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Oligomerization of Recombinant and Endogenously Expressed Human Histamine H_4 Receptors

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ABSTRACT

In this study, we report the homo- and hetero-oligomerization of the human histamine H_4R by both biochemical (Western blot and immobilized metal affinity chromatography) and biophysical [bioluminescence resonance energy transfer and time-resolved fluorescence resonance energy transfer (*tr*-FRET)] techniques. The H_4R receptor is the most recently discovered member of the histamine family of G-protein-coupled receptors. Using specific polyclonal antibodies raised against the C-terminal tail of the H_4R, we demonstrate the presence of H_4R oligomers in human embryonic kidney 293 and COS-7 cells heterologously overexpressing H_4Rs and putative native H_4R oligomers in human phytohaemagglutinin blasts endogenously expressing H_4Rs. Moreover, we show that H_4R homo-oligomers are formed constitutively, are formed at low receptor densities (300 fmol/mg of protein), and are present at the cell surface, as detected by *tr*-FRET. The formation of these oligomers is independent of *N*-glycosylation and is not modulated by H_4R ligands, covering the full spectrum of agonists, neutral antagonists, and inverse agonists. Although we show H_4R homo-oligomer formation at physiological expression levels, the detection of H_1R-H_4R hetero-oligomers was achieved only at higher H_1R expression levels and are most likely not physiologically relevant.

The human histamine H_4 receptor (hH_4R), a prototypical member of the superfamily of G-protein-coupled receptors (GPCRs), has been identified recently through the use of bioinformatics by several groups simultaneously (Oda et al., 2000; Liu et al., 2001; Morse et al., 2001; Nguyen et al., 2001; Zhu et al., 2001). The H_4R couples to members of the $G_{i/o}$ family of heterotrimeric G-proteins to mediate the inhibition of adenylyl cyclase. In addition, the receptor may activate phospholipase C and induce calcium mobilization (de Esch et al., 2005). The H_4R expression is almost exclusively restricted to hematopoietic cells and is suggested to mediate functions of the immune system. As such, the H_4R is a target for the development of anti-inflammatory drugs (Hofstra et al., 2003; Thurmond et al., 2004; de Esch et al., 2005).

The use of various biochemical and biophysical approaches has revealed recently that members of the GPCR family may exist as homo- and hetero-oligomers at the cell surface. When considering the heterotrimeric G protein, which is approximately twice the size of the GPCR (Lambright et al., 1996), it seems reasonable that GPCRs need to oligomerize to interact with the G protein, as suggested for the leukotriene B_4 receptor (Baneres and Parello, 2003). Hetero-oligomerization has been shown to be pivotal for the $GABA_BR1$, which needs to associate with $GABA_BR2$ receptors to be transported to the cell membrane (Jones et al., 1998), and for the T1R taste receptors, which require hetero-oligomerization to form receptors that can recognize sweets (Nelson et al., 2001) or amino acids (Nelson et al., 2002). In other cases, hetero-oligomerization may change the ligand binding characteristics, potentially giving rise to a new dimension in GPCR drug

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ABBREVIATIONS: hH_4R, human histamine H_4 receptor; GPCR, G-protein-coupled receptor; BRET, bioluminescence resonance energy transfer; *tr*-FRET, time-resolved fluorescence resonance energy transfer; HA, hemagglutinin; HEK, human embryonic kidney; eYFP, enhanced yellow fluorescent protein; *R*luc, *Renilla reniformis* luciferase; PCR, polymerase chain reaction; APC, allophycocyanin; PHA, phytohemagglutinin; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; Ni^{2+}-NTA, nickel-nitrilotriacetic acid; JNJ 7777120, 1-[(5-chloro-1*H*-indol-2-yl)carbonyl]-4-methylpiperazine.

discovery (Devi, 2001; Terrillon and Bouvier, 2004; Waldhoer et al., 2005).

We have reported previously the detection of homo-oligomers of the human histamine H_1 receptor (H_1R) by applying biochemical and *tr*-FRET experiments and by the formation of H_1R radioligand binding sites upon the coexpression of two ligand binding-deficient mutant H_1Rs (Bakker et al., 2004). The H_1R is a well-known target for the treatment of seasonal allergies but has also been shown to mediate inflammatory responses in keratinocytes (Giustizieri et al., 2004; Matsubara et al., 2005). The H_1R is ubiquitously expressed and is coexpressed together with the H_4R in leukocytes, including monocytes and T lymphocytes (Cameron et al., 1986; Morse et al., 2001), suggesting that on these cells, histamine may modulate inflammatory actions through the action on both H_1Rs and H_4Rs. We therefore investigated the potential homo-oligomerization of the H_4R and the hetero-oligomerization of the H_1R with the H_4R using heterologous expression systems.

Herein, we report on the generation of specific antibodies raised against the H_4R, the detection of homo-oligomers of the H_4R, and the potential formation of H_1R-H_4R hetero-oligomers by using biochemical and BRET and *tr*-FRET approaches. Using these methodologies, we show the human H_4R to constitutively form homo-oligomers at the cell surface and that the oligomerization is independent of ligand stimulation of the receptors. Furthermore, *N*-glycosylation of the H_4R receptor is not a prerequisite for oligomer formation. Although we can detect H_4R homo-oligomers at physiologically relevant H_4R expression levels and in endogenously H_4R expressing PHA blast cells, the detection of H_1R-H_4R hetero-oligomers requires higher receptor expression levels.

Materials and Methods

Materials. Reagents for *tr*-FRET were from Cis Bio International (Bagnols-sur-Cèze Cedex, France). Coelenterazine was purchased from Chemicon International (Temecula, CA). Sheep anti-mouse IgG horseradish peroxidase was from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Bovine serum albumin, chloroquine diphosphate, DEAE-dextran (chloride form), histamine (2-[4-imidazolyl-]ethylamine hydrochloride), mepyramine (pyrilamine maleate), monoclonal mouse anti-FLAG (DYKDDDDK), and polyethyleneimine were purchased from Sigma (St. Louis, MO). Calf serum (Integro BV, Dieren, The Netherlands). Cell culture media, penicillin, and streptomycin were obtained from Invitrogen (Merelbeke, Belgium). Cell culture plastics were from Greiner Bio-one (Wemmel, Belgium). Tris was from AppliChem (Darmstadt, Germany). [^{3}H]Histamine (12.40 and 18.10 Ci/mmol) and [^{3}H]mepyramine (23.00 Ci/mmol) were purchased from PerkinElmer Life Science (Boston, MA). Oligonucleotides were purchased from Isogen Biocience (Maarsen, The Netherlands). *Pfu* Turbo DNA polymerase was purchased from Stratagene (La Jolla, CA). Restriction enzymes were from MBI Fermentas (St. Leon-Rot, Germany). Thioperamide, iodophenpropit, clobenpropit, and JNJ 7777120 were synthesized at the Department of Medicinal Chemistry, Vrije Universiteit Amsterdam (Amsterdam, The Netherlands). Gifts of mouse anti-hemagglutinin (anti-HA) antibody (Dr. J. van Minnen), pcDNA3.1-eYFP vector (Dr. T. Schmidt), pRL-CMV vector (Dr. G. Milligan), pCR3.1-HA-H_1R and pcDEF$_3$-c-myc-H_1R (Dr. S Hill), expression vector pcDEF$_3$ (Dr. J. Langer) (Goldman et al., 1996), and mianserin (Organon NV, Oss, The Netherlands) are greatly acknowledged.

Wild-type human H_4R in pcDNA3.1 was purchased from Guthrie cDNA resource center (Sayre, PA). The vector was subcloned into the pcDEF$_3$ using BamHI/XbaI sites.

Construction of Epitope-Tagged Proteins for *tr*-FRET. An N-terminally FLAG (DYKDDDDK) epitope-tagged H_4R was created by PCR. The coding sequence of the hH_4 gene was amplified using the sense oligonucleotide primer 5′-GGGAAGCTTGCCACCATGGACTACAAGGACGACGATGACAAGGATCCAGATACTAATAGCAC-3′ and the antisense primer 5′-GGAAGG CACGGGGGAGGGC-3′. The amplified gene was first cloned into the pCRII-Topo vector by TOPO TA cloning (Invitrogen BV, Breda, The Netherlands) and subsequently subcloned into the pcDEF$_3$ expression vector using EcoRV/XbaI sites.

An N-terminally HA (YPYDVPDYA) epitope-tagged H_4R was created by PCR in two steps. The H_4R gene was amplified by PCR with a 5′ SacII site and without start codon using the sense primer 5′-ACCGCGGCCCCAGATACTA ATAGCACAATC-3′and the antisense primer 5′-GGAAGGCACGGGGAGGGC-3′. The fragment was directly cloned to the pCRII-Topo vector. The gene was subsequently subcloned using SacII/XbaI sites into the pcDNA3.1-HA-rH_{3A}R vector (Bakker et al., 2006). The HA-H_4 gene was finally subcloned using BamHI/XbaI sites to pcDEF$_3$. The HA-H_1R gene was subcloned from the pCR3.1-HA-H_1R into the pcDEF$_3$ using Bsp1407I/SpeI restriction sites.

