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Cloning and characterization of dominant negative splice variants of the human histamine H₄ receptor.¹

Richard M. van Rijn^{*2}, André van Marle^{*3}, Paul L. Chazot[†], Ellen Langemeijer^{*}, Yongjun Qin[‡], Fiona C. Shenton[†], Herman D. Lim^{*}, Obbe P. Zuiderveld^{*}, Kamonchanok Sansuk, Michel Dy[§], Martine J. Smit^{*}, Cornelis P. Tensen[‡], Remko A. Bakker^{*4}, and Rob Leurs^{*}

^{*}Leiden/Amsterdam Center for Drug Research, Department of Medicinal Chemistry, Vrije Universiteit Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands,

[†]Centre for Integrative Neuroscience (CINS), School of Biological & Biomedical Sciences, Durham University, South Road, Durham DH1 3LE, England,

[‡]Leiden University Medical Center, Skin Research Lab, Einthovenweg 20, 2300 RC Leiden, The Netherlands,

[§]UMR 8147, Centre National de la Recherche Scientifique, Faculte de Medecine Rene Descartes, Paris V, Hopital Necker, 75015 Paris

Running title: Dominant negative isoforms of the histamine H₄ receptor.

Address correspondence to: Rob Leurs, tel: ++31-20-5987600, fax ++31-20-5987610. E-mail: leurs@few.vu.nl

¹ The sequence of the identified hH₄R₍₃₀₂₎ and hH₄R₍₆₇₎ have been deposited in the GenBank® database (accession number DQ835186 and DQ835187, respectively)

² Current address: Department of Neuroscience, Ernst Gallo Clinic and Research Center, University of California San Francisco, 5858 Horton St. Ste 200, Emeryville, CA 94608, United States

³ Current address: Biofocus DPI, Archimedesweg 4, 2333 CN Leiden, The Netherlands

⁴ Current address: Boehringer Ingelheim Pharma GmbH & Co. KG, Department of Metabolic Diseases, 88397 Biberach, Germany (*RAB*).

Synopsis

The histamine H₄ receptor (H₄R) is the latest identified member of the histamine receptor subfamily of G protein-coupled receptors with potential functional implications in inflammatory diseases and cancer. The H₄R is primarily expressed in eosinophils and mast cells and shares highest homology with the H₃R. The occurrence of at least twenty different hH₃R isoforms led us to investigate the possible existence of H₄R splice variants. Herein we report on the cloning of the first two alternatively spliced H₄R isoforms from CD34⁺ cord blood cell derived eosinophils and mast cells. These H₄R splice variants are localized predominantly intracellularly when expressed recombinantly in mammalian cells. We failed to detect any ligand binding, H₄R-ligand induced signaling or constitutive activity for these H₄R splice variants. However, when co-expressed with the full-length H₄R (H₄R₍₃₉₀₎), the H₄R splice variants have a dominant negative effect on the surface expression of the H₄R₍₃₉₀₎. We detect H₄R₍₃₉₀₎-H₄R splice variant hetero-oligomers by employing both biochemical (immunoprecipitation, cell-surface labeling) and biophysical (*time-resolved* FRET) techniques. Messenger RNAs encoding the H₄R splice variants are detected in various cell types and are expressed at similar levels as the full-length H₄R₍₃₉₀₎ mRNA in for example premonocytes. We conclude that the herein described H₄R splice variants have a dominant negative effect on H₄R₍₃₉₀₎ functionality, being able to retain the H₄R₍₃₉₀₎ intracellularly and to inactivate a population of H₄R₍₃₉₀₎ presumably via hetero-oligomerization.

Key words: Histamine, H₄ receptor, g protein-coupled receptor, isoform, alternative splicing, oligomerization,

List of nonstandard abbreviations: hH₄R, human histamine H₄ receptor; H₃R, histamine H₃ receptor; GPCR, G protein-coupled receptor; COS-7, African green monkey kidney; HEK, human embryonic kidney; FRET, fluorescence energy transfer; *tr*, *time-resolved*; PBS, phosphate-buffered saline; HA, hemagglutinin; Q-PCR, Quantitative-Polymerase Chain Reaction; IL, interleukin; TM, transmembrane; JNJ 7777120, 1-[(5-chloro-1*H*-indol-2-yl)carbonyl]-4-methylpiperazine.

Introduction

The human histamine H₄ receptor (hH₄R) is the fourth and most recently discovered member of the G-protein coupled receptor (GPCR) subfamily of histamine receptors [1-6]. The H₄R is predominantly expressed in hematopoietic cells and is suggested to play a role in inflammation [7, 8] and allergy [9]. Interestingly, recently connections of the H₄R with rheumatoid arthritis [10], colon cancer [11, 12] and breast cancer [13] have also been proposed. Consequently, the H₄R is currently seen as a potential new drug target [14] and is getting much attention from pharmaceutical industry.

The hH₄R gene has been cloned on the basis of its relatively high homology with the histamine H₃ receptor (H₃R) [1-6]. The H₄R gene shows the same genomic organization as the H₃R gene, with the positions of two introns at identical sites as found for the H₃R gene [4, 15], potentially resulting in the existence of differentially spliced isoforms, as have been detected for both human and rat H₃Rs [16, 17]. A large variety of genes encoding GPCRs have been found to be alternatively spliced [18] thereby introducing additional diversity to GPCR pharmacology. Natural occurring splice variants may have practically identical pharmacological properties as the full-length receptor [19], but can also have altered functionality or lack any functional responses [18, 20, 21]. Moreover, the expression of full-length receptors and receptor isoforms can be differentially regulated as was recently demonstrated for the expression of the neurokinin-1 receptor and a C-terminally truncated neurokinin-1 receptor isoform in undifferentiated and differentiated THP-1 cells [22].

For the H₃R a large number of alternatively spliced isoforms have been described [16, 17]. Many human H₃R isoforms, with deletions in the third intracellular loop and potentially different signal transduction, have been reported. However, also a variety of non-7TM H₃R isoforms are found in brain mRNA [16, 17]. Recently, we have shown that several natural occurring 6TM rH₃R splice variants interfered with trafficking of the full-length rH₃R to the cell membrane, hence reducing its signaling properties [23]. Such dominant negative behavior of alternatively spliced isoforms has for example also been described for the α_{1A} -adrenergic receptor [20], the dopamine D_{3nf} receptor [21], an alternatively spliced rat vasopressin V₂ receptor [24] and a naturally occurring splice variant of the calcitonin receptor lacking exon 13 [25].

In view of the great interest in the H₄R as a pharmacological target and the large diversity of GPCR isoforms for the related H₃R, the identification of H₄R isoforms is of utmost importance for the future understanding of H₄R pharmacology and target validation strategies. In the present study we report on the identification and pharmacological characterization of two new splice variants of the hH₄R. Although translated and present at the cell surface, albeit to a lesser extent than the H₄R₍₃₉₀₎, these receptor isoforms are non-functional when individually expressed in recombinant mammalian cells. However, when co-expressed with the hH₄R₍₃₉₀₎ the two H₄R isoforms are able to decrease [³H]histamine binding to the hH₄R₍₃₉₀₎. Surface labeling experiments showed a reduction in H₄R₍₃₉₀₎ surface expression when co-expressed with either of the H₄R isoforms. The apparent mechanism underlying the dominant negative effect might be attributed to hetero-oligomerization between the hH₄R₍₃₉₀₎ and the H₄R splice variants as determined by biophysical and biochemical techniques.

Experimental:

Cloning and sequence analysis of the hH₄(₃₀₂) and H₄(₆₇) splice variants cDNAs.

In order to identify and isolate H₄R splice variants, CD34⁺ cells were isolated from cord blood mononuclear cells and were cultured in 100 ng/ml stem cell factor (SCF) and 80 ng/ml interleukin 6 (IL6) for 8-10 weeks, in 5 ng/ml IL3 and 1 ng/ml transforming growth factor- β for 3-4 weeks or in 2 ng/ml IL3 and 5 ng/ml IL5 for 4-5 weeks, to differentiate the CD34⁺ cells to mast cells, basophils or eosinophils, respectively [26-28]. Peripheral blood mononuclear cells were cultured in 100 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 10 ng/ml IL4 to create immature dendritic cells, which were subsequently activated with either lipopolysaccharide (LPS) or a combination of tumor

necrosis factor α and IL1 β [3, 29]. Total RNA was isolated using the nucleo spin RNA II purification kit (Clontech Laboratories, Palo Alto, USA) and cDNA was obtained by random primed cDNA synthesis. A set of (nested) sense and anti-sense oligonucleotide primers, spanning the intron/exon junctions of the H₄R gene sequence, were used in order to identify potential H₄R splice variants. Use of the following sense primers 5'-TGTGATGCCAGATACTAATA-3', 5'-ATGCCAGATACTAATAGCAC-3', 5'-TCTTTGTGGGTGTGATCTCC-3' and anti-sense primers 5'-TGTGAT GGCAAGGATGTACC-3', 5'-TTAAGAAGATACTGACCGAC-3', 5'-TCTTTAAGAAGATACTGACC-3' resulted in the amplification of at least two different products. The generated PCR fragments were cloned in pCRII-TOPO vectors (Invitrogen) and the cDNAs were sequenced on both DNA strands. The sequence of the identified hH₄R₍₃₀₂₎ and hH₄R₍₆₇₎ have been deposited in the GenBank® database (accession number DQ835186 and DQ835187, respectively). The cloned cDNAs were transferred to the mammalian expression vector pcDEF3 [30], either untagged or tagged N-terminally with a FLAG-epitope (DYKDDDDK).

