$ 9.1	1.20 $\pm$ 0.28	7.22 $\pm$ 0.08	5.56 $\pm$ 0.12	6.64 $\pm$ 0.06
Chimera 9	9.2 $\pm$ 2.3	3.28 $\pm$ 0.53	7.75 $\pm$ 0.04	6.22 $\pm$ 0.04	7.48 $\pm$ 0.11
Chimera 10	79.4 $\pm$ 6.5	1.60 $\pm$ 0.58	6.93 $\pm$ 0.19	5.40 $\pm$ 0.04	7.05 $\pm$ 0.08

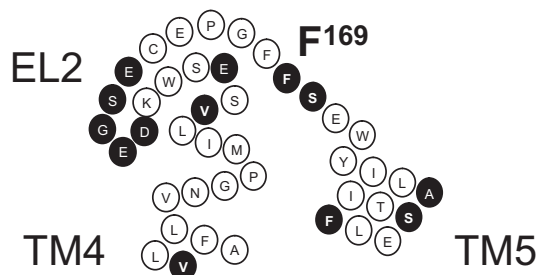
**Phenylalanine 169 in the EL2 As Determining Structural Element.** As can be seen in Table 4, in the domain between the extracellular halves of TM4 and TM5, eight single amino acid residues are different, and a stretch of six

amino acid residues (DEGSE<sup>159–163</sup>, located adjacent to the conserved cysteine residue in the EL2) in the human H<sub>4</sub>R is replaced by the eight amino acid stretch NSTNTKD<sup>159–165</sup> of the mouse protein sequence. Therefore, nine new mutants of

TABLE 4

Pharmacological analysis of mutant human H<sub>4</sub>R proteins, using [<sup>3</sup>H]histamine saturation analysis and displacement studies

Equilibrium  $K_d$  and  $B_{max}$  values for [<sup>3</sup>H]histamine (picomole per milligram protein) and  $pK_i$  of ligands for the indicated H<sub>4</sub>R mutants are presented as average  $\pm$  S.E.M. of results of at least three independent experiments. The figure in the panel shows the human H<sub>4</sub>R amino acid sequence that was replaced by the mouse H<sub>4</sub>R sequence as in chimera 10. The residues that are identical to those of the mouse H<sub>4</sub>R are indicated in white, whereas the ones that differ are shown in black. The amino acid residues of the human H<sub>4</sub>R indicated in this table are altered into the corresponding residues of the mouse H<sub>4</sub>R.



Receptor	$K_d$	$B_{max}$	$pK_i$		
			Histamine	Clozapine	VUF 8430
	<i>nM</i>				
hH <sub>4</sub> R	9.0 $\pm$ 0.1	2.71 $\pm$ 1.00	7.89 $\pm$ 0.05	6.35 $\pm$ 0.03	7.46 $\pm$ 0.02
mH <sub>4</sub> R	77.5 $\pm$ 11	1.48 $\pm$ 0.24	7.09 $\pm$ 0.04	5.49 $\pm$ 0.07	6.75 $\pm$ 0.10
V141I	10.5 $\pm$ 1.4	2.00 $\pm$ 0.39	7.66 $\pm$ 0.08	6.19 $\pm$ 0.05	7.59 $\pm$ 0.05
V153A	12.9 $\pm$ 3.2	1.04 $\pm$ 0.11	7.72 $\pm$ 0.14	6.23 $\pm$ 0.05	7.46 $\pm$ 0.09
E155D	11.1 $\pm$ 3.4	1.97 $\pm$ 0.24	7.61 $\pm$ 0.04	6.23 $\pm$ 0.02	7.41 $\pm$ 0.06
DEGSE $\rightarrow$ NSTNTKD	10.2 $\pm$ 2.1	1.52 $\pm$ 0.31	7.65 $\pm$ 0.07	6.44 $\pm$ 0.04	7.52 $\pm$ 0.05
F169V	42.4 $\pm$ 9.7	1.88 $\pm$ 0.34	7.12 $\pm$ 0.10	5.52 $\pm$ 0.05	7.05 $\pm$ 0.05
S170T	11.9 $\pm$ 2.0	1.13 $\pm$ 0.23	7.82 $\pm$ 0.06	6.02 $\pm$ 0.03	7.60 $\pm$ 0.05
A176T	8.5 $\pm$ 1.3	1.54 $\pm$ 0.33	7.76 $\pm$ 0.05	6.18 $\pm$ 0.03	7.52 $\pm$ 0.06
S179M	18.0 $\pm$ 1.3	2.73 $\pm$ 0.58	7.57 $\pm$ 0.06	6.27 $\pm$ 0.05	7.52 $\pm$ 0.06
F180L	7.8 $\pm$ 1.0	3.10 $\pm$ 0.38	8.06 $\pm$ 0.07	6.37 $\pm$ 0.04	7.65 $\pm$ 0.09