Construction of Fusion Proteins for BRET. For the BRET assay, H_4Rs were C-terminally fused to either a *Renilla reniformis* luciferase (H_4R-*R*luc) or a yellow fluorescent protein (H_4R-eYFP) in two steps. The coding sequence of the hH_4R gene was amplified without its stop codon using the sense primer 5′-TCGGATCCACCATGCCAGATACTAATAGC-3′ and the antisense primer 5′-CCGCGGC CGCACTAGTAGAAGATACTGACCGAC-3′, harboring unique BamHI and NotI restriction sites, respectively. The gene was cloned directly into the pCRII-Topo vector and subsequently subcloned to a pcDEF$_3$ vector using BamHI/NotI sites [pcDEF$_3$-H_4R (Del stop)].

The coding sequence for the *R*luc gene was amplified from the p*R*L-CMV vector lacking a start codon and harboring a NotI restriction site using the sense primer 5′-AGCGGCCGCGACTTCGAAAGTTTATGATCC-3′ and the antisense primer 5′-TCTAGAATTATTGTTCATTTTTGAG-3′. The gene was directly cloned to the pCRII-Topo vector and subsequently subcloned in frame using NotI/XbaI sites into the pcDEF$_3$-H_4R (Del stop) vector.

The coding sequence for the eYFP gene was amplified from the pcDNA3.1-eYFP vector lacking a start codon and harboring a NotI restriction site using the sense primer 5′-CGCGGCCGCGGTGAGCAAGGGCGAGGAG-3′ and the antisense primer 5′-GTCTAGATTACTTGTACAGCTCGTCCATG-3′. The gene was directly cloned to the pCRII-Topo vector and subsequently subcloned in frame using NotI/XbaI sites into the pcDEF$_3$-H_4R (Del stop) vector.

An hH_1R-eYFP fusion was generated by PCR using the sense primer 5′-AAGAGAATTCTGCATATTCGCTCCATGGTGAGCAAGGGCG-3′ and the antisense primer 5′-TTCTCTAGATTACTTGTACAGCTCGTCC-3′, harboring unique EcoRI and XbaI restriction sites, using pcDNA3.1eYFP as template. The PCR fragment was digested using EcoRI and XbaI, and the purified fragment was subsequently ligated together with the fragment that was obtained by digestion of the pcDEF$_3$-hH_1R plasmid using EcoRI/XbaI sites.

An hH_1R-*R*luc fusion was generated by PCR using the sense primer 5′-AAGAGAATTCTGCATATTCGCTCCATGACTTCGAAAGTTTATGATCC-3′ and the antisense primer 5′-CGCTCTAGAATTATTGTTCATTTTTGAGAACTCGC-3′, harboring unique EcoRI and XbaI restriction sites. The PCR fragment was digested using EcoRI and XbaI and the purified fragment was subsequently ligated together with the fragment that was obtained by digestion of the pcDEF$_3$-hH_1R plasmid using EcoRI/XbaI sites. Each construct was fully sequenced before its expression and analysis.

Construction of His_{10}-Tagged Proteins for Immobilization. An N-terminally c-myc (EQKLISEEDL) and C-terminally His_{10}-epitope-tagged H_4R was created as follows. First, a c-myc epitope-tagged H_4R was created by PCR in two steps. The c-myc tag was amplified by PCR using a pcDEF$_3$-c-myc-H_1R vector as template

with a 3′-NheI site using the sense primer 5′-GGGTGGAGAC TGAAGTTAGGCC-3′and the antisense primer 5′-GTGCTAGCAG-GTCCTCCTCGGAG-3′. The fragment was directly cloned to the pCRII-Topo vector (pCRII-topo-myc). The H_4R gene was amplified without start codon and contained a 5′-NheI restriction site using the following sense 5′-CCGCTAGCCAGATACTAATAGCAC-3′ and the antisense primer 5′- TCTTTAAGAAGATACTGACC-3′. The gene was directly cloned to the pcDNA3.1/V5-His-Topo vector. The H_4R gene was subsequently subcloned in frame using NheI/NotI into the pCRII-topo-c-myc vector (pCRII-topo-c-myc-H_4R). The c-myc-H_4R gene was subsequently subcloned into the pcDEF$_3$ expression vector using the BamHI/XbaI sites.

Second, the gene of the wild-type H_4R was amplified by PCR without a start and stop codon with a 5′-BamHI site and a 3′-SpeI site using the sense primer 5′-CCGG ATCCCCAGATACTAATAG-CACAATCAA-3′ and the antisense primer 5′-CCGCGGCCG CAC-TAGTAGAAGATACTGACCGAC-3′ and directly cloned into the pC-RII-Topo vector. The H_4R gene was then subcloned in frame from the pCRII-topo-vector using BamHI/SpeI sites in the pSFV2genB vector. An N-terminally tagged FLAG and C-terminally tagged H_4R-His_{10} gene was subcloned from the pSFV2genB-FLAG-H_4R-His_{10} behind the p10 promoter of the pFastbac_DUAL vector using NcoI/NheI restriction sites.

The H_4R-His_{10} gene was amplified by PCR from the pFastbac_ DUAL-FLAG-H_4R-His_{10} vector without start codon and a 3′-XbaI site using the sense primer 5′-CATCTAGATTAATTACCCACT-GGGCCC-3′and the antisense primer 5′-GAGGATCCGCCAGATAC-TAATAGCACAATC-3′ and directly cloned into the pcDNA3.1/V5-His-Topo vector by TOPO TA cloning and subsequently subcloned into the pcDEF$_3$-c-myc-H_4R vector using BoxI/XbaI restriction sites. Each construct was fully sequenced before its expression and analysis.

Cell Culture and Transfection of COS-7 Cells. COS-7 African green monkey kidney cells were maintained at 37°C humidified in 5% CO_2/95% air atmosphere in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) fetal calf serum, 50 IU/ml penicillin and 50 μg/ml streptomycin and grown in 100-mm dishes. Cells were transiently transfected using the DEAE-dextran method as described previously (Bakker et al., 2001). The total amount of DNA transfected was maintained constant by the addition of pcDEF$_3$.

Cell Culture and Transfection of HEK 293 Cells. HEK 293 cells were maintained at 37°C humidified in 5% CO_2/95% air atmosphere in Dulbecco's modified Eagle's medium/F-12 (Cambrex, Nottingham, UK) supplemented with 10% (v/v) fetal calf serum, 50 IU/ml penicillin, and 50 μg/ml streptomycin and grown in 100-mm dishes. HEK 293 cells were transfected with pcDEF$_3$-H_4R receptor essentially using the Lipofectamine Plus method described by Shenton et al. (2005). In brief, for each cDNA, two microtubes were prepared: tube 1 contained 2 μg of cDNA, 6 μl of Lipofectamine Plus reagent (Invitrogen), and 150 μl of Opti-MEM I media (Invitrogen); tube 2 contained 5 μl of Lipofectamine reagent (Invitrogen) and 150 μl of Opti-MEM I media. The mixtures were incubated at room temperature for 15 min, after which the contents of tube 2 were added to tube 1, followed by a further 15-min incubation. In the meantime, the HEK 293 cells at 50 to 80% confluence in 2-ml Petri dishes were washed three times with Opti-MEM I media. At the end of the second incubation period, the contents of tube 1 were increased to 1.5 ml with Optimem-I media and added to the washed HEK 293 cells. The cells were incubated at 37°C for 6 h. The transfection mixture was then removed and replaced with growth media. The cells were harvested 48 h after transfection, and cell homogenates were prepared for immunoblotting.

[^{3}H]Histamine Binding Studies. Cells used for radioligand binding studies were harvested 48 h after transfection and homogenized in ice-cold H_4R binding buffer (50 mM Tris, pH 7.4). For saturation isotherms, cell membrane homogenates were incubated at 37°C for 60 min with 0 to 125 nM [^{3}H]histamine in a total assay volume of 200 μl. Nonspecific binding was determined by incubation in the presence of 10 μM JNJ 7777120. For competition binding assays, the cell homogenates were incubated at 37°C for 60 min with 0.1 to 10,000 nM ligand in the presence of ~15 nM [^{3}H]histamine in a total volume of 200 μl. The incubations were stopped by rapid dilution with ice-cold H_4R binding buffer. The bound radioactivity was separated by filtration through GF/C filter plates (Whatman, Maidstone, UK) that had been treated with 0.3% polyethyleneimine. Filters were washed four times with H_4R binding buffer, and radioactivity retained on the filters was measured by liquid scintillation counting.

[^{3}H]Mepyramine Binding Studies. Cells used for radioligand binding studies were harvested 48 h after transfection and homogenized in ice-cold H_1R binding buffer (50 mM Na^{2+}/potassium phosphate buffer, pH 7.4). For saturation isotherms, cell membrane homogenates were incubated at room temperature for 30 min with 0 to 25 nM [^{3}H]mepyramine in a total assay volume of 200 μl. Nonspecific binding was determined by incubation in the presence of 1 μM mianserin. For competition binding assays, the cell homogenates were incubated at room temperature for 30 min with 0.1 to 10,000 nM in the presence of ~1.5 nM [^{3}H]mepyramine in a total volume of 200 μl. The incubations were stopped by rapid dilution with ice-cold H_1R binding buffer. The bound radioactivity was separated by filtration through Whatman GF/C filter plates that had been treated with 0.3% polyethyleneimine. Filters were washed four times with H_1R binding buffer, and radioactivity retained on the filters was measured by liquid scintillation counting.