Culture and differentiation of HL-60 cells.

HL-60 suspension cells were maintained at 37°C humidified in 5% CO₂/95% air atmosphere in RPMI 1640 + L-Glutamine supplemented with 10% (v/v) fetal bovine serum and antibiotics. Cells were differentiated to granulocytes and eosinophils. HL60 cells were incubated for 5 days with 0.65% (v/v) N, N-dimethylformamide to differentiate the cells to granulocytes. Eosinophilic differentiation was performed by culturing HL-60 cells in the presence of 0.5 mM butyric acid for 48h. IL-5 was added to a concentration of 10 ng/ml, and the cells were cultured for 72h [1]. Total RNA was isolated using TRIzol according to manufacturer's protocol. cDNA was synthesized using Iscript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The cDNA was subsequently used in Q-PCR.

Quantitative PCR.

The integrity and quality of isolated total RNA was determined by agarose gelelectrophoresis and concentration was determined spectrophotometrically. Next, 1 μ g of total RNA was DNase treated at 37°C for 15 min (RQ1 DNase, Promega), and the reaction terminated at 60°C for 10 min. The treated RNA was directly subjected to 1st strand cDNA synthesis (Iscript, Bio-Rad) in 20 μ l. The synthesized cDNA was diluted to 500 μ l with distilled water and then used for Q-PCR (MyIQ, Bio-Rad) in a 20 μ l reaction (2x SYBR buffer 10 μ l, 25 pM primers, and cDNA 5.0 μ l). The reaction was stopped after the 40th PCR cycle was completed.

Primers of H₄R₍₃₉₀₎ and two splice variants H₄R₍₃₀₂₎ and H₄R₍₆₇₎ were designed using Beacon designer software (Bio-Rad). A sense primer was designed that recognizes both H₄R₍₃₉₀₎ and the H₄R splice variants 5'-TGCTAGGAAATGCTTTGGTC-3'. Isoform specificity was obtained by designing anti-sense primers that are able to bind only to the cDNA of either the H₄R₍₃₉₀₎ 5'-GCGTGTGAGGGATGTACAAA-3', the H₄R₍₃₀₂₎ 5'-TCCAAGACTCTGAAACCACAA A-3' or the H₄R₍₆₇₎ 5'-TCCAAGACTCTGAAACCACAAA-3'. Specificity of primers was evaluated by checking dissociation curve and amplification slope. For data analysis, the nuclear ribosome protein 11 (rps11, NM001015) was used as reference to H₄R expression. Expression level was analyzed based on the 2^{- $\Delta\Delta$ Ct} value method [31].

Cell culture and transfection.

COS-7 and HEK 293T cells were maintained at 37°C humidified in 5% CO₂/95% air atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal calf serum, 50 IU/ml of penicillin and 50 μ g/ml streptomycin and grown in 100 mm dishes. COS-7 Cells were transiently transfected using the DEAE-dextran method as previously described [32]. HEK 293T cells were

transfected using the polyethyleneimine (PEI) transfection method adapted from Durocher et al [33]. Briefly, 5 µg DNA was diluted in 500 µl 0.9% NaCl, and 10 µl of a 1 mg/ml PEI (linear MW~25.000) was added. Following ten minutes of incubation the mixture was added to the HEK 293T cells. 24 hours after transfection, cells were transferred into 96 well plates. In all experiments the total amount of DNA transfected was maintained constant by addition of the empty expression vector pcDEF₃.

Radioligand binding studies.

Cells used for radioligand binding studies were harvested 48h post transfection and homogenized in ice cold hH₄R binding buffer (50 mM Tris, pH 7.4). For saturation isotherms cell membrane homogenates were incubated at 37°C for 60 minutes with 0-150 nM [³H]histamine or 0-30 nM [³H]JNJ 7777120 [34] in a total assay volume of 200 µl. For competition binding assays the cell homogenates were incubated at 37°C for 60 minutes with 0.1-10.000 nM ligand in the presence of ~15 nM [³H]histamine in a total volume of 200 µl. The incubations were stopped by rapid dilution with ice cold hH₄R binding buffer. To determine H₁R binding was determined using [³H]mepyramine as radioligand and hH₁R binding buffer (50 mM phosphate buffer pH 7.4). To determine non-selective binding non-labelled JNJ 7777120 and mianserin were used for H₄R and H₁R, respectively. The bound radioactivity was separated by filtration through GF/C filterplates (Perkin Elmer) that had been treated with 0.3% PEI. Filters were washed four times with ice cold hH₄R binding buffer and radioactivity retained on the filters was measured by liquid scintillation counting.

CRE-luciferase reporter gene assay.

HEK 293T cells transiently co-transfected with 2.5 µg pTNLC-121 (containing 21 cAMP-responsive elements upstream of the luciferase cDNA) [35] and 5 µg pcDEF3-H4R, pcDEF3-H4R(302), pcDEF3-H4R₍₆₇₎ were seeded in 96-well white-plates in culture medium and after 42h incubated with histamine or thioperamide and 0.5 µM forskolin for 6h. After 48h, cells were assayed for luminescence by aspiration of the medium and the addition of 25 µl/well luciferase assay reagent [0.83 mM ATP, 0.83 mM D-luciferin, 18.7 mM MgCl₂, 0.78 µM Na₂H₂P₂O₇, 38.9 mM Tris, pH 7.8, 0.39% (v/v) glycerol, 0.03% (v/v) Triton X-100, and 2.6 µM dithiothreitol]. After 30 min, luminescence was measured for 3 s/well in a Victor² 1420 Multilabel Counter (PerkinElmer, Boston, USA).

Enzyme linked immunosorbent assay (ELISA).

48h after transfection, receptor expression in COS-7 cells was measured using an ELISA assay as previously described [36]. To detect HA (hemagglutinin)-epitope tagged receptors a rat anti-HA antibody was used as primary antibody, and a rabbit anti-rat-horseradish peroxidase conjugate as secondary antibody. To detect FLAG-epitope tagged receptors a mouse anti-FLAG antibody was used as primary antibody and a goat anti-mouse-horseradish peroxidase conjugate as secondary antibody. The 3', 3', 5, 5'-tetramethylbenzidine liquid substrate system for ELISA was used as substrate and the optical density was measured at 450 nm using a Victor² 1420 Multilabel Counter.

***tr*-FRET assay.**

tr-FRET assays were performed using a combination of both Eu³⁺-labelled and allophycocyanin-labelled anti-FLAG and anti-HA antibodies as described previously [37]. Briefly, *tr*-FRET was assessed in 5 x 10⁵ intact COS-7 cells, transiently expressing the appropriate HA and FLAG tagged receptors. Cells were incubated in PBS containing 50% FCS (v/v), 0.8 nM of Eu³⁺-labelled antibody, and 8 nM of allophycocyanin-labelled antibody for two hours at room temperature. After two wash steps the cells were resuspended in 50 µl PBS and transferred to a 384-microtiter plate. Energy transfer was measured by

exciting the Eu^{3+} label at 320 nm and monitoring the allophycocyanin emission for 500 μs at 665 nm using a Novostar (BMG Labtechnologies, Offenberg, Germany) configured for *tr*-fluorescence after a 100 μs delay.

Surface biotinylation assay.

HEK 293 cells were transfected with clones encoding individual H_4R isoforms or co-transfected with $\text{H}_4\text{R}_{(390)}$ with variable amounts of green fluorescent protein (GFP, control), $\text{H}_4\text{R}_{(302)}$ or $\text{H}_4\text{R}_{(67)}$ isoforms (1:1 or 1:5). The medium was carefully removed 24-40 hours post transfection and cells were washed with ice-cold PBS/4 % (w/v) sucrose (3x1 ml per 35mm dish). The cells were then incubated for 30 min at 4°C with 1mg/ml Sulfo-NHS-SS-Biotin (Pierce, Rockford, IL, USA) in ice-cold PBS/4 % (w/v) sucrose (0.5 ml/dish) with gentle shaking. Subsequently, cells were washed once with ice-cold PBS/4 % (w/v) sucrose (1ml/dish), and incubated for 10 min at 4°C with quenching buffer (192mM glycine, 4% (w/v) sucrose in 50mM Tris-HCl pH 8.0) (0.5 ml/dish). Cells were scraped into PBS/4 % (w/v) sucrose and spun at 6000 rpm for 2 min. This wash procedure was repeated on a further occasion and the cells were homogenised in 1% (w/v) SDS in lysis buffer (50mM Tris-HCl, pH 8.0/ 2 mM EDTA/1% Triton-X100). The samples were then diluted five-fold in lysis buffer (without SDS), and incubated with 20 μl 50% (w/v) slurry of streptavidin beads for 2 hrs at 4°C. Following incubation, beads were centrifuged at 9000rpm at 4°C for 5 min. The supernatant (intracellular fraction) was retained and the beads washed in lysis buffer. The bound fraction was isolated by elution in SDS PAGE Sample buffer/50mM DTT at 50°C for 30 min. Supernatants following centrifugation at 9000 rpm were retained (surface fraction) for analysis.

Immunoprecipitation of H_4R isoforms.