the human H<sub>4</sub>R were constructed, including V141I, V153A, E155D, DEGSE<sup>159–163</sup> $\rightarrow$ NSTNTKD<sup>159–165</sup>, F169V, S170T, A176T, S179M, and F180L (Table 4). All human H<sub>4</sub>R mutants were well expressed after transient transfection of HEK 293T cells, and all proteins bound [<sup>3</sup>H]histamine in a saturable manner (Table 4). From the nine tested H<sub>4</sub>R mutants, the human H<sub>4</sub>R F169V was the only receptor protein with a shift in agonist binding profile. Replacement of phenylalanine 169 in the human H<sub>4</sub>R protein with a valine residue, as found in the mouse and rat H<sub>4</sub>R, resulted in a clear change in binding profile (Table 4). The agonist affinities for the human H<sub>4</sub>R F169V mutant are significantly different from the wild-type human H<sub>4</sub>R. The observed  $pK_i$  values for the H<sub>4</sub>R F169V of 7.12 (histamine), 5.52 (clozapine), and 7.05 (VUF8430) are similar to those observed for the mouse H<sub>4</sub>R (Table 4). Furthermore, the F169V mutant behaves similarly to the mouse H<sub>4</sub>R in response to histamine in a G $\alpha_{q15}$ -driven NFAT-luciferase reporter gene assay (Supplemental Fig. 2), with  $pEC_{50}$  values of  $5.86 \pm 0.09$  and  $5.82 \pm 0.03$ , respectively, which are lower than the potency at the human H<sub>4</sub>R ( $pEC_{50} = 6.29 \pm 0.05$ ). Despite the gaps between  $pK_i$  and  $pEC_{50}$  values, the order of histamine potency at these receptors is maintained.

## Discussion

Soon after the cloning of the human H<sub>4</sub>R (Oda et al., 2000), rat, mouse, and guinea pig orthologs were reported as well (Liu et al., 2001). Mouse H<sub>4</sub>R shows a substantial lower affinity for H<sub>4</sub>R agonists, like histamine, VUF 8430 (Lim et al., 2006), and clozapine, whereas the H<sub>4</sub>R antagonist JNJ 7777120 does not distinguish between the human and mouse H<sub>4</sub>R (Thurmond et al., 2004; this study). Considering the

relative low homology between the human and mouse H<sub>4</sub>R protein sequences (67%), the observed pharmacological differences between human and mouse H<sub>4</sub>R are not surprising. Despite the fact that such species differences might hamper preclinical evaluation of H<sub>4</sub>R ligands, such a natural genetic variation also offers an opportunity to investigate receptor-ligand interactions.

In this study, we employed a chimeric receptor approach to localize receptor domains that could be responsible for the observed pharmacological differences between the human and mouse H<sub>4</sub>R. Such an approach has been proven to be quite successful for a number of other GPCRs (Yin et al., 2004). All chimeric human/mouse H<sub>4</sub>R proteins were expressed in HEK 293T cells at detectable levels as measured by [<sup>3</sup>H]histamine radioligand binding. The ability of the chimeras to act as functional receptors is most probably due to the reasonable homology between the human and mouse H<sub>4</sub>R within the TM domains. A systematic analysis of a set of 10 chimeric H<sub>4</sub>R enabled us to localize the receptor domain responsible for the difference in agonist binding to a region that spans from the extracellular half of TM4 to the extracellular half of TM5, including the EL2 (Table 3).