Anti-H_4R Antibody Generation. The unique peptide corresponding to the amino acids CIKKQPLPSQHSRSVSS of the human H_4R subtype was conjugated to thyroglobulin by the cysteine-coupling method (Chazot et al., 1998). The resultant conjugate was used to generate polyclonal antibodies in rabbits. Antibody production and affinity purification was performed as described previously (Chazot et al., 2001).

Production of Human PHA Blasts. Human peripheral blood mononuclear cells (PBMCs) stimulated with phytohemagglutinin (PHA blasts) were generated essentially as described previously (Bradford et al., 2005). In brief, heparinized human whole blood was obtained from healthy volunteers (with local ethical approval), and PBMCs were separated using Lymphoprep (Axis-Shield Poc AS, Oslo, Norway) and centrifuged at 400*g* for 25 min. The PBMCs were isolated from the interfacial layer, washed twice in RPMI 1640 medium without L-glutamine (Invitrogen), and resuspended in RPMI 1640 medium complemented with 10% (v/v) fetal calf serum, 1% (v/v) penicillin and streptomycin, and 1% (v/v) l-glutamine. Cell density was adjusted accordingly to 1×10^6 cells/ml with RPMI 1640 medium. Next, 100 μl of PHA (Lectin; Sigma, Poole, Dorset, UK) was added to the cells to make PHA blasts. These were grown in culture for 24 h, harvested, and a cell homogenate was prepared in the presence of protease inhibitors (Protease Inhibitor Cocktail III; Calbiochem, Beeston, Nottingham, UK).

Immunoblotting. SDS-polyacrylamide gel electrophoresis was carried out using 6 or 7.5% polyacrylamide slab gels under reducing conditions. Samples of HEK 293 cells, COS-7 cells, and PHA blasts (20–50 μg of protein) were prepared using a chloroform/methanol method of protein precipitation, and immunoblotting was performed as described previously (Chazot et al., 2001; Bakker et al., 2006). Immunoblots were probed with anti-H_4 374–390 antibody at a concentration of 0.5 μg/ml.

Blots containing FLAG or c-myc-tagged receptors were probed with primary antibodies, mouse anti-FLAG (1.5 μg/ml), or mouse anti-c-myc (1 μg/ml), respectively. Horseradish peroxidase-conjugated goat anti-mouse antibodies (1:2000–5000) were used as secondary antibodies.

Immunoprecipitation. HEK 293 cells were transfected with HA-H_4 receptor and solubilized with 1% Triton X-100/0.15 M NaCl for 30 min at 4°C. Immunoprecipitation was performed essentially as described previously (Chazot et al., 1994). Solubilized HEK 293 cell extracts were incubated with 5 μg of rat anti-HA antibody (Roche

Diagnostics, Mannheim, Germany) or rat nonimmune Ig (ADI, San Antonio, TX) at 4°C for 1 h. Prechilled, washed Protein G agarose slurry (50 μl; Sigma) was added and incubated for 1 h at 4°C on a rocking platform. Precipitation pellets were collected by centrifugation at 10,000*g* for 30 s at 4°C, washed with 3× PBS, resuspended in sample buffer, vortex-mixed, and heated to 90 to 100°C for 3 min. The sample was then recentrifuged, and the supernatant was subjected to immunoblotting. Control experiments were performed using untransfected HEK 293 cells.

Cross-Linking Experiments. The cross-linking method used was essentially as described by Shenton et al. (2005; Bakker et al., 2006). In brief, aliquots of COS-7 cells expressing c-myc-H_4Rs were pelleted, and the suspension buffer was removed and replaced with 150 μl of cross-linking buffer (150 mM NaCl, 100 mM sodium HEPES, 5 mM EDTA, pH 7.5, and 5 mM dithiothreitol) to give a final protein concentration of approximately 0.5 mg/ml. The cross-linker [bis(sulfosuccinimidyl) suberate sodium salt] was dissolved in 20 mM HCl to give a 100 mM stock solution. The tubes were incubated at room temperature with continual mixing for 12 min with 0.25, 0.5, 1.0, and 2 mM cross-linker, centrifuged at 10,000 rpm for 5 min, the cross-linking mixture was removed, and the resultant pellet was prepared for immunoblotting.

Tunicamycin Experiments. HEK 293 cells expressing H_4Rs were incubated with 2, 4, 6, and 8 μg/ml tunicamycin (stock dissolved in dimethyl sulfoxide at 2 mg/ml) immediately after transfection and were harvested 48 h after transfection, homogenized, and subjected to immunoblotting (Chazot et al., 1995). Cells grown in the absence of tunicamycin were incubated with the respective volume of dimethyl sulfoxide.

Deglycosylation of Native H_4 Receptor. Human PHA blast cell suspensions were resuspended in deglycosylation buffer (50 μM sodium phosphate, pH 6.0, containing 0.1% SDS, 0.1% β-mercaptoethanol, and 20 mM EDTA) and incubated with either water (control) or PNGase F enzyme (Sigma) at a final enzyme concentration of 400 IU/ml (test) for 16 h at 37°C. The samples were then subjected to immunoblotting and probed with anti-H_4 374–390 antibody at a concentration of 2 μg/ml. The NMDAR1 transfected into HEK 293 cells was used as a positive control essentially as described by Chazot et al. (1992).

Receptor Immobilization. Membranes of COS-7 cells transiently expressing c-myc-H_4R-His and FLAG-H_4R or HA-H_1R-His and FLAG-H_4R receptors were homogenized, solubilized, and subsequently immobilized on Ni^{2+}-NTA columns (Invitrogen) as described previously (Bakker et al., 2006). Immobilized receptors were eluted using 250 mM imidazole. Samples were prepared for immunoblotting and were subjected to chloroform/methanol extraction loaded on a 7.5% SDS page gel and subsequently blotted on nitrocellulose paper (GE Healthcare).

BRET Assay. Forty-eight hours after transfection, cells were detached with trypsin and washed twice with PBS. Approximately 50,000 cells per well were distributed in white-bottomed 96-well microplates (Corning BV, Schiphol-Rijk, The Netherlands). Coelenterazine was added to a final concentration of 5 μM, and readings were collected immediately after this addition using a $Victor_2$ allowing signal detection at 460 and 530 nm.

tr-FRET Assay. *tr*-FRET assays were performed using Europium (Eu^{3+})-labeled and allophyocyanin anti-FLAG and anti-HA antibodies as described by Bakker et al. (2006). In brief, *tr*-FRET was assessed in 1×10^6 whole COS-7 cells transiently expressing the appropriate HA- and FLAG-tagged receptors. Cells were incubated in PBS containing 50% fetal calf serum (v/v), 0.8 nM Eu^{3+}-labeled antibody, and 8 nM allophycocyanin-labeled antibody for 2 h at room temperature on a rotating wheel, after which the membranes were washed twice with PBS. The final pellet was resuspended in 50 μl of PBS and transferred to a 384-microtiter plate. Energy transfer was measured by exciting the Eu^{3+} at 320 nm and monitoring the XL-665 allophycocyanin emission for 500 μs at 665 nm using a Novostar (BMG LabTechnologies, Offenburg, Germany) configured for time-resolved fluorescence after a 100-μs delay.

Analytical Methods. Binding data were evaluated by a nonlinear least-squares curve-fitting program using Prism software (GraphPad Software Inc., San Diego, CA). Protein concentrations were determined according to Bradford's method (1976) using bovine serum albumin as standard. All data are represented as mean ± S.E.M. from at least three independent experiments performed in triplicate. Statistical significance was determined by a Student's unpaired *t* test ($p < 0.05$ was considered statistically significant).

Results

Pharmacological Characterization of the hH_4R and hH_1R Expressed in COS-7 Cells. We have used COS-7 cells previously successfully for the heterologous expression of the hH_1R and for the identification of H_1R oligomers (Bakker et al., 2004). To investigate the potential oligomerization of the hH_4R, we therefore expressed the hH_4R heterologously in COS-7 cells. Transfection of these cells with cDNA coding for the hH_4R resulted in the expression of a high-affinity [^{3}H]histamine binding site (Table 1). Subsequent displacement studies using [^{3}H]histamine as a radioligand revealed these binding sites to display a characteristic H_4R pharmacological profile (Table 1). The H_1R and H_4R constructs used in the *tr*-FRET, BRET, and immobilization assays were also characterized by radioligand binding (saturation and displacement) assays. COS-7 cells were transiently transfected with cDNA encoding the HA-H_4R, FLAG-H_4R, H_4R-*R*luc, H_4R-eYFP, c-myc-H_4R-His, HA-H_1R, H_1R-*R*luc, or the H_1R-eYFP. [^{3}H]Histamine bound to the H_4Rs according to a one-site saturable model with Hill slopes of approximately 1 and dissociation constants (K_d) similar to those of the wild-type hH_4R, although the B_{max} value is affected by fusion of the *R. reniformis* luciferase enzyme (Table 1). Bound [^{3}H]histamine

TABLE 1

Characterization of epitope-tagged and H_4R fusion constructs

The pK_i values of histamine and thioperamide for the H_4R constructs used in the experiments were determined by [^{3}H]histamine saturation and displacement binding assays. The pEC_{50} values of histamine and thioperamide were determined using a CRE-luciferase reporter gene assay. The values are expressed as mean ± S.E.M. of at least three separate experiments performed in triplicate.