HEK 293 cells were transfected with $\text{H}_4\text{R}_{(390)}$ with or without FLAG- $\text{H}_4\text{R}_{(302)}$ or FLAG- $\text{H}_4\text{R}_{(67)}$. Cell samples were solubilised with 1% Triton-X100 in 50 mM Tris-HCL pH 7.4 and incubated with 50 μl 50% (w/v) slurry of anti-FLAG Sepharose beads (Sigma, UK) for 2 hrs at 4°C. Following incubation, beads were centrifuged at 9000 rpm at 4°C for 5 min. The supernatant was retained (unbound fraction) and the beads washed in 50 mM Tris-HCL pH7.4/Triton X100 and bound fraction isolated by elution in SDS PAGE Sample buffer/50mM DTT at 50°C for 30 min. Supernatant following centrifugation at 9000 rpm was retained (bound fraction) for analysis.

Immunoblotting.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 6 % or 7.5 % polyacrylamide slab gels under reducing conditions. Samples of HEK 293 cells (20-50 μg protein) were prepared using a chloroform/methanol method of protein precipitation, and immunoblotting was performed as previously described [23, 37, 38]. Immunoblots were probed with anti- H_4R 374-390 antibody [37] at a concentration of 0.5 μg /ml. Blots containing FLAG- or HA-tagged receptors were probed with primary antibodies, mouse anti-FLAG (1.5 μg /ml) or rat anti-HA (1 μg /ml), respectively. Horseradish peroxidase conjugated goat anti-mouse or anti-rat antibodies (1:2000-5000) were used as secondary antibodies (Little Chalfont, Buckinghamshire, England).

Drugs and Reagents.

Reagents for *tr*-FRET were from Cis Bio International (Bagnols-sur-Cèze Cedex, France). Chloroquine diphosphate, DEAE-dextran (chloride form), dithiotreitol, histamine(2-[4-imidazolyl]ethylamine hydrochloride), clozapine, mianserin, 3,3',5,5'tetramethyl benzidine (TMB) monoclonal mouse M2 anti-FLAG (DYKDDDDK), N,N-dimethylformamide, pertussis toxin, triton X-100 and polyethyleneimine was purchased from Sigma (St.Louis, MO). High affinity rat anti-HA (YPYDVPDYA) was from Roche

Applied Science (Basel, Switzerland). Goat anti-mouse IgG (H+L) HRP was obtained from Bio-Rad (Hercules, CA, USA), polyclonal rabbit anti-Rat IgG HRP was from Dakocytomation (Glostrup, Denmark). Calf serum was purchased from Integro BV (Dieren, The Netherlands). Polyethyleneimine (linear, MW~25.000) was from Polysciences Inc (Warrington, PA, USA). D-luciferin was from Duchefa (Haarlem, The Netherlands). Cell culture media, penicillin, streptomycin and TRIzol were obtained from Invitrogen (Breda, The Netherlands). Cell culture plastics were from Greiner Bio-one (Wemmel, Belgium). Tris was from AppliChem (Darmstadt, Germany). [³H]histamine (18.10 Ci/mmol) and [³H]mepyramine (23 Ci/mmol) were purchased from Perkin-Elmer Life Science, Inc. (Boston, MA, USA). Oligonucleotides were purchased from Isogen Bioscience (Maarsen, The Netherlands). PfuTurbo[®] DNA polymerase was purchased from Stratagene (La Jolla, CA, USA). Restriction enzymes were from MBI Fermentas (St. Leon-Rot, Germany). The human H₄R₍₃₉₀₎ cDNA in pcDNA3.1 was purchased from the Guthrie cDNA resource center (Sayre, PA, USA). The hH₄R was subcloned into the pcDEF₃ using *Bam*HI/*Xba*I sites. Thioperamide fumarate, 4-methylhistamine and JNJ 7777120, were synthesized at the Department of Medicinal Chemistry, Vrije Universiteit Amsterdam, Amsterdam. Gifts of expression vector pcDEF₃ (Dr. J Langer) and [³H]JNJ 7777120 (Dr. R. Thurmond) are greatly acknowledged.

Data Analysis.

All radioligand binding data were evaluated by a non-linear least squares curve fitting program using Graphpad Prism[®] (Graphpad Software Inc, San Diego, CA). Protein concentrations were determined according to Bradford, using BSA as standard. All data are represented as mean ± S.E.M. from at least three independent experiments in triplicate. Statistical significance was determined by an ANOVA-tukey test ($p < 0.05$ was considered statistically significant).

Results.

Cloning of human H₄R splice variants. Using mRNA isolated from mast cells, eosinophils, basophils and dendritic cells derived from CD34+ cord blood cells, we investigated the occurrence of hH₄R isoforms. Our RT-PCR approach using specific primers (see *Materials and Methods*) identified two new H₄R splice variants. The first splice variant encodes a protein consisting of 302 amino acids (H₄R₍₃₀₂₎), compared to 390 amino acids of the H₄R₍₃₉₀₎ and was cloned from mRNA from eosinophils. Alternative splicing of mRNA encoding H₄R₍₃₀₂₎ results in the deletion of exon 2. A previously unidentified exon/intron junction within exon 3 of the hH₄R gene, however, allows for the use of an alternative splicing acceptor maintaining the gene in its original frame. Consequently, the H₄R₍₃₀₂₎ isoform has a deletion of 88 amino acids between TMII and TMIV (Figure 1). The second splice variant was cloned independently from mRNA from both mast cells and eosinophils. Alternative splicing of mRNA encoding this H₄R isoform also results in the removal of exon 2. However, in this case the H₄R₍₃₉₀₎ splice acceptor site of exon 3 is recognized, causing a frame-shift and the introduction of an alternative stop codon. This results in a severely truncated H₄R₍₆₇₎ receptor containing only the first 67 amino acids of the hH₄R (Figure 1).

Endogenous occurrence of H₄R splice variants mRNA in human cells. Quantitative PCR (Q-PCR) was subsequently used to investigate the presence of mRNA encoding the genes of the hH₄R₍₃₉₀₎ and the hH₄R splice variants in several cell types. To this end, mRNA of undifferentiated HL-60 cells (pre-monocytes) and HL-60 cells, differentiated to produce eosinophils or granulocytes, were collected and subjected to Q-PCR analysis. As control, a gene, encoding for ribosomal protein S11 was also amplified to correct obtained mRNA expression levels. RPS11 mRNA expression is not affected by the various experimental conditions used, and Q-PCR efficiency of RPS11 is similar to that of the H₄R₍₃₉₀₎ and the H₄R splice variants (data not shown). Of all tested cells, eosinophils expressed the highest amounts of mRNA of the H₄R isoforms (Table 1). In pre-monocytes the H₄R₍₆₇₎ mRNA levels were almost 2-fold higher compared to the H₄R₍₃₉₀₎ mRNA levels, whereas the H₄R₍₃₂₀₎ expression was very low in this cell

type. In the other tested cells, the mRNA levels of the H₄R₍₃₉₀₎ were considerably higher compared to the levels of the 2 splice variants (Table 1).

Pharmacological characterization of newly identified H₄R splice variants

We have previously successfully employed COS-7 cells for the heterologous expression of the H₄R as well as for the identification of H₄R oligomers [37]. To study their pharmacology we therefore also expressed the two H₄R splice variants heterologously in COS-7 cells. The H₄R agonist radioligand [³H]histamine and the H₄R antagonist radioligand [³H]JNJ 7777120 bound to the H₄R₍₃₉₀₎ according to a one site saturable model with dissociation constants (K_D) of 23.9 ± 3.6 nM (n=3) and 11.1 ± 3.6 nM (n=3), respectively. The obtained B_{max}-values for both radioligands are approximately 1 pmol/mg protein. However, transfection of COS-7 cells with cDNAs coding for the H₄R splice variants did not result in the detection of significant specific radioligand binding using either [³H]histamine (see Supplementary Figure 1A) or [³H]JNJ 7777120 (see Supplementary Figure 1B).

To determine whether the H₄R splice variants were constitutively active and/or able to signal via G_{i/o}-proteins, the cDNA encoding the H₄R₍₃₉₀₎, H₄R₍₃₀₂₎ or H₄R₍₆₇₎ were heterologously transfected in HEK 293T cells together with a CRE-luciferase reporter gene. Histamine acts as a full agonist (pEC₅₀ = 8.6 ± 0.1, n=5) on the H₄R₍₃₉₀₎. Moreover thioperamide acts as a full inverse agonist (pEC₅₀ = 6.7 ± 0.1, n=4), revealing the known constitutive activity of the H₄R₍₃₉₀₎ (see Supplementary Figure 2A). No effect of either histamine or thioperamide could be observed for both the H₄R₍₃₀₂₎ and the H₄R₍₆₇₎ isoforms over a range of 0.1-10.000 nM (see Supplementary Figure 2C and 2E). Whereas pretreatment with 0.1 µg/ml pertussin toxin (PTX) abolishes the constitutive signaling of the H₄R₍₃₉₀₎ (see Supplementary Figure 2B) in the CRE-reporter gene assay, no effect of PTX was observed for the H₄R₍₃₀₂₎ and the H₄R₍₆₇₎ isoforms compared to cells not treated with PTX (see Supplementary Figure 2D and 2F).