To identify specific amino acid residues between the extracellular half of TM4 and the extracellular half of TM5 responsible for the pharmacological differences, we employed site-directed mutagenesis of the human H<sub>4</sub>R at all divergent positions. Our detailed mutagenesis studies identified Phe<sup>169</sup> in EL2 as the single amino acid responsible for the differences in affinity between the human and mouse H<sub>4</sub>R. Mutation of Phe<sup>169</sup> of the human H<sub>4</sub>R into the corresponding residue of the mouse H<sub>4</sub>R, Val<sup>171</sup>, results in the mutant F169V human H<sub>4</sub>R protein, which binds H<sub>4</sub>R agonists like

the mouse H<sub>4</sub>R does. Val<sup>171</sup> is conserved between mouse and rat H<sub>4</sub>R, whereas the Phe<sup>169</sup> found in the human H<sub>4</sub>R is conserved in the monkey receptor. As expected on the basis of our mutagenesis results, the rat H<sub>4</sub>R indeed shows a low affinity for histamine (Liu et al., 2001), whereas the monkey H<sub>4</sub>R resembles the human H<sub>4</sub>R in this respect (Oda et al., 2005).

The EL2 has been suggested to be involved in the binding of diverse types of ligands, such as aminergic receptor ligands (Laurila et al., 2007; Scarselli et al., 2007), nicotinic acid (Tunaru et al., 2005), leukotriene B<sub>4</sub> (Basu et al., 2007), or vasopressin (Conner et al., 2007). The involvement of EL2 in the binding pocket of aminergic GPCRs has originally been suggested for the dopamine D<sub>2</sub> receptor on the basis of detailed substituted-cysteine accessibility analysis (Shi and Javitch, 2004). Residues Ile<sup>184</sup> and Asn<sup>186</sup>, which are, respectively, located +2 and +4 relative to the highly conserved Cys<sup>182</sup> in EL2, were proposed to be directly involved in the binding pocket of the D<sub>2</sub> receptor. These studies were supported by the observation that, in the X-ray crystal structure of bovine rhodopsin, EL2 forms a  $\beta$  hairpin located on top of the seven transmembrane pore protruding into the ligand binding cavity (Palczewski et al., 2000) and, therefore, may become part of the ligand binding pocket.