Receptor	[^{3}H]Histamine		pK_i		pEC_{50}	
	K_d	B_{max}	Histamine	Thioperamide	Histamine	Thioperamide
	nM	*pmol/mg protein*				
Wild-type H_4R	19.9 ± 1.4	1.0 ± 1.4	7.6 ± 0.1	7.3 ± 0.1	8.8 ± 0.2	6.4 ± 0.3
HA-H_4R	23.2 ± 0.6	2.0 ± 0.5	7.4 ± 0.1	7.0 ± 0.1	8.9 ± 0.2	6.4 ± 0.2
FLAG-H_4R	26.3 ± 5.5	1.3 ± 0.3	7.6 ± 0.1	7.4 ± 0.1	9.0 ± 0.1	6.5 ± 0.4
H_4R-*R*luc	30.8 ± 2.3	0.1 ± 0.02	7.6 ± 0.1	7.4 ± 0.1	9.0 ± 0.1	6.0 ± 0.3
H_4R-eYFP	57.4 ± 3.6	1.1 ± 0.3	7.2 ± 0.1	7.3 ± 0.2	8.6 ± 0.3	5.7 ± 0.1
c-myc-H_4R-His_{10}	33.3 ± 2.0	2.6 ± 0.5	7.3 ± 0.1	7.6 ± 0.1	9.0 ± 0.1	6.3 ± 0.3

could be displaced from all of the N- and/or C-terminally tagged H_4Rs by the agonist histamine and the inverse agonist thioperamide with affinity values (pK_i) comparable with the wild-type (Table 1). Likewise, the H_1R radioligand [^{3}H]mepyramine bound the various hH_1R constructs according to a one-site saturable binding model with K_d values similar to those of the wild-type hH_1R (data not shown). The agonist histamine and H_1R inverse agonist mepyramine were able to displace the radioligand with affinities equal to those of the wild-type H_1R (data not shown). The aforementioned H_4R constructs were functionally characterized using a cAMP response element-luciferase-luciferase reporter gene assay. In these assays, histamine behaved as a full H_4R agonist and thioperamide as a full inverse H_4R agonist for each H_4R construct, with pEC_{50} values comparable with those obtained for the wild-type H_4R (Table 1).

Generation of hH_4R-Specific Antibodies. To enable our biochemical approaches and to study H_4R function in native tissue, we raised a rabbit polyclonal anti-hH_4 (374–390) receptor antibody, which represents the first published selective immunological probe for the hH_4R. The antibody was generated against the last 17 amino acids of the C-terminal tail of the H_4R (Fig. 1A). The selectivity of the anti-hH_4R antibody was confirmed by blockade with the C-terminal peptide of the H_4R (Fig. 1B, lane 3) and a lack of cross reactivity with the human H_3R, the most related GPCR (de Esch et al., 2005) (Fig. 1B, lane 1). In transfected HEK 293 cells, the antibody detects two major reactive species at 34 to 36 and 65 to 72 kDa (Fig. 1B, lane 2). The lower bands most likely represent monomeric H_4Rs. An additional band (approximately 45 kDa) was occasionally detected; such bands are likely to represent a proteolytic fragment. We suspect the 34-kDa species to be the unglycosylated product of the species at 36 kDa. The higher molecular mass species could either represent a heavily glycosylated form of the H_4R or dimeric H_4Rs.

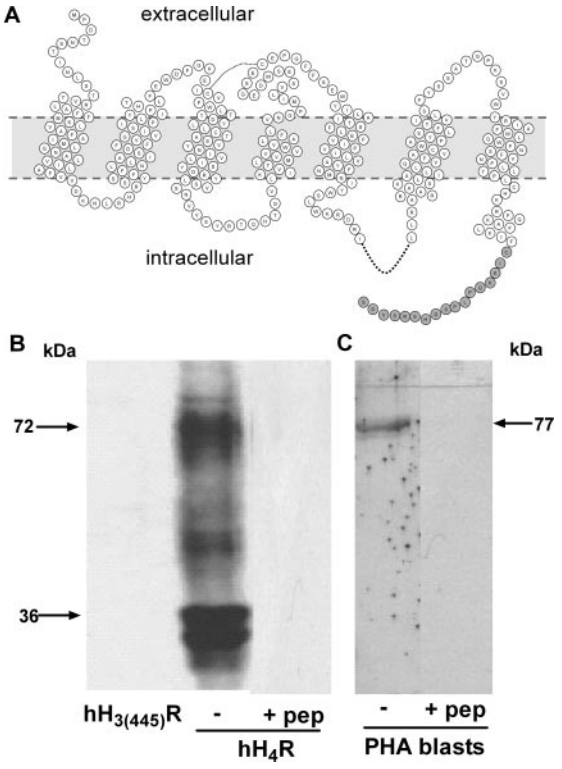

Fig. 1. Characterization of specific polyclonal H_4R antibodies. A, snake plot of the hH_4R; the region of the C-terminal tail (374–390) against which the antibody was raised is marked in gray. HEK 293 cells expressing $hH_{3(445)}$Rs or hH_4Rs (B), and human PHA blasts (C) were probed by immunoblotting using the anti-H_4 (374–390) antibody (0.5 μg/ml) either alone or preincubated for 16 h at 4°C with 500 μg/ml (374–390) peptide. The major immunoreactive species labeled in the HEK 293 hH_4R and the human PHA blasts were greatly suppressed by preincubation with the antigen peptide (B, lane 3; and C, lane 2, respectively), demonstrating the sequence selectivity of the antibody. Furthermore, no significant labeling of the $hH_{3(445)}$R (B, lane 1) or in untransfected HEK 293 cells (data not shown) was detected.

Evidence that Native H_4R Are Robust Dimers. The H_4R clearly plays a role as an immune modulator, with mRNA expression shown in human mast cells, neutrophils, eosinophils, and T lymphocytes (Nakamura et al., 2000; Oda et al., 2000; Morse et al., 2001; Zhu et al., 2001; Gantner et al., 2002; Hofstra et al., 2003). A single major diffuse immunoreactive species (approximately 77 kDa) coincident with the putative recombinant dimeric hH_4R species expressed in COS-7 cells was detected in human PHA blasts (Fig. 1C, lane 1). This species was abolished by preincubation with the 374–390 peptide, again demonstrating the peptide selectivity of the antibody. Little or no protein monomers were detected in the native preparation, consistent with our previous data with the H_3R (Chazot et al., 2001; Bakker et al., 2006). It is noteworthy that these experiments were performed under reducing conditions, indicating the robust nature of the dimeric species in native tissue. An identical labeling pattern was detected with the anti-hH_4 374–390 antibody probing human spleen lysates (data not shown). The putative dimeric recombinant hH_4R species expressed in HEK 293 cells was consistently smaller (approximately 72 kDa), which may reflect differential glycosylation in the two cell lines (Fig. 3). Coincident protein species were detected by the anti-hH_4 374–390 and the anti-epitope-tagged antibodies in the respective cell lines, further confirming that the hH_4 receptor is being labeled by the anti-hH4 374–390 antibody (data not shown). No signal was detected in either COS-7 or HEK 293 cell lines, further supporting the selectivity of the anti-hH_4 374–390 antibody. These data identify for the first time the H_4R protein in human T lymphocytes.

Immunoprecipitation of Recombinantly Expressed HA-H_4Rs from HEK 293 Cells. To further characterize the selectivity of the H_4R antibody, an immunoprecipitation assay was performed. HA-H_4Rs expressed transiently in HEK 293 cells (Fig. 2, lanes 3–5) were immunoprecipitated using anti-HA antibodies (Fig. 2, lane 4) or a nonimmune Ig (Fig. 2, lane 3). As negative controls, nontransfected HEK 293 cells (Fig. 2, lane 1) and nontransfected HEK 293 cells immunoprecipitated with anti-HA antibodies (Fig. 2, lane 2) were used. As positive control, solubilized HEK 293 cells expressing HA-H_4Rs (Fig. 2, lane 5) was used. All samples were subjected to immunoblotting using the anti-H_4R antibodies. Immunoreactive species were only detected for the HEK 293 cells expressing the HA-H_4R, which had been anti-HA-immunoprecipitated (Fig. 2, lane 4). The immunoreactive species represent the putative monomeric and dimeric H_4R and are identical with the reactive species in the positive control (Fig. 2, lane 5).

Cross-Linking of H_4Rs. To further investigate the homo-oligomeric structure of the H_4R, a cross-linking study was performed using N-terminally c-myc-tagged H_4R expressed

in COS-7 cells. Upon application of increasing concentrations of the cell-impermeable cross-linker BS_3, a progressive reduction in the monomeric doublet species (34 and 36 kDa) was observed (Fig. 3A). Concomitant the appearance of, initially, a diffuse species of 77 kDa (putative glycosylated and unglycosylated dimers) and then higher molecular mass species (>175 kDa) at 0.25 mM and 2 mM BS_3, respectively, was noticed (Fig. 3A). These data are highly consistent with hH_4Rs expressed in HEK 293 cells (data not shown).