H₄R splice variants are translated and present predominantly intracellularly. The absence of radioligand binding and G_{i/o}-coupled signaling of the H₄R splice variants could simply be due to the fact that receptor proteins are not expressed. In order to determine whether the H₄R splice variants are translated into protein and to determine the localization of these splice variants an ELISA was performed with COS-7 cells expressing HA epitope-tagged the H₄R₍₃₉₀₎ (HA-H₄R₍₃₉₀₎), FLAG epitope-tagged H₄R₍₃₀₂₎ (FLAG-H₄R₍₃₀₂₎) or FLAG-H₄R₍₆₇₎ (Figure 2A). The localization (cell surface or intracellular) of the receptors was determined by permeabilization of the cells using NP-40. Whereas the HA-H₄R₍₃₉₀₎ is readily observed on the cell membrane, both H₄R splice variants are located predominantly intracellularly, although a small fraction of each was clearly detected on the cell membrane (Figure 2A).

Surface labeling experiments provided additional evidence for the intracellular localization of the H₄R splice variants (Figure 2B). HEK 293 cells expressing the FLAG-H₄R₍₃₀₂₎ or the FLAG-H₄R₍₆₇₎ were biotinylated to distinguish intracellularly localized H₄R isoforms from receptors present at the cell surface. Similar to the ELISA results, the majority of the H₄R isoforms can be detected intracellularly (Figure 2B). We could only detect β-actin in the intracellular fraction, suggesting that the H₄R splice variant protein detected in the surface fraction is not due to contamination from the intracellular fraction (Figure 2B). In both assays the presence of H₄R splice variants at the cell surface is more evident for the FLAG-H₄R₍₆₇₎ than for the FLAG-H₄R₍₃₀₂₎ (Figure 2B). No anti-FLAG reactivity was observed in mock transfected HEK 293 cells (data not shown).

H₄R splice variants are able to down-regulate H₄R₍₃₉₀₎ binding. Natural occurring splice variants of for example the rH₃R have a dominant negative effect on receptor cell-surface expression of the full-length rH₃R [23]. To investigate the effect of the H₄R splice variants on the H₄R pharmacology, [³H]histamine radioligand binding was performed on membranes of COS-7 cells co-expressing the H₄R₍₃₉₀₎ together with either the H₄R₍₃₀₂₎ or the H₄R₍₆₇₎ splice variant. [³H]histamine bound to the COS-7 cells co-expressing the H₄R₍₃₉₀₎ with the H₄R splice variants according to a one site, saturable model with dissociation constants (K_D) similar to the value obtained for the H₄R₍₃₉₀₎ expressed individually in COS-7 cells (Figure 3A, Table 2). However, the maximal amount of H₄Rs labelled by [³H]histamine (B_{max})

decreased upon co-transfection of individual cDNAs coding for the H₄R₍₃₉₀₎ and the H₄R splice variants in a 1:1 cDNA ratio compared to cells transfected only with cDNA encoding the H₄R₍₃₉₀₎. A decrease in B_{max} of 30 ± 5% (n=3) was observed when the H₄R₍₃₉₀₎ was co-expressed with the H₄R₍₆₇₎. An even larger decrease of 55 ± 3% (n=3) was observed when co-expressed with the H₄R₍₃₀₂₎ (Figure 3A, Table 2). We next tested a set of typical H₄R ligands for their affinity in cells expressing the H₄R₍₃₉₀₎ alone or when co-expressed with the H₄R₍₃₀₂₎ and H₄R₍₆₇₎. No difference in K_i values for the selective H₄R agonist 4-methylhistamine, the H₄R inverse agonist thioperamide, the non-imidazole H₄R neutral antagonist JNJ 777120 and the non-imidazole H₄R agonist clozapine [1, 39] were observed (Figure 3B, Table 2). However, we still observed the decrease in apparent H₄R₍₃₉₀₎ B_{max} when co-expressed with either one of the splice variants. We continued to explore this by transfecting a fixed amount of H₄R₍₃₉₀₎ with increasing amounts of H₄R₍₃₀₂₎ cDNA, as the observed decrease in B_{max} was strongest with this splice variant. The decrease in B_{max} is directly related to the ratio of H₄R₍₃₉₀₎ and H₄R₍₃₀₂₎ cDNA that was transfected i.e. transfecting more H₄R₍₃₀₂₎ cDNA results in a concomitant decrease of the B_{max} value for [³H]histamine (Figure 3C). To determine if the effect on B_{max} was selective for the H₄R₍₃₉₀₎ we transfected a fixed concentration of H₁R cDNA with increasing amounts of H₄R₍₃₀₂₎ cDNA. No significant effect on H₁R B_{max} could be observed even when expressing 5-fold more H₄R₍₃₀₂₎ than H₁R cDNA (Figure 3D). Moreover the observed decrease in H₄R₍₃₉₀₎ B_{max} was not a result of saturation of the translation system, as co-expression of H₁R, which does not physically interact with the H₄R [37] had no negative influence on H₄R expression (Figure 3E).

Co-expression of the H₄R₍₃₉₀₎ with the H₄R splice variants results in reduced surface expression of the H₄R₍₃₉₀₎. To determine the location of the H₄R₍₃₉₀₎ upon co-expression with either of the two H₄R isoforms a biotinylation-surface labeling assay was performed in combination with Western blot analysis using our previously described anti-H₄R antibody [37] on HEK 293 cells either individually expressing the H₄R₍₃₉₀₎ or co-expressing the H₄R₍₃₉₀₎ with the H₄R₍₃₀₂₎ or the H₄R₍₆₇₎. In agreement with the ELISA results, the H₄R₍₃₉₀₎ is located predominantly on the cell surface when individually expressed (Figure 4, lane 1). As reported previously [37], the H₄R₍₃₉₀₎ is detected both as a monomer and as a dimeric receptor. However, upon co-expression of the H₄R₍₃₉₀₎ with the H₄R₍₃₀₂₎ or H₄R₍₆₇₎ a clear reduction in intensity in anti-H₄R reactivity is observed in the cell surface fraction (Figure 4, lanes 4 and 7). This reduction in apparent H₄R surface expression is clearly dependent on the amount of splice variant co-transfected with the H₄R₍₃₉₀₎. Increasing the amount of H₄R isoform 5-fold causes a stronger reduction in anti-H₄R reactivity (Figure 4, lanes 5 and 8). This reduction was not observed when co-transfecting identical amounts of GFP (Figure 4, lane 2). Blotting of intracellular fractions showed that the total amount of H₄R₍₃₉₀₎ was unaffected by co-expression of either H₄R splice variant (see Supplemental Figure 3).

Hetero-oligomerization between H₄R₍₃₉₀₎ and H₄R splice variants. We hypothesized that hetero-oligomerization between the H₄R₍₃₉₀₎ and the H₄R splice variants might be responsible for intracellular retention of the H₄R₍₃₉₀₎ and the observed decrease in [³H]histamine binding to the co-expressed H₄R₍₃₉₀₎. We therefore initially performed *tr*-FRET measurements to measure hetero-oligomerization. For this purpose, COS-7 cells co-expressing N-terminally HA-tagged histamine H₄R₍₃₉₀₎ (HA-H₄R₍₃₉₀₎) and either FLAG-H₄R₍₃₀₂₎ or FLAG-H₄R₍₆₇₎ were incubated with Europium (Eu³⁺) labeled anti-HA antibodies or a combination of the Eu³⁺ labeled anti-HA and allophycocyanin (APC) labeled anti-FLAG antibodies. The resultant data were normalized against *tr*-FRET signals obtained from COS-7 cells individually expressing the FLAG-tagged H₄R splice variants and the HA-H₄R₍₃₉₀₎s that were mixed and exposed to the two antibodies. Upon co-expression of the HA-H₄R₍₃₉₀₎ in a 0.2:1 cDNA ratio with the FLAG-H₄R₍₃₀₂₎ or the FLAG-H₄R₍₆₇₎ isoforms a significant *tr*-FRET signal could be observed compared to the *tr*-FRET signal obtained from a mixture of cells individually expressing the two receptors (Figure 5). Increasing the amount of HA-H₄R₍₃₉₀₎ co-transfected with the H₄R splice variant 5-fold to a 1:1 cDNA ratio, resulted in an further increase in *tr*-FRET signal for the H₄R₍₃₉₀₎-H₄R₍₆₇₎ hetero-oligomer (Figure 5). As a control, we used COS-7 cells co-expressing the FLAG-H₄R₍₃₉₀₎ and triple HA-tagged sphingosine-1-phosphate (EDG1) receptor (3xHA-S₁P₁R). Although the S₁P₁R and the H₄R₍₃₉₀₎ are co-localized in a variety of cell

types [40] and the S_1P_1R is known to form homo- and hetero-oligomers [41], no hetero-oligomerization occurred between these two receptors in COS-7 cells (Figure 5).