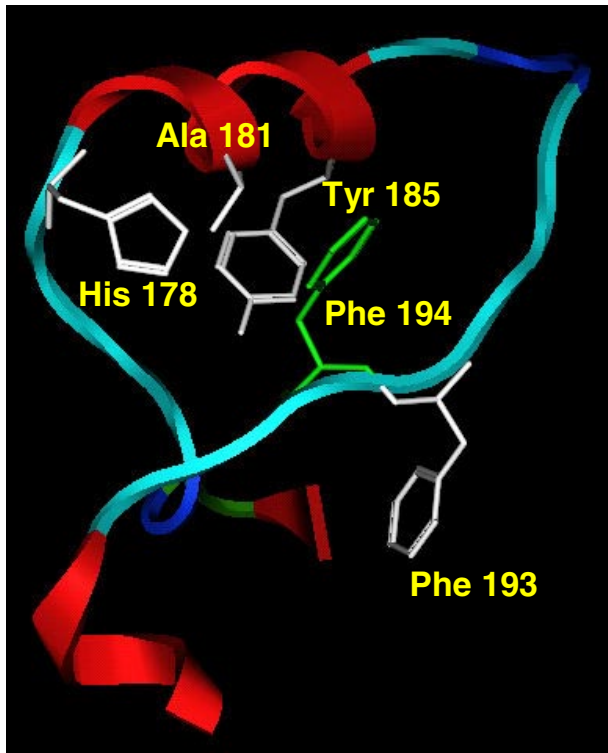
Recently, new structural information on the family of aminergic GPCRs has been obtained after the successful crystallization of the inactive conformation of the  $\beta_2$  adrenergic receptor ( $\beta_2$ AR) (Cherezov et al., 2007; Rasmussen et al., 2007). The high-resolution crystal structure of the human  $\beta_2$ AR shows a highly intricate structure for the EL2 (Cherezov et al., 2007), which is clearly different from the EL2 in bovine rhodopsin. As found in the H<sub>4</sub>R, the  $\beta_2$ AR contains a Phe-Phe motif in the EL2 (residues Phe<sup>193</sup>-Phe<sup>194</sup>). The crystal structure demonstrates the importance of Phe<sup>193</sup>, as it protrudes deep into the binding pocket where it directly interacts with one of the aromatic rings of the 9H-carbazole group of the  $\beta_2$ AR antagonist carazolol (Cherezov et al., 2007; Rosenbaum et al., 2007). It is interesting that residue Phe<sup>193</sup> of the  $\beta_2$ AR aligns with Ile<sup>184</sup> of the D<sub>2</sub> receptor, which was shown to be involved in the binding pocket of D<sub>2</sub> receptor (Shi and Javitch, 2004). On the other hand, Phe<sup>194</sup> does not have any direct interactions with the ligand in the  $\beta_2$ AR crystal structure (Cherezov et al., 2007; Rosenbaum et al., 2007). The present study, however, clearly shows the importance of the analogous H<sub>4</sub>R residue Phe<sup>169</sup> for agonist binding. In the  $\beta_2$ AR crystal structure, Phe<sup>194</sup> has a strong lipophilic interaction with Tyr<sup>185</sup>, Ala<sup>181</sup>, and His<sup>178</sup> (Fig. 3A). We propose that these interactions are important to properly orient Phe<sup>194</sup> and hence position EL2 in the observed orientation. In agreement with the observations in the crystal structure of the  $\beta_2$ AR, our homology model of the H<sub>4</sub>R clearly shows that the side chain of Phe<sup>169</sup> is also stabilized by lipophilic interactions, i.e., with residues Trp<sup>157</sup>, Pro<sup>166</sup>, Leu<sup>241</sup>, Ser<sup>245</sup>, Pro<sup>250</sup>, and Tyr<sup>255</sup> (Fig. 3B). A mutation to a smaller substituent, such as valine, the focus of the current study, would loosen the orienting interactions and increase the flexibility of the EL2 loop and concomitantly Phe<sup>168</sup>. The concurrent increase in flexibility would result in a reduced binding affinity, which is in agreement with the observed binding differences between the human and mouse H<sub>4</sub>Rs. Amino acids in this position, therefore, do not directly interact with receptor-bound agonists but rather indirectly fine-tune the tightness of binding.