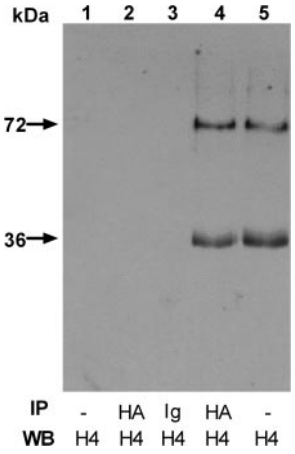

Fig. 2. The anti-H_4R antibodies recognize anti-HA immunoprecipitated (IP) HA-H_4Rs. HEK 293 cells alone (lanes 1 and 2) or transfected with cDNA encoding the HA-H_4R (lanes 3–5) were subjected to immunoprecipitation with an anti-HA antibody (lanes 2 and 4) or a nonimmune Ig (lane 3). The precipitates (lanes 2–4) or solubilized cells (lanes 1 and 5) were immunoblotted using the anti-H_4R antibody.

Biochemical Evidence that the hH_4Rs Is an *N*-Glycosylated Homodimer. In the N terminus of the hH_4R, Asn^5 and Asn^9 are potential sites for *N*-glycosylation (Fig. 3B). To study whether the higher molecular mass species are the *N*-glycosylated forms of the hH_4R, we expressed H_4Rs in the presence of the *N*-glycosylation inhibitor tunicamycin. In the absence of tunicamycin, two major putative monomeric species, 34 and 36 kDa, and a diffuse 65- to 72-kDa species were detected as in Fig. 1 (also see Fig. 3C, lane 1). In the presence of 2 μg/ml tunicamycin, a complete loss of the 36-kDa species and concomitant increase in intensity of the 34-kDa species and an additional species at 32 kDa were observed (Fig. 3C, lane 2). Furthermore, the diffuse 65- to 72-kDa species, detected in the absence of tunicamycin, was reduced to a single 65-kDa species. It is noteworthy that an increase in tunicamycin concentration had no further effect on either the 34- or 65-kDa species (Fig. 3C, lanes 3–5). The 32-kDa species observed upon tunicamycin treatment is probably a breakdown

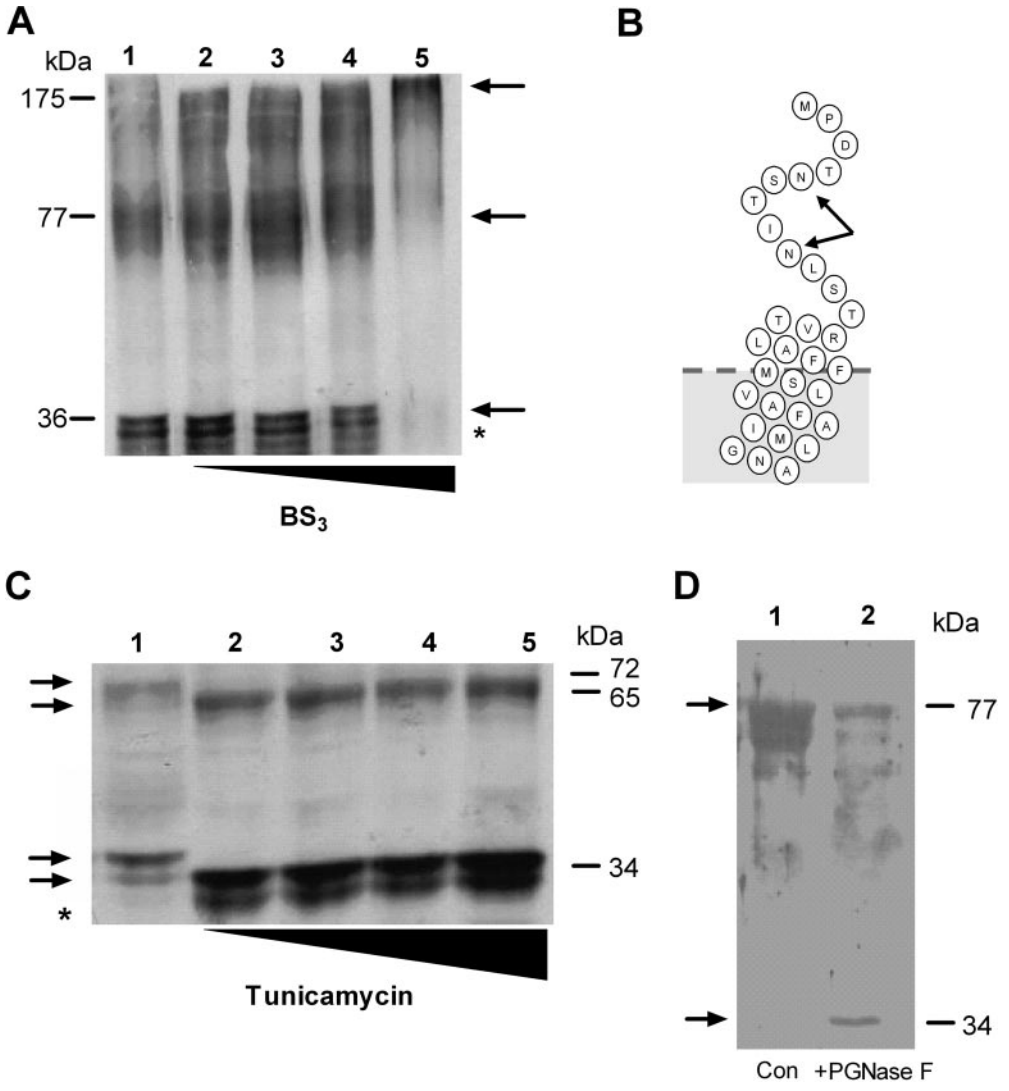

Fig. 3. Evidence for hH_4R dimers and higher oligomers and glycosylation of the hH_4R dimers. A, COS-7 cells transfected with cDNA encoding the hH_4R were subjected to cross-linking using increasing concentrations of BS_3 (0.12–2 mM). The resultant pellets were subjected to immunoblotting and probed with the anti-hH_4 (374–390) antibody (0.5 μg/ml). Lane 1, COS-7 cells expressing hH_4Rs as control; lanes 2 to 5, COS-7 cells expressing hH_4Rs treated with 0.12, 0.5, 1, and 2 mM BS_3, respectively. *, a species that is likely to be a proteolytic fragment of the hH_4R (observed in both host cells). B, snake plot of the N-terminal tail and beginning of transmembrane 1 of the H_4R; arrows indicate possible *N*-glycosylation sites. C, HEK 293 cells transfected with the hH_4R were grown in the absence and presence of 2, 4, 6, and 8 μg/ml tunicamycin for 48 h. The cells were harvested, and homogenates were prepared and subjected to immunoblotting. Immunoblots were probed with the anti-hH_4 (374–390) receptor antibody. Lane 1, hH_4Rs in absence of tunicamycin; lanes 2 to 5, hH_4Rs in presence of 2, 4, 6, and 8 μg/ml tunicamycin, respectively. D, PHA blasts were subjected to *N*-deglycosylation with PNGase F enzyme at a final enzyme concentration of 400 IU/ml for 16 h at 37°C. Control PHA blasts were incubated in parallel with deglycosylation buffer alone. Samples were then subjected to immunoblotting, and immunoblots were probed with the anti-hH_4 (374–390) receptor antibody. Lane 1, control; lane 2, in the presence of PNGase F. Enzymatic deglycosylation resulted in the reduction in intensity of the 77-kDa species and appearance of the 34-kDa putative monomer.

product of the glycosylated 36-kDa species in untreated cells. These data strongly suggest that the recombinant hH_4R is *N*-glycosylated and forms dimers. This last process is independent of post-translational *N*-glycosylation. It is noteworthy that upon enzymic *N*-deglycosylation of PHA blasts, the 77-kDa species was greatly reduced in intensity, and a new 34-kDa species was detected, consistent with the monomeric hH_4R (Fig. 3D, lane 2).

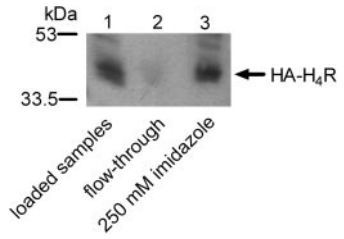

Fig. 4. Biochemical detection of homodimeric H_4Rs. Cells coexpressing H_4Rs with an N-terminal c-myc- and C-terminal His_{10}-tag (c-myc-H_4R-His_{10}) and an N-terminally HA-tagged H_4Rs (HA-H_4R) receptors were solubilized and loaded onto an Ni^{2+}-NTA column. Samples were taken of the solubilized receptors before loading onto the column (lane 1), of the unbound fraction (lane 2), and of the bound fraction that was eluted using 250 mM imidazole (lane 3). Samples were resolved by SDS-polyacrylamide gel electrophoresis and then immunoblotted using anti-HA antibodies.