Additional support for hetero-oligomerization between $H_4R_{(390)}$ and the two H_4R splice variants arose from co-immunoprecipitation studies. HEK 293 cells co-expressing either the $H_4R_{(390)}$ with FLAG- $H_4R_{(390)}$, FLAG- $H_4R_{(302)}$ or FLAG- $H_4R_{(67)}$ were solubilized and immunoprecipitated with anti-FLAG sepharose beads and subsequently immunoblotted using the anti- H_4R antibody. In all three cases the anti- H_4R antibody was able to detect the $H_4R_{(390)}$ (Figure 6, lane 2, 4 and 6). As the anti- H_4R antibody recognizes the C-terminal tail of the $H_4R_{(390)}$ the antibody can still react with the $H_4R_{(302)}$ but not with the $H_4R_{(67)}$. Western blot analysis on immunoprecipitated FLAG- $H_4R_{(302)}$ clearly shows the presence of the full length $H_4R_{(390)}$ as well as the shorter $H_4R_{(302)}$. However, we do not consistently detect the $H_4R_{(302)}$ on western blot, suggesting that the antibody interaction with the $H_4R_{(302)}$ may be weaker than with the $H_4R_{(390)}$. It is possible that small differences in receptor folding causes the C-terminal tail of the $H_4R_{(302)}$ to be less available for the H_4R antibody compared to the $H_4R_{(390)}$. Additionally, a broad high molecular weight species can be distinguished, most likely consisting of homo-dimeric $H_4R_{(390)}$ and FLAG- $H_4R_{(302)}$ and hetero-dimeric $H_4R_{(390)}$ -FLAG- $H_4R_{(302)}$ (Figure 6, lane 4). The FLAG- $H_4R_{(67)}$ also physically interacts with the $H_4R_{(390)}$ as we can co-immunoprecipitate the $H_4R_{(390)}$ with the FLAG- $H_4R_{(67)}$. Interestingly, no monomeric $H_4R_{(390)}$ was detected in the immunoprecipitated FLAG- $H_4R_{(67)}$ sample (Figure 6, lane 6). As control, transfected HEK 293 cells were immunoprecipitated with sepharose beads. In these samples no detectable H_4R was precipitated (Figure 6, lanes 1, 3 and 5).

Discussion

The hH_4R , the latest member of the histamine receptor family [1-6], is expressed abundantly on hematopoietic cells [1, 3-5] and its role in eosinophil and mast cell functions [7, 42, 43] points to a potential role in various allergic and inflammatory conditions [7, 9, 14]. Within the histamine receptor family the H_4R shares highest homology with the H_3R ; up to 58% within the transmembrane (TM) domains. Moreover, the organization of the gene encoding the H_4R closely resembles that of the H_3R gene, as both genes consist of three exons and two introns [4, 15]. In recent years, a large number of alternatively spliced isoforms have been discovered for the H_3R [16, 17]. In our attempts to determine if alternative splice variants also exist for the H_4R , we describe here for the first time two isoforms of the human H_4R , which we cloned from cDNA from human CD34+ cord blood cells derived eosinophils and mast cells. The first splice variant, lacks part of TMII, TMIII and TMIV ($H_4R_{(302)}$), while the second splice variant is a heavily truncated H_4R and contains only TMI and the first half of TMII ($H_4R_{(67)}$). Quantitative PCR showed that in different cell types mRNA encoding the $H_4R_{(390)}$ the H_4R splice variants are differentially expressed. The H_4R splice variant mRNA was most abundant in eosinophils, which is in agreement with the fact that both isoforms were cloned from eosinophils. Interestingly, in pre-monocytes the expression of $H_4R_{(67)}$ mRNA resembles that of the $H_4R_{(390)}$. The lower abundance for mRNA of H_4R splice variants in other cell types, could be due to reduced mRNA stability compared to the mRNA of the full-length receptor and appears to be common for other GPCR splice variants as well [44, 45]. Yet, it is possible that in different cells or under different (patho) physiological conditions the H_4R splice variants are expressed more abundantly, resulting in the dominant negative effect seen in our recombinant cell systems. Evidence supporting this possibility has been reported recently for the H_3R isoforms in the rat brain [23]. Future studies should address these important questions.

In March 2003 Merck registered a patent (WO 03/020907 A2) in which they describe the discovery of two splice variants of the hH_4R , $H_{4b}R$ and $H_{4c}R$, cloned from human spleen cDNA. While the alternative splicing of the H_4R that leads to the formation of the $H_{4b}R$ and the $H_{4c}R$ is identical, the $H_{4c}R$ appears to result from alternatively splicing event after exon 2. The $H_{4c}R$ is the result of the recognition of an alternative acceptor site in exon 3, resulting in a 33 amino acid deletion near TMIV. Interestingly, the reported sequences of both the $H_{4b}R$ and $H_{4c}R$ contain additional amino acids, which we have not observed in the identified $H_4R_{(67)}$ or $H_4R_{(302)}$ and can also not be explained based on the H_4R genomic

sequence. According to the patent (WO 03/020907 A2), the H_{4b}R and H_{4c}R are able to bind [³H]histamine and to signal via several functional pathways, such as Ca²⁺-mobilization, inhibition of cAMP production and the activation of mitogen activated protein kinase (MAPK). In our search for H₄R isoforms we did not detect the H_{4b}R and H_{4c}R in immature dendritic cells (DC), differentially activated DCs (LPS or TNF α /IL β 1), mast cells, eosinophils or basophils. Using PCR, we constructed both the putative H_{4b}R and H_{4c}R isoforms, but in contrast to the findings of Merck, we were not able to detect any specific [³H]histamine binding using these splice variants (data not shown).

Both the H_{4R}₍₃₀₂₎ and H_{4R}₍₆₇₎ lack TMIII, which harbors the key aspartate residue, D⁹⁴ (3.32), presumed to be crucial for histamine binding, as well as for the high affinity binding of other known orthosteric H₄R ligands [46]. As expected, the two novel H₄R splice variants were unable to bind the agonist radioligand [³H]histamine or the non-imidazole antagonist radioligand [³H]JNJ 7777120 [34]. Although the two isoforms apparently are unable to bind ligands with high affinity, it was still possible that the two receptor isoform could signal constitutively or at high histamine concentrations. Previous studies have shown that the H_{4R}₍₃₉₀₎ is constitutively active and can be further activated by H₄R agonists [1, 39]. However, whereas PTX treatment or the inverse agonist thioperamide inhibit the constitutive activity of the H_{4R}₍₃₉₀₎, no effect of thioperamide or PTX treatment was observed for both new H₄R isoforms. Moreover, also high concentrations of histamine were unable to modulate H₄R signal transduction in cells expressing either one of the two H₄R isoforms. These data indicate that neither the H_{4R}₍₃₀₂₎ nor H_{4R}₍₆₇₎ alone are able to (constitutively) activate the G_{i/o}-coupled signaling pathway by themselves.

Since both the H_{4R}₍₃₀₂₎ and H_{4R}₍₆₇₎ isoforms are unable to signal and are expressed predominantly intracellular as determined by ELISA and Western blot experiments, we decided to explore the effects of these splice variants upon co-expression with the H_{4R}₍₃₉₀₎. Dominant negative splice variants of GPCRs, influencing the functionality (for example signaling or trafficking) of the full-length receptor have been shown for a variety of GPCRs such as the histamine rH₃R [23], the dopamine D₃R [21], the gonadotropin-releasing hormone receptor GnRH [47] and the growth hormone receptor GHR [48]. In all these cases, the reported dominant negative effect of the receptor isoforms is presumably due to hetero-oligomerization between the full-length receptor and the receptor isoform. Upon co-expression of the H_{4R}₍₃₉₀₎ with the H_{4R}₍₃₀₂₎ or the H_{4R}₍₆₇₎ isoforms a clear decrease in B_{max} for [³H]histamine was observed. However, the ligand binding properties of the remaining [³H]histamine binding sites were unaffected as the K_D value for [³H]histamine as well as K_i values of other H₄R ligands did not change when the H_{4R}₍₃₉₀₎ is co-expressed with either of the H₄R splice variants. The apparent reduction in B_{max} could be explained by reduced cell-surface expression of H_{4R}₍₃₉₀₎. We have recently shown that three non-functional rH₃R splice variants all have a dominant negative effect on the cell-surface expression of full-length H₃Rs, presumably by rH₃ isoform hetero-dimerization [23]. We have previously shown by various methods that also the H_{4R}₍₃₉₀₎ can readily form homo-oligomers [37]. In the present study *time-resolved* FRET as well as co-immunoprecipitation experiments revealed that the H_{4R}₍₃₉₀₎ can form cell-surface hetero-oligomers with the H_{4R}₍₃₀₂₎ as well as with the H_{4R}₍₆₇₎ isoforms. Similar to our previous report on H₄R homo-dimers, we find that the homo-dimers and hetero-dimers are (partially) insensitive to SDS and DTT, suggesting that these dimers may interact via strong hydrophobic interactions, as has been reported for for example the H₃R receptor [23] and the μ -opioid receptor- α_{2A} -adrenergic receptor hetero-oligomer [49]. Co-expression of the H₄R splice variants resulted in a clear reduction in the surface expression of the H_{4R}₍₃₉₀₎ as determined in a surface labeling assay. The reduction in H₄R binding and surface expression is not merely caused by saturation of the expression system, as both co-transfection of H₁R and GFP did not have any negative influence on the expression of H₄R. These findings resemble our previous findings with the rH_{3DEF} isoforms, which trap all full-length rH₃R intracellularly [23]. However, the herein described H₄R splice variants resemble more the D_{3nr}R than the rH₃R isoforms, which seem to be expressed exclusively intracellularly [23]. Similar to the H₄R isoforms, the D_{3nr}R is non-functional, expressed at the cell surface to some extent and reduces the binding of radioligand to the D₃R through hetero-oligomerization [21].