Analysis of literature data supports our hypothesis for a role of the second phenylalanine residue in the Phe-Phe motif in agonist binding to the H<sub>4</sub>R. Of 325 nonolfactory GPCRs containing a cysteine residue in EL2, only the muscarinic M<sub>2</sub>, the  $\beta_2$ AR, the histamine H<sub>3</sub>R and H<sub>4</sub>R, GP116, and the trace-amine associated receptor 1 contain the Phe-Phe motif downstream of the conserved cysteine residue. Furthermore, there are 15 GPCRs with Phe-Tyr, Tyr-Phe, or Tyr-Tyr motifs downstream of the conserved cysteine residue, including the H<sub>1</sub>R protein (de Graaf et al., 2008). If one restricts the analysis to the identified aminergic GPCR subfamilies (i.e.,  $\beta$ -adrenergic, muscarinic, and histaminergic GPCRs), it is clear that the histamine receptor family (with the exception of the H<sub>2</sub>R) shows a high conservation of this aromatic-aromatic motif in EL2 (Fig. 3C). In contrast, within the GPCR subfamily of muscarinic receptors, the M<sub>2</sub> receptor uniquely possesses the Phe-Phe motif in EL2 (Fig. 3C). The other subfamily members (M<sub>1</sub>, M<sub>3-5</sub>) all contain a Phe-Leu motif in EL2 (Fig. 3C). Mutagenesis of the Phe residue of the Phe-Leu motif in the M<sub>1</sub> receptor only marginally affects the binding of the antagonist *N*-methylscopolamine (Goodwin et al., 2007). However, mutation of this conserved Phe residue in the M<sub>3</sub> receptor results in an 8-fold reduction in the affinity of the agonist carbachol (Scarselli et al., 2007). Furthermore, mutation of Leu of the Phe-Leu motif of the M<sub>3</sub> receptor to alanine (M<sub>3</sub> L225A) also results in a reduced affinity for the agonist carbachol (Scarselli et al., 2007). These mutagenesis data on the M<sub>3</sub> receptor support the suggestion for a role of Phe-Phe/Leu motifs in the binding of agonists to certain aminergic GPCRs. In line with this hypothesis is the notion that the sequence variation in EL2 within the class of muscarinic receptor (Phe-Phe/Leu motif) might explain observed differences in agonist affinity at the five muscarinic receptors (Ford et al., 2002). Among the muscarinic receptors, acetylcholine and carbachol show a clear preference for binding to the M<sub>2</sub> receptor (Phe-Phe motif) over the other four muscarinic receptor subtypes (Phe-Leu motif) (Ford et al., 2002). At the same time, previously identified amino acid residues important for acetylcholine binding, including Asp<sup>3.32</sup>, Tyr<sup>3.33</sup>, Trp<sup>6.48</sup>, Phe<sup>6.51</sup>, Asn<sup>6.52</sup>, Tyr<sup>7.39</sup>, and Tyr<sup>7.43</sup> (Ballesteros-Weinstein numbering system) (Ballesteros and Weinstein, 1995; Goodwin et al., 2007) are all conserved in the five different muscarinic receptor subtypes. The Phe-Phe and Phe-Leu motif of the muscarinic receptors is perfectly aligned with the Phe-Phe motif in EL2 of H<sub>4</sub>R (Fig. 3C). Based on the correlation with our finding with the F169V mutation of the human H<sub>4</sub>R, we propose a role of the second Phe residue in the Phe-Phe motif in the high-affinity binding of acetylcholine and carbachol to the muscarinic M<sub>2</sub> receptor. Future modeling, mutagenesis, and ultimately structural studies should further address these issues.

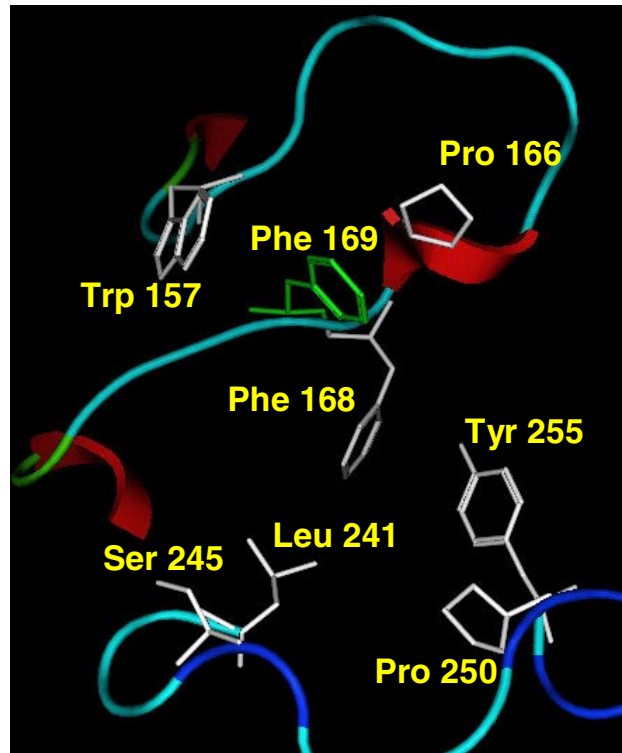
In summary, following the observation of species differences between the human and mouse H<sub>4</sub>R for binding agonists, we identified phenylalanine 169 in the EL2 as the single amino acid responsible for the differences in agonist binding. Phenylalanine 169 is part of a Phe-Phe motif, which is also present in the recently crystallized  $\beta_2$ AR. These results point to an important role of the EL2 in the agonist binding to the H<sub>4</sub>R and provide a molecular explanation for the species differences between human and rodent H<sub>4</sub>Rs. Moreover, our findings also shed some light on the known differences in ligand binding in the family of muscarinic receptor subtypes.