HA-H_4Rs Associate with c-myc-H_4R-His_{10}. To further investigate whether the H_4Rs can associate with each other to form homo-oligomers, membranes of COS-7 cells coexpressing N-terminally c-myc and C-terminally His_{10}-tagged hH_4Rs (c-myc-H_4R-His_{10}) and N-terminally HA-tagged hH_4Rs (HA-H_4R) were solubilized and loaded on an Ni^{2+}-resin column. The HA-H_4Rs, when coexpressed with the c-myc-H_4R-His_{10}, were retained on the Ni^{2+}-column and could be eluted with 250 mM imidazole, as detected with anti-HA antibodies (Fig. 4, lane 3). When cells individually expressing c-myc-H_4R-His_{10} and HA-H_4Rs were mixed before solubilization and subsequently loaded on the column, no HA-H_4Rs were found to interact with the Ni^{2+} resin (data not shown).

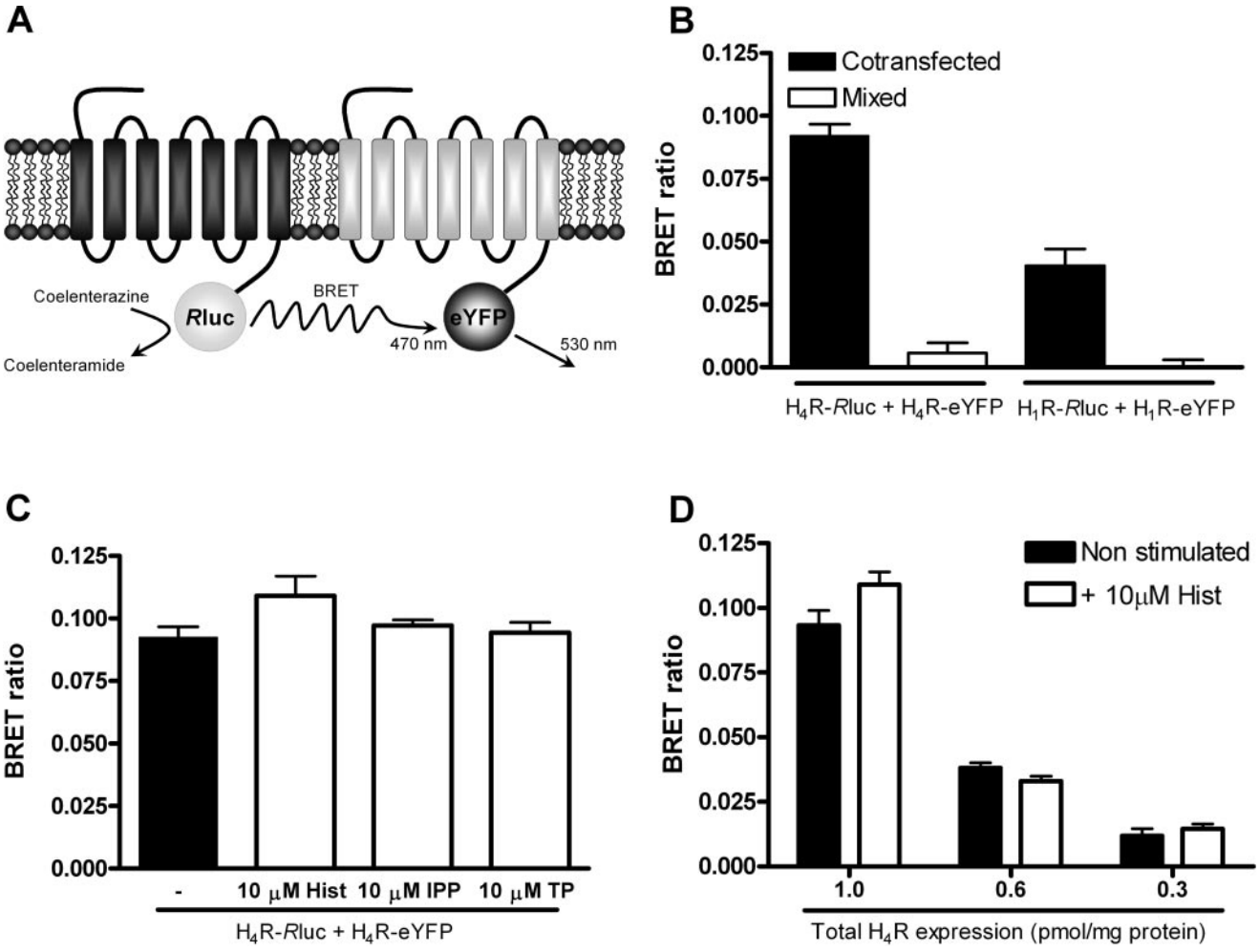

Fig. 5. Evaluation of homo-oligomerization of the H_4R and homo-oligomerization of the H_1R by BRET using the coexpression of *R*luc and eYFP C-terminal receptor-fusion proteins. A, schematic representation of BRET. After addition, coelenterazine is converted by the *R*luc enzyme fused to the C terminus of a receptor into light of a wavelength of 470 nm, which when in close proximity (<100 Å) can excite the eYFP protein fused to the C terminus of another receptor, leading to the emission of light at a wavelength of 530 nm. B, BRET ratios for the hH_4R homo-oligomers compared with the hH_1R homo-oligomers. Cells expressing the indicated receptor-fusion proteins were exposed to 5 μM coelenterazine, after which energy transfer was measured. Cells individually expressing either H_4R-*R*luc or H_1R-*R*luc were mixed before exposure to coelenterazine with cells individually expressing H_4R-eYFP or H_1R-eYFP, respectively (□). C, effects of a 15-min stimulation of 10 μM histamine (Hist), iodophenpropit (IPP), or thioperamide (TP) on the BRET ratios for the hH_4R homo-oligomers. D, effects of a 15-min stimulation of 10 μM histamine on H_4Rs homo-oligomers. Cells were expressed with a constant amount of H_4R-*R*luc (~0.2 pmol/mg of protein) and a decreasing amount of H_4R-eYFP. Total H_4R expression was 1.0, 0.6, and 0.3 (pmol/mg of protein). Ratios are expressed as the mean ± S.E.M. from at least three experiments performed in triplicate.

BRET Shows Constitutive Ligand-Independent Homo-Oligomerization of hH_4Rs. The use of biophysical techniques has been of great value to the study of GPCR oligomerization. We have used BRET to study in further detail the homo-oligomerization of the H_4R. BRET was performed on COS-7 cells expressing either the H_4R-*R*luc or coexpressing the H_4R-*R*luc with the H_4R-eYFP. After the addition of coelenterazine, a robust BRET signal could be observed in the cells coexpressing the two H_4Rs (Fig. 5B). As a negative control, cells individually expressing either of the H_4R constructs were mixed before adding coelenterazine (Fig. 5B). Previous studies have reported the ability of H_1Rs to oligomerize (Carrillo et al., 2003; Bakker et al., 2004) Therefore, cells in which the H_1R-*R*luc and the H_1R-eYFP were coexpressed were taken as a positive control. In these cells, a BRET signal was detected that was approximately 2-fold lower than that observed for the H_4Rs (Fig. 5B).

To investigate the effect of ligands on H_4R oligomerization, cells coexpressing the H_4R-*R*luc with the H_4R-eYFP were incubated with a 10 μM concentration of the agonist histamine, the neutral antagonist iodophenpropit (Lim et al., 2005) or the inverse agonist thioperamide for 15 min before the actual BRET measurement. No significant change was observed in BRET signal between stimulated and nonstimulated cells (Fig. 5C).

Agonist-induced increase in oligomerization of somatostatin receptors occurs at physiological expression levels (160 fmol/mg of protein) but not after overexpression (Patel et al., 2002). We therefore also tested the effect of histamine stimulation on H_4R oligomerization at different expression levels. While maintaining H_4R-*R*luc expression level constant (approximately 0.2 pmol/mg of protein), we reduced the amount of expressed H_4-eYFP. The concomitant reduction of the donor/acceptor ratio resulted in an expected decrease in BRET signal. At total H_4R expression levels of 1.0, 0.6, or 0.3 pmol/mg, a significant BRET signal was observed. However, histamine also did not effect the H_4R oligomerization at lower H_4R expression levels (Fig. 5D).

***tr*-FRET Shows the Presence of hH_4R Oligomers at Cell Surface.** To study whether the H_4Rs oligomers are actually present at the cell surface, we performed *tr*-FRET assays on COS-7 cells coexpressing N-terminally FLAG-tagged H_4Rs (FLAG-H_4R) and HA-H_4Rs. These cells were incubated with Eu^{3+}-labeled anti-FLAG antibodies or a combination of the Eu^{3+}-labeled anti-FLAG and allophycocyanin (APC)-labeled anti-HA antibodies. As control, cells individually expressing the FLAG-H_4Rs and the HA-H_4Rs were mixed and exposed to the two antibodies. Only from the cells coexpressing the FLAG-H_4Rs and HA-H_4Rs was a pronounced signal observed (Fig. 6B). This FRET signal can only be explained as a result of the resonance energy transfer from Eu^{3+} anti-FLAG antibodies bound to FLAG-H_4Rs to APC anti-HA antibodies bound to HA-H_4Rs. Because this resonance energy transfer can only take place within 100 Å, the data indicate the formation of H_4R oligomers at the cell surface of living cells. Stimulation of the COS-7 cells with 10 μM histamine or 10 μM thioperamide preceding *tr*-FRET measurement did not result in a significant change in signal (Fig. 6B). The used antibodies did not have an influence on the ligand binding to the H_4Rs because no significant difference was found in [^{3}H]histamine binding in the absence or presence of the antibodies (data not shown).