Hetero-oligomerization between the H₄R₍₃₉₀₎ and the H₄R₍₆₇₎ appears to have a less pronounced effect on the cell surface location of the H₄R₍₃₉₀₎ compared to the H₄R₍₃₀₂₎ (B_{max} reduced to 70% vs. 45% at similar cDNA ratios). Co-immunoprecipitation data of the H₄R₍₆₇₎ with the H₄R₍₃₉₀₎ indicate the assembly of predominantly high molecular weight species. One might speculate that the small H₄R₍₆₇₎ is able to form higher oligomeric complexes with H₄R₍₃₉₀₎ (trimers, tetramers) more readily than the larger H₄R₍₃₀₂₎ isoform, but has less cytoplasmic (ER) retention properties. The fact that we see an increase in *tr*-FRET signal when co-expressing more H₄R₍₆₇₎ with H₄R₍₃₉₀₎ may support a hypothesis for the formation oligomeric H₄R₍₆₇₎-H₄R₍₃₉₀₎ complexes. However, more detailed studies such as for example employing triple BRET/FRET assays using bimolecular fluorescence complementation [50], are required to further investigate this hypothesis.

The observed hetero-oligomerization between the H₄R₍₃₉₀₎ and either of the two H₄R splice variants also offers insight into the domains involved in H₄R oligomerization. In general, family A GPCRs are thought to oligomerize by interaction of TM domains, either via the formation of contact-dimers or by domain-swapping [51, 52]. When focusing on aminergic receptors, several TM domains have been reported to be involved in the mechanism of oligomerization. For example the involvement of TMIV in the oligomer interface has been clearly demonstrated for the dopamine D2 receptor, [53]. However, our data from the heavily truncated H₄R₍₆₇₎ reveals that for the H₄R TMIV is not crucial for the formation of H₄R dimers, whereas TMI-II must be involved. This is similar to the α_{1B} -adrenergic receptor for which TMI is important for homo-oligomerization [54, 55]. Trans membrane domain II of H₄R₍₆₇₎ is partly truncated, therefore it will most likely be part of the C-terminal tail of H₄R₍₆₇₎. Consequently, we propose TMI as an important domain involved in H₄R oligomerization.

In summary, we have cloned and characterized two new splice variants of the human H₄R from CD34+ cord blood derived eosinophils. Although both H₄R splice variants are non-functional and mainly localized intracellularly when recombinantly expressed in mammalian cells, they can hetero-oligomerize with the H₄R₍₃₉₀₎, thereby reducing the ability of the H₄R₍₃₉₀₎ to reach the cell surface and functionally bind histamine. As mRNA of these splice variants is differentially expressed in different cell types, we propose that under certain conditions, these newly discovered H₄R splice variants may have a role in the regulation of H₄R₍₃₉₀₎ function. Future research will focus on finding more areas where these splice variants are abundantly expressed, investigating the mechanism of hetero-oligomerization in greater detail and on determining whether the observed dominant effect of the splice variants seen in our recombinant systems can be detected in native tissue.

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Table 1. Expression of H₄R₍₃₉₀₎ and H₄R isoforms messenger RNA in a variety of differentiated HL-60 cells.

HL-60 cells were cultured under various conditions (see *Materials and Methods*) to differentiate the cells to pre-monocytes (pH 7.4 or pH 8.0), granulocytes or eosinophils. The amount of mRNA encoding H₄R₍₃₉₀₎, H₄R₍₃₀₂₎ and H₄R₍₆₇₎ were determined by Q-PCR. Messenger RNA expression values were normalized against the validated control gene encoding ribosomal protein S11 (RPS11).

	Pre-monocytes	Pre-monocytes (pH 8)	Granulocytes	Eosinophils
H ₄ R ₍₃₉₀₎	11.5 ± 10	4.7 ± 1.5	46.8 ± 23	179 ± 30
H ₄ R ₍₃₀₂₎	<<1	<<1	<1	<1
H _{4c} R ₍₆₇₎	<1	9.1 ± 5.9	<<1	29.0 ± 7.1

Fold expression (relative to rps11, *10⁻⁶)

Table 2. Characterization of the hH₄R₍₃₉₀₎ expressed individually or co-expressed with either the H₄R₍₃₀₂₎ or the H₄R₍₆₇₎ splice variant in transiently transfected COS-7 cells.

The K_D values, the H₄R expression (B_{max}) and the pK_i values of 4-methylhistamine (4-Me-HA), clozapine, JNJ 7777120 and thioperamide were determined by [³H]histamine saturation and displacement binding assays. COS-7 cells were transfected in a 1:1 ratio with cDNA encoding the H₄R₍₃₉₀₎ and the H₄R splice variants. The values are expressed as mean ± SEM of at least three independent experiments.

Receptor	K _D (nM)	B _{max} (%)	[³ H]histamine			
			4-Me-HA	Clozapine	JNJ 7777120	Thioperamide
H ₄ R ₍₃₉₀₎	23.9 ± 2.8	100	7.1 ± 0.1	6.5 ± 0.1	7.7 ± 0.1	7.3 ± 0.1
H ₄ R ₍₃₉₀₎ + H ₄ R ₍₃₀₂₎	24.4 ± 2.0	45 ± 3	7.1 ± 0.1	6.5 ± 0.1	7.7 ± 0.1	7.2 ± 0.1
H ₄ R ₍₃₉₀₎ + H ₄ R ₍₆₇₎	20.2 ± 1.9	70 ± 5	7.1 ± 0.1	6.4 ± 0.1	7.7 ± 0.1	7.3 ± 0.1

Figure Legends

Figure 1: Sequence alignment of human H₄R splice variants and genomic arrangement of the H₄R gene. (A). Amino acid alignment of the hH₄R with the two identified H₄R splice variants. Indicated underneath the amino acids are seven transmembrane domains as proposed for the hH₄R₍₃₉₀₎ (TM1 through TM7). Two arrows indicate where intron 1 and intron 2 is splice out of the H₄R₍₃₉₀₎ mRNA. The H₄R₍₃₀₂₎ splice variant passes over exon 2 and recognizes an alternative acceptor site (arrow between TMIV and TMV) within exon 3. The H₄R₍₆₇₎ splice variant also skips exon 2 but recognizes the typical acceptor site after intron 2, resulting in a frameshift and the introduction of an alternative, premature stop codon. (B). Overview of the exon/intron structure of the H₄R gene on chromosome 18q11.2 (Genbank accession number NM_021624 (GI:14251204)). The exon/intron junctions for the H₄R₍₃₉₀₎, H₄R₍₃₀₂₎ and H₄R₍₆₇₎ are portrayed. Exon sequences are depicted in upper case, and intron sequences in lower case.

Figure 2: The H₄R splice variants are expressed predominantly intracellularly when recombinantly expressed in COS-7 cells. (A). ELISA assays were performed on transiently transfected COS-7 cells individually expressing HA-H₄Rs, FLAG-H₄R₍₃₀₂₎ or FLAG-H₄R₍₆₇₎. Receptor expression and localization were determined using anti-HA and anti-FLAG antibodies on intact (open bars) and permeabilized cells (closed bars). Data has been normalized against COS-7 cells transfected with empty vector. (B). HEK 293 cells were transfected with either FLAG-H₄R₍₃₀₂₎ or FLAG-H₄R₍₆₇₎ receptor isoforms. Intact cells were biotinylated for 15 min at 4°C with 1mg/ml Sulfo-NHS-SS-Biotin, washed and homogenised. Biotinylated surface fraction was isolated by streptavidin chromatography, and analyzed by immunoblotting using anti-FLAG antibodies. Lanes 1 and 3: Intracellular fraction; Lanes 2 and 4: Surface fractions, where lanes 1 and 2 are FLAG- H₄R₍₃₀₂₎ samples, and lanes 3 and 4 and FLAG- H₄R₍₆₇₎ samples. The lower panel shows the same blot reprobed with anti-β actin antibody as a control to confirm that intracellular proteins are not detected in the surface fraction and equal amounts of sample were used. Representative experiments are shown (n≥3).

Figure 3: Dominant negative effect of H₄R splice variants on [³H]histamine binding to H₄R₍₃₉₀₎. (A). Saturation of [³H]histamine to H₄R receptors expressed individually (●) or co-expressed with the H₄R splice variants H₄R₍₃₀₂₎ (■) or H₄R₍₆₇₎ (○) in COS-7 cells. (B). Displacement of [³H]histamine from H₄R expressed individually (●) or co-expressed with the H₄R splice variants H₄R₍₃₀₂₎ (■) or H₄R₍₆₇₎ (○) in COS-7 cells by the H₄R antagonist JNJ 777120 (dotted line) or H₄R agonist clozapine (solid line). (C). Dose dependent decrease of [³H]histamine binding to H₄R₍₃₉₀₎ by increasing the amount of H₄R₍₃₀₂₎ cDNA transfected transiently in COS-7 cells (D). No decrease in [³H]mepyramine binding to H₁R with increasing amounts of H₄R₍₃₀₂₎ cDNA transfected transiently in COS-7 cells (E). Co-expression of H₄R₍₃₉₀₎ with H₁R in a 2:1 ratio has no negative influence on H₄R₍₃₉₀₎ binding in COS-7 cells Representative experiments are shown (n=3).