A



B



C

	W <sup>4.50</sup>		F <sup>5.47</sup>
BR	WVMA-LACAAPPLVGSRYIPE-----GMQCSCGIDYYTPHEETNNESFVIYMFVVHF		
$\beta_1$	WAISALVSFLPILMHWRAESD-EARRCYNDPKCC--DFVT-----NRAYAIASSVVSF		
$\beta_2$	WIVSGLTSFLPIQMHWYRATHQ-EAINCYANETCC--DFFT-----NQAYAIASSIVSF		
$\beta_3$	WVSAAVSFAPIMSQWWRVGADAEARCHSNPRCC--AFAS-----NMPYVLLSSSVSF		
M <sub>1</sub>	WLVS-FVLWAPAILFWQYLVGE-----RTVLAGQCYIQFLS-----QPIITFGTAMAAF		
M <sub>2</sub>	WVLS-FILWAPAILFWQFIVGV-----RTVEDGECYIQFFS-----NAAVTFGTAIAAF		
M <sub>3</sub>	WVIS-FVLWAPAILFWQYFVGK-----RTVPPGECFIQFLS-----EPTITFGTAMAAF		
M <sub>4</sub>	WVLS-FVLWAPAILFWQFVVGK-----RTVPDNQCFIQFLS-----NPAVTFGTAMAAF		
M <sub>5</sub>	WLIS-FILWAPAILCWQYLVGK-----RTVPLDECQIQFLS-----EPTITFGTAMAAF		
H <sub>1</sub>	WFLS--FLWVIPILGWNHFMQQ---TSVRREDKCE <sup><b>TF</b></sup> DFYD-----VTWFKVMTAIINF		
H <sub>2</sub>	WVISITLSFLSIHLGWN <sup><b>SR</b></sup> NET---SKGNHTT <sup><b>SK</b></sup> C--KVQV-----NEVYGLVDGLVTF		
H <sub>3</sub>	WVLA-FLLYGPAILSWEYLSGG-----SSIPEGHCYAEFFY-----NWYFLITASTLEF		
H <sub>4</sub>	WVLA-FLVNGPMILVSES <sup><b>WK</b></sup> DE-----GSE----CEPGF <sup><b>FS</b></sup> -----EWYILAIT <sup><b>S</b></sup> FLEF		

**Fig. 3.** Close-up of the EL2 as observed in the recent X-ray structure of the  $\beta_2$ -adrenergic receptor (Cherezov et al., 2007) (A) and homology model of the human H<sub>4</sub>R (B). Phe<sup>193</sup> points inwards into the GPCR binding pocket, whereas Phe<sup>194</sup> interacts with various residues in the EL2 of the  $\beta_2$ -adrenergic receptor, thereby stabilizing the conformation of the EL2 (Cherezov et al., 2007). Phe<sup>168</sup> and Phe<sup>169</sup> of the human H<sub>4</sub>R might play similar roles as Phe<sup>193</sup> and Phe<sup>194</sup> residues, respectively. The images were created with Molecular Operating Environment, version 2007.09 (Chemical Computing Group, Inc.). C, partial alignment of amino acid sequences of human  $\beta_{1-3}$  adrenergic, muscarinic M<sub>1-5</sub>, and histaminergic H<sub>1-4</sub> receptors and bovine rhodopsin (BR). The sequences start at residue Trp<sup>4.50</sup> and end at Phe<sup>5.47</sup> (Ballesteros-Weinstein numbering system) (Ballesteros and Weinstein, 1995) and were aligned by ClustalW (Higgins et al., 1994). Residue Phe<sup>169</sup> of the H<sub>4</sub>R is printed in boldface.

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