Lack of Hetero-Oligomerization between H_4R and H_1Rs. We have subsequently used *tr*-FRET to investigate whether hetero-oligomerization occurs between H_4Rs and H_1Rs. *tr*-FRET was performed on COS-7 cells coexpressing the FLAG-H_4Rs and N-terminally HA-tagged histamine H_1Rs (HA-H_1Rs). As a control, cells individually expressing the FLAG-H_4Rs and the HA-H_1Rs were mixed and exposed to the two antibodies. No significantly increased *tr*-FRET signal could be observed compared with the signal obtained from cells individually expressing the two receptors that were mixed before incubation with the antibodies (Fig. 6C). The ratio and total amount of antibodies was maintained equal between experiments with H_1R-H_4Rs and H_4R-H_4Rs to ensure proper comparison.

Comparable results were obtained using Eu^{3+} anti-HA antibodies and APC anti-FLAG antibodies (data not shown).

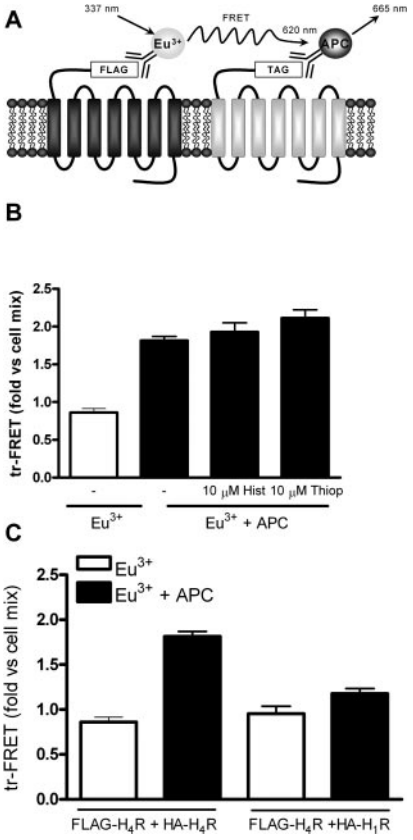

Fig. 6. Evaluation of homo-oligomerization of the H_4R and hetero-oligomerization of the H_4R with the H_1R by *tr*-FRET using coexpression of differentially epitope-tagged receptors. A, schematic representation of *tr*-FRET. Excitation at 337 nm of anti-FLAG Eu^{3+} antibody bound to the FLAG-epitope-tagged receptor leads to the emission of light at a wavelength of 620 nm, which, when in close proximity (<100 Å), can excite the anti-FLAG APC antibody bound to another FLAG-epitope-tagged receptor, leading to the emission of light at a wavelength of 665 nm. B, *tr*-FRET using cells coexpressing FLAG- and HA-tagged H_4Rs. Cells were incubated for 2 h with the Eu^{3+}-labeled anti-FLAG antibodies (□, Eu^{3+}) or with both Eu^{3+}-labeled anti-FLAG and APC-labeled anti-HA antibodies (■, APC) in the presence or absence of 10 μM histamine (Hist) or 10 μM thioperamide (Thiop). C, *tr*-FRET using cells coexpressing FLAG-tagged H_4Rs (FLAG-H_4R) and either HA-tagged H_4Rs (HA-H_4R) or HA-tagged H_1Rs (HA-H_1R). Cells were incubated for 2 h with the Eu^{3+}-labeled anti-FLAG antibodies (□, Eu^{3+}), or with both Eu^{3+}-labeled anti-FLAG and APC-labeled anti-HA antibodies (■, APC). Data are normalized for the *tr*-FRET signal obtained from a mixture of cells that was obtained by mixing of cells that have been incubated with Eu^{3+}-labeled anti-FLAG antibodies with cells that have been incubated with APC-labeled anti-FLAG antibodies. Data shown are from a representative experiment.

Stimulation of cotransfected cells with 10 μM histamine did not lead to a change in FRET signal (data not shown).

Homo-Oligomerization of H_4Rs versus Hetero-Oligomerization between H_4Rs and H_1Rs. To further investigate hetero-oligomerization between H_4Rs and H_1Rs, BRET saturation curves were produced for both the H_4R homo-oligomer and the H_1R-H_4R hetero-oligomer. Experiments were performed in which COS-7 cells were transfected with a fixed amount of H_4-*R*luc and increasing amounts of either H_4R-eYFP or H_1R-eYFP cDNA. Expression levels were determined by radioligand binding. Expression of the H_4R-*R*luc was maintained at approximately 0.2 pmol/mg of protein. Expression levels of H_4R-*R*luc were correlated with luminescence and expression levels for the eYFP fused H_1R and H_4R were correlated with fluorescence. A linear correlation was obtained for all three constructs. Expression for the H_4*R*-eYFP ranged from 0.3 to 2.5 pmol/mg of protein, whereas expression levels of the H_1R-eYFP ranged from 0.5 to 16 pmol/mg of protein. For the H_4R homo-oligomers, a steep increase in BRET signal is observed, showing detectable BRET when total H_4R expression is 0.3 pmol/mg of protein. For the H_1R-H_4R hetero-oligomers, a more gradual increase in BRET signal is observed upon increased expression of the H_1R-eYFP. A BRET signal is observed for the first time at expression levels of 1 pmol/mg of protein of the H_1R-eYFP. The H_4R-H_4R homo-oligomer showed a 2-fold lower $BRET_{50}$ value (0.77 versus 1.6) and a 2.5-fold higher B_{max} (0.1 versus 0.04) than the H_1R-H_4R hetero-oligomer as determined from the BRET saturation curve (Fig. 7).

Discussion

GPCR oligomerization has become a generally accepted phenomenon and has been reported to occur in all GPCR classes (George et al., 2002). Data obtained from atomic force microscopy (Fotiadis et al., 2003), electron microscopy, and Western blot analysis (Suda et al., 2004) have provided compelling evidence that the light-sensitive rhodopsin is predominantly present as a dimer in the retinal disc membrane. For histamine receptors, oligomerization has been shown convincingly for the hH_1Rs (Carrillo et al., 2003; Bakker et al., 2004), the hH_2Rs (Fukushima et al., 1997), and H_3Rs (Shenton et al., 2005; Bakker et al., 2006). In view of the emerging role of GPCR oligomerization in GPCR function and our interest in the H_4R as a new target for inflammatory conditions (de Esch et al., 2005), we investigated oligomerization of the human H_4R by various means. Combining biophysical measurements like *tr*-FRET and BRET (Angers et al., 2002; Boute et al., 2002) with biochemical approaches, like Western blot analysis and histidine-tag-based affinity chromatography, we provide compelling evidence for homo- and hetero-oligomer formation of hH_4Rs.

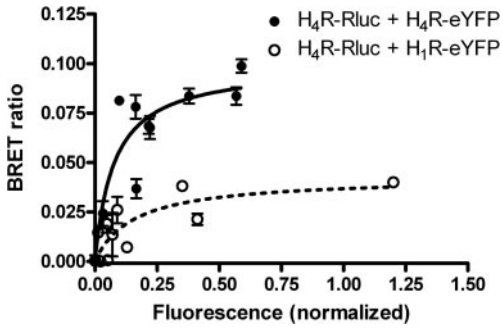

Fig. 7. Evaluation of receptor-expression dependence of the detection of H_4R homo-oligomers and H_1R-H_4R hetero-oligomers using BRET. BRET saturation curves for the hH_4R homo-oligomers (H_4R-*R*luc + H_4*R*-eYFP, solid line) compared with H_1R-H_4R hetero-oligomers (H_4R-*R*luc + H_1R-eYFP, broken line) at increasing expression levels of the eYFP-tagged receptor. COS-7 cells were transfected with a fixed amount DNA encoding for the H_4R-*R*luc and increasing amounts of DNA encoding for the H_4*R*-eYFP or the H_1R-eYFP. Plotted on the *x*-axis is the fluorescence obtained from the eYFP, which has been correlated to the expression of H_4*R*-eYFP (●) and H_1R-eYFP (○). Expression level of the H_4R-*R*luc was maintained at approximately 200 fmol/mg of protein, as determined from the luminescence, which has been correlated to the expression of the H_4R-*R*luc.

To enable our biochemical approaches and to study H_4R function in native tissues, we report in this study on the first polyclonal H_4R antibody that can successfully be used for Western blot analysis. This new molecular tool is directed against the C-terminal tail of the H_4R and detected monomeric and potential dimeric H_4R species after Western blot analysis of membranes from HEK 293 and COS-7-transfected cells. The selectivity of the new H_4R antibody was confirmed by blockade with the C-terminal peptide used to raise the antibody and the lack of cross-reactivity toward the highly related human hH_3R. Furthermore, through an immunoprecipitation study, the H_4R antibody was shown to detect the same HA-H_4Rs as detected by a commercially available anti-HA antibody.