Figure 4: Reduced surface expression of H₄R₍₃₉₀₎ when co-expressed with H₄R isoforms. HEK 293 cells individually expressing H₄R₍₃₉₀₎ or co-expressing H₄R₍₃₉₀₎ with either GFP (control), H₄R₍₃₀₂₎ or H₄R₍₆₇₎ with cDNA ratios of 1:1 and 1:5, respectively. Intact cells were biotinylated for 30 min at 4°C with 1mg/ml Sulfo-NHS-SS-Biotin, washed and homogenised. Biotinylated surface fractions were isolated by streptavidin chromatography, and analyzed by immunoblotting. Surface fractions: lane 1: H₄R₍₃₉₀₎ alone; lane 2: H₄R₍₃₉₀₎+ GFP (1:5 ratio); H₄R₍₃₉₀₎+ H₄R₍₃₀₂₎ (1:1 ratio); lane 3: H₄R₍₃₉₀₎ alone; lane 4: H₄R₍₃₉₀₎+ GFP; lane 5: H₄R₍₃₉₀₎+ H₄R₍₃₀₂₎ (1:5 ratio); lane 6: H₄R₍₃₉₀₎ alone; lane 7: H₄R₍₃₉₀₎+ H₄R₍₆₇₎ (1:1 ratio); lane 8: H₄R₍₃₉₀₎+ H₄R₍₆₇₎ (1:5 ratio). All lanes were probed with anti-H₄R receptor antibody. A representative experiment is shown (n=3).

Figure 5: Evaluation of hetero-oligomerization between the HA-tagged H₄R₍₃₉₀₎ and FLAG-tagged H₄R splice variants H₄R₍₃₀₂₎ and H₄R₍₆₇₎ by *tr*-FRET using co-expression of differentially epitope-tagged receptors. Transiently transfected COS-7 cells co-expressing the HA-H₄R₍₃₉₀₎ and the FLAG-

H₄R₍₃₀₂₎ or FLAG-H₄R₍₆₇₎ in a 0.2:1 or 1:1 cDNA ratio were incubated for 2 hours with the Eu³⁺-labeled anti-FLAG and APC-labeled anti-HA antibodies. As a controls *tr*-FRET was performed on COS-7 cells co-expressing the FLAG-H₄R₍₃₉₀₎ and triple HA-tagged S₁P₁R or HA-H₄(₃₉₀)R. Data are normalized for the *tr*-FRET signal obtained from a mixture of COS-7 cells that individually expressing the indicated constructs. Fluorescence at 665 nm caused by FRET was in the order of 3800 RLU when co-expressing H₄R₍₃₉₀₎ with the H₄R₍₃₀₂₎ or H₄R₍₆₇₎ in a 1:1 ratio and 2450 RLU in a 0.2:1 ratio. When individually expressing cells were mixed fluorescence values was on average 2300 RLU and 2000 RLU when mixing cells expressing H₄R₍₃₉₀₎ with H₄R₍₃₀₂₎ or H₄R₍₆₇₎ in a 1:1 ratio, respectively. For the 0.2:1 ratio values were on average 1800 RLU. Data shown are average of at least three experiments each performed in triplicate.

Figure 6: Immunoprecipitation evidence for H₄R₍₃₉₀₎ interaction with hH₄R isoforms, H₄R₍₃₀₂₎ and H₄R₍₆₇₎. HEK 293 cells were co-transfected with equal amounts of H₄R₍₃₉₀₎ and either FLAG-H₄R₍₃₉₀₎, FLAG-H₄R₍₃₀₂₎ or FLAG-H₄R₍₆₇₎. Cells were harvested 40h post transfection, solubilised with 1% Triton-X100 and subjected to immunoprecipitation with anti-FLAG sepharose or sepharose control beads for 2h at 4°C. Following washing, bound material was collected using SDS-PAGE sample buffer and analyzed by immunoblotting. Immunoblots were probed with anti-H₄R antibody. Lane 1, 3 and 5 are control precipitations with sepharose beads; Lane 2, 4, and 6 are precipitations with anti-FLAG sepharose. Protein species identified were consistent with the dimeric and monomeric H₄R₍₃₉₀₎ or H₄R₍₃₀₂₎ species, respectively. The H₄R₍₆₇₎ was not detected by this antibody. A representative experiment is shown (n=3).

Figure 1

A

```

hH4R (390)  MPDNTNSTINLSLSTRVTLAFFMSLVAFAIMLGNALVILAFVVDKDLRHRSSYFFLNLAIS 60
hH4R (302)  MPDNTNSTINLSLSTRVTLAFFMSLVAFAIMLGNALVILAFVVDKDLRHRSSYFFLNLAIS 60
hH4R (67)   MPDNTNSTINLSLSTRVTLAFFMSLVAFAIMLGNALVILAFVVDKDLRHRSSYFFLNLAIS 60
              TM1                                     TM2

hH4R (390)  DFFVGVVISIPLYIPHTLFEWDFGKEICVFWLTTDYLLCTASVYVNIVLISYDRYLSVSNV 120
hH4R (302)  DFFV----- 64
hH4R (67)   DFFV----- 67
              ↑                                     ↑
            Intron 1                               Intron 2

hH4R (390)  SYRTQHTGVLKIVTLMVAVVLAFLVNGPMLVSESWKDEGSECEPGFFSEWYILAITSF 180
hH4R (302)  -----VSESWKDEGSECEPGFFSEWYILAITSF 92
hH4R (67)   GVLX-----
              TM4             ↑             TM5
                        Alter. acceptor

hH4R (390)  LEFVIPVILVAYFNMNIIYWSLWKRDLHLSRCQSHPLTAVSSNICGHSFRGRLSSRRLSA 240
hH4R (302)  LEFVIPVILVAYFNMNIIYWSLWKRDLHLSRCQSHPLTAVSSNICGHSFRGRLSSRRLSA 152
hH4R (67)   -----

hH4R (390)  STEVPASFHSERQRRKSSLMFSSRTKMNSNTIASKMGSPSQSDSVALHQREHVLLRARR 300
hH4R (302)  STEVPASFHSERQRRKSSLMFSSRTKMNSNTIASKMGSPSQSDSVALHQREHVLLRARR 212
hH4R (67)   -----

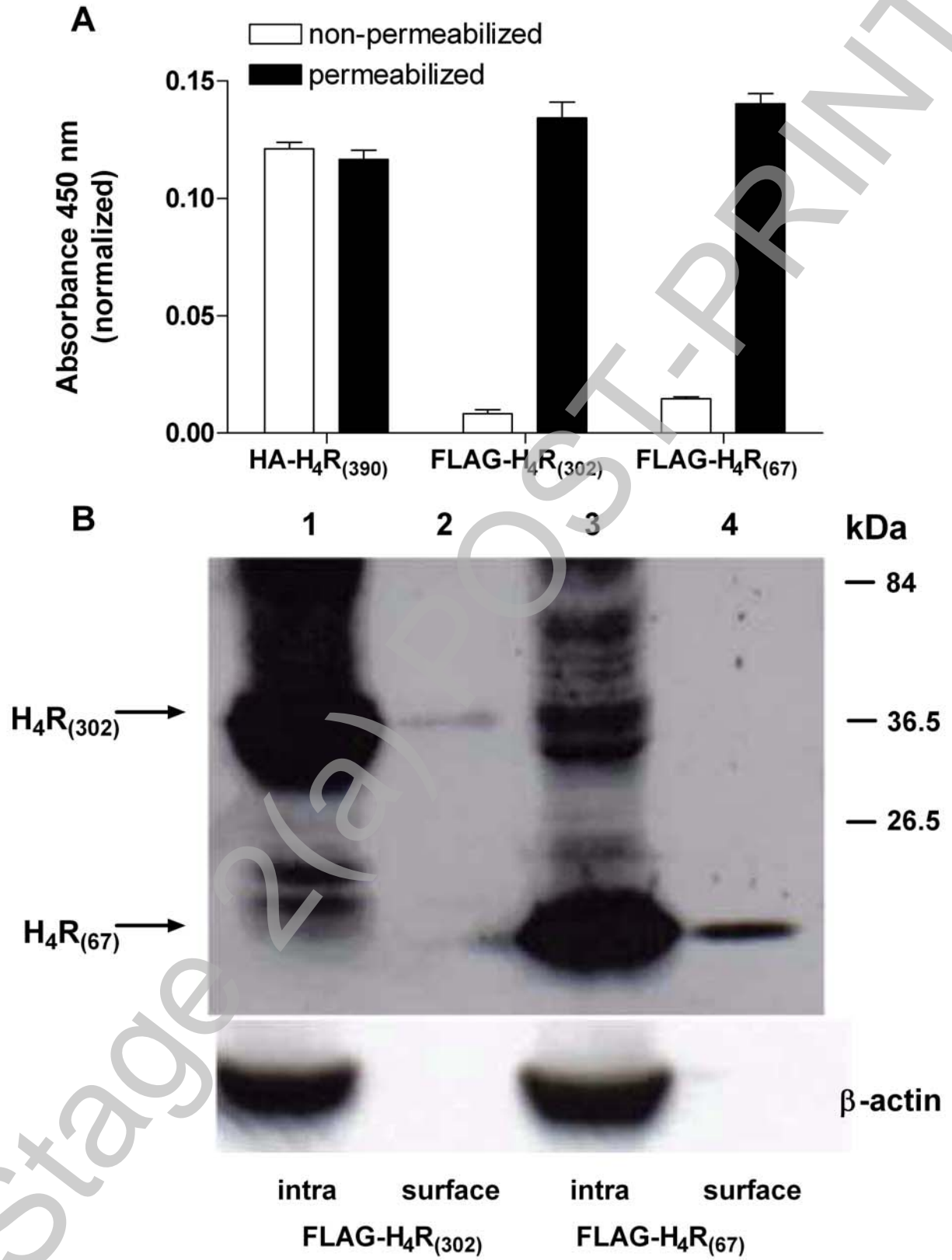
hH4R (390)  LAKSLAILLGVFAVCWAPYSLEFTIVLSFYSSATGPKSVWYRIAFLWLFNSFVNPLLYPL 360
hH4R (302)  LAKSLAILLGVFAVCWAPYSLEFTIVLSFYSSATGPKSVWYRIAFLWLFNSFVNPLLYPL 272
hH4R (67)   -----
              TM6                                     TM7

hH4R (390)  CHKRFQKAFLEKIFCIKKQPLPSQHSRSVSS 390
hH4R (302)  CHKRFQKAFLEKIFCIKKQPLPSQHSRSVSS 302
hH4R (67)   -----
  
```

B

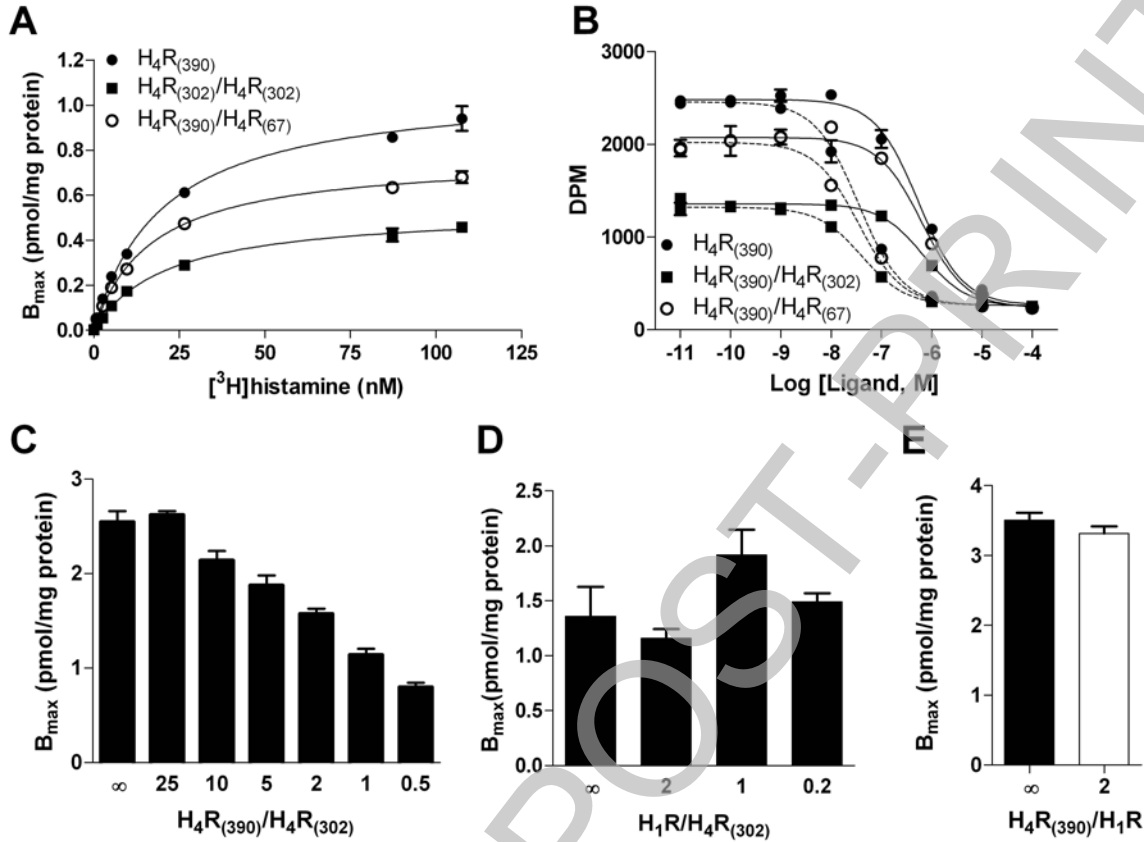
	Ex1/In1	In1/Ex2	Ex2/In2	In2/Ex3	In2/Ex3 (alter.)
H ₄ R ₍₃₉₀₎	TTGTGG/gtaagt	gtgcag/GTGTGA	AATGCT/gtaagt	ttctag/GTGTCT	
H ₄ R ₍₃₀₂₎	TTGTGG/gtaagt				ttctag/TTTCAG
H ₄ R ₍₆₇₎	TTGTGG/gtaagt			ttctag/GTGTCT	

Figure 2



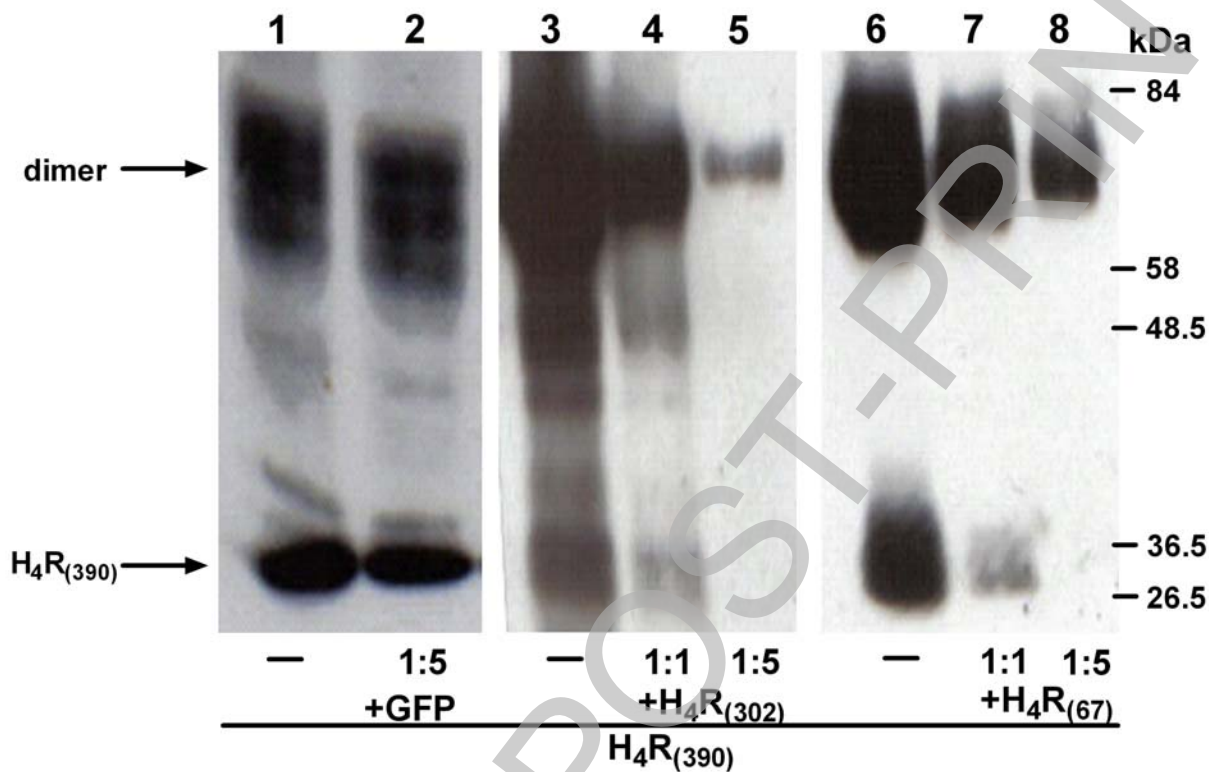
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Figure 3



THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071583

Figure 4



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Stage 2(a) POST-PRINT

Figure 5

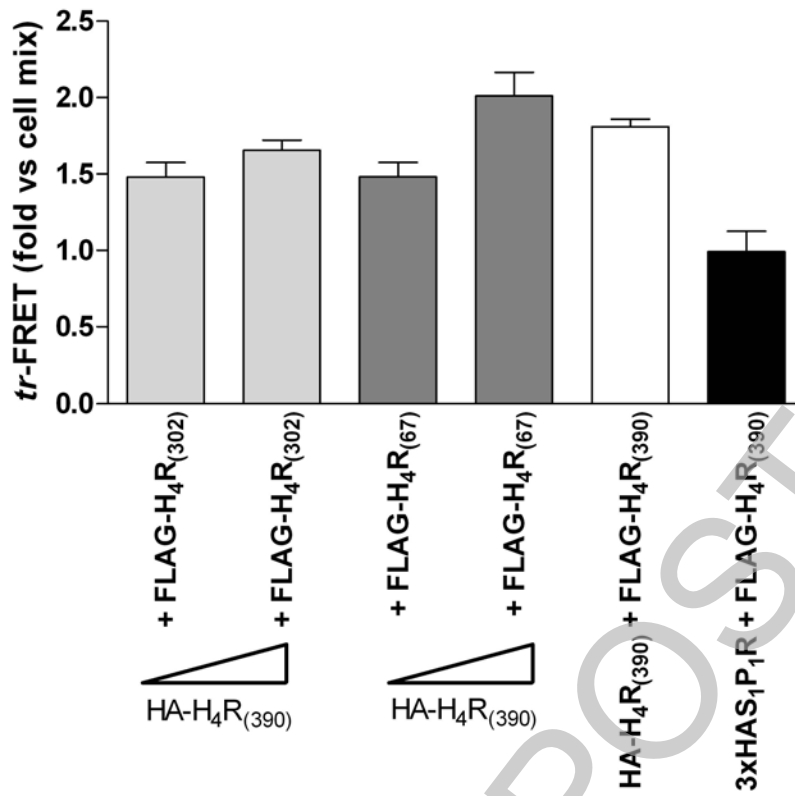