Western blot analysis of the H_4R expressed in tunicamycin-treated cells indicates that the H_4R normally is *N*-glycosylated, most likely at Asn^5 and/or Asn^9 of the extracellular N terminus of the H_4R (Nguyen et al., 2001). However, inhibition of *N*-glycosolyation did not affect the presence of putative dimeric H_4R species on the Western blot. To show that H_4R proteins are in close proximity of each other, a requirement for oligomerization, a cross-linking experiment, using BS_3 was performed. With increasing concentrations of the cross-linker BS_3, the bands representing the monomeric H_4R disappeared. At the same time, bands representing oligomeric H_4Rs became more apparent. These data indicate that the H_4Rs are in close enough proximity for cross-linking by BS_3 and suggests that the 65- to 72-kDa species might represent dimerized H_4R proteins. Finally, the polyclonal H_4R antibody allowed us to study the presence of H_4R proteins in human PHA blasts. High-level H_4R mRNA expression has been shown in various white blood cells, including T-lymphocytes (Nakamura et al., 2000; Oda et al., 2000; Morse et al., 2001; Zhu et al., 2001; Hofstra et al., 2003), but H_4R protein expression so far has not been shown. Western blot analysis of membranes of PHA blasts with our polyclonal anti-H_4R antibody indeed revealed the presence of H_4R protein in PHA blasts. It is interesting to note that the endogenously expressed H_4R was only detected as a high molecular mass species. Enzymatic deglycosylation of the native H_4R protein resulted in a partial reduction of the high molecular mass species (77 kDa) to monomeric H_4Rs (34 kDa). These data in themselves do not directly exclude a heavily glycosylated (approximately 33 kDa) H_4R protein. However, the hH_4Rs in human HEK 293 cells is only moderately glycosylated (approximately 2 kDa), and the high molecular mass species coincide with the putative dimeric H_4Rs when recombinantly expressed in COS-7 (77 kDa) and HEK 293 cells (72 kDa). These data can be explained by assuming that in human PHA blasts, the hH_4R functions predominantly as a dimer.

We hypothesize that *N*-glycosylation is not a prerequisite for dimerization, but it helps to stabilize the H_4R dimers. A similar stabilizing effect of glycosylation on receptor dimers has recently been shown for the human bradykinin B_2 receptors (Michineau et al., 2006). The putative H_4R dimerization in native tissue clearly warrants further investigation.

As an alternative biochemical method, we used an immobilized metal (Ni^{2+}) affinity chromatography approach with a histidine-tagged H_4R protein. To this end, we coexpressed c-myc-H_4R-His_{10} and HA-H_4Rs receptors to study oligomer formation via affinity column chromatography. In contrast to c-myc-H_4R-His_{10} receptors, HA-H_4Rs are not robustly retained onto an Ni^{2+}-NTA resin when expressed alone. Yet c-myc-H_4R-His_{10} receptors immobilized onto Ni^{2+}-NTA resin were shown also to retain coexpressed HA-H_4Rs on the Ni^{2+}-NTA column, as determined by HA-immunoreactivity detected after elution of histidine-tagged proteins with high imidazole concentrations. These findings indicate that c-myc-H_4R-His_{10} receptors physically interact with the coexpressed HA-H_4Rs to form oligomers that can be retained on the Ni^{2+}-NTA resin through the C-terminal His_{10} tag.

We continued our investigation of the oligomerization of the H_4Rs in living cells using BRET and *tr*-FRET assays. Using the BRET assay, a clear signal could be detected when coexpressing H_4R-*R*luc with H_4R-eYFP in nonstimulated cells, suggesting constitutive homo-oligomerization of H_4Rs. Because the oligomers detected in the immobilization and BRET assays do not necessarily have to be present at the cell surface, we also studied H_4R oligomerization on the cell membrane of living cells by *tr*-FRET. The *tr*-FRET approach uses antibodies that do not permeate the cell membrane and detects cell surface H_4R oligomers present at the cell surface. Similar to the BRET assay, we detected a robust signal, indicating the constitutive presence of H_4R homo-oligomers at the cell surface.

A number of studies have investigated the effect of agonists on receptor oligomerization. However, at present, the effects of ligand stimulation on GPCR oligomerization are not consistent. It has been found that agonists can promote or reduce GPCR oligomerization or are without effect on GPCR oligomerization (Angers et al., 2002; George et al., 2002; Pfleger and Eidne, 2005). In the case of the H_4R, we did not detect any significant difference in BRET signal if cells were treated with either the H_4R agonist histamine, the neutral H_4R antagonist iodophenpropit (Lim et al., 2005), or the inverse H_4R agonist thioperamide (Morse et al., 2001; Lim et al., 2005), suggesting that H_4R ligands do not modulate H_4R homo-oligomerization. Likewise, no agonist- or inverse agonist-induced modulation of H_4R oligomerization was detected in the *tr*-FRET assay. Patel et al. (2002) reported that agonist-induced oligomerization of somatostatin receptors was only detected at physiological expression levels but not after overexpression. We therefore performed BRET experiments at various H_4R expression levels. First, it is noteworthy that already at an H_4R expression level of approximately 300 fmol/mg of protein, significant BRET signals can be observed. These data indicate that at physiological expression levels, the H_4R can indeed homo-oligomerize and corroborate our findings of H_4R dimers, detected on PHA blasts with our anti-H_4R antibody. Second, from the BRET experiments, we also conclude that also at low expression levels of the H_4Rs, homo-oligomerization is not affected by agonist stimulation. Nevertheless, one should be aware that results concerning ligand effects on dimerization obtained with these biophysical assays can be difficult to interpret, because agonist-induced changes in H_4R conformation could potentially influence the energy transfer between the energy acceptor and donor (Angers et al., 2000).

The H_4R has been linked to play a role in inflammation based on its expression pattern and recent findings, showing that the H_4R induces chemotaxis of eosinophils (O'Reilly et al., 2002; Buckland et al., 2003) and mast cells (Hofstra et al., 2003) and stimulates the release of interleukin-16 from $CD8^+$ T cells (Gantner et al., 2002) and the release of leukotriene B_4 in zymosan-challenged mice (Takeshita et al., 2003). The H_1R is colocalized with the H_4R in several white blood cells (Cameron et al., 1986; Morse et al., 2001) and plays a prominent role in inflammatory conditions (Giustizieri et al., 2004; Matsubara et al., 2005). Because both the H_1Rs (Carrillo et al., 2003; Bakker et al., 2004) and the H_4R (this study) are able to form homo-oligomers, we were prompted to study whether the H_4Rs can form hetero-oligomers with the H_1Rs. In fact, previous work with hetero-oligomeric opioid receptors has revealed that GPCR hetero-oligomerization brings an additional layer of complexity to the class of GPCR proteins (Bouvier, 2001; Devi, 2001; Franco et al., 2003; Waldhoer et al., 2005) but also offers opportunities to develop hetero-oligomeric-selective ligands (Waldhoer et al., 2005). Yet using *tr*-FRET assays, we were unable to detect H_1R-H_4R hetero-oligomers, suggesting that such GPCR hetero-oligomers are not present at the cell surface. In contrast to the *tr*-FRET experiments, we were able to detect an expression level-dependent formation of H_1R-H_4R hetero-oligomers using BRET. Distinct from the detection of H_4R homo-oligomers, H_1R-H_4R hetero-oligomers were only detected at high-expression levels, and we failed to detect hetero-oligomers at physiologically relevant conditions. Results from BRET saturation studies demonstrate a higher propensity for the formation of H_4R-H_4R homo-oligomers over H_1R-H_4R hetero-oligomers. We presume that the signal observed with BRET at high expression levels possibly originates from intracellular H_1R-H_4R hetero-oligomers. Whereas some receptors, such as the 5-hydroxytryptamine-1A receptor seem to readily form heteromeric receptors (Salim et al., 2002), our present H_4R data corroborate the idea that GPCR hetero-oligomerization is highly selective, as reported for the adrenergic receptors (Stanasila et al., 2003; Uberti et al., 2005) and the thyrotropin-releasing hormone receptors (Kroeger et al., 2001). Although hetero-oligomerization has been shown to occur even between receptors from different classes (Ferre et al., 2002), the relatively low homology (23%) between H_1R and H_4R (Oda et al., 2000) is apparently too low to readily form hetero-oligomers.

In conclusion, we have developed specific antibodies against the C terminus of the H_4R which allowed the detection of endogenously expressed H_4R proteins. This anti-hH_4R antibody is an important new molecular tool for studying the localization and function of the H_4R. Moreover, we determined by various methods that the H_4R constitutively forms cell surface homo-oligomers. Homodimeric H_4Rs are not only found using heterologous ex-

pression systems but are also present in PHA blasts and spleen lysates endogenously expressing H_4Rs. The formation of H_4R oligomers is not dependent on *N*-glycosylation or affected by ligand stimulation but is possibly destabilized by deglycosylation. Although H_1R-H_4R hetero-oligomers could be detected using BRET upon receptor overexpression, these hetero-oligomers are probably not present at the cell surface. Moreover, H_1R-H_4R hetero-oligomers were not found at physiologically relevant expression levels. Future studies will have to reveal whether the H_4R can form hetero-oligomers with other GPCR family members or if it preferentially exists as homo-oligomer.

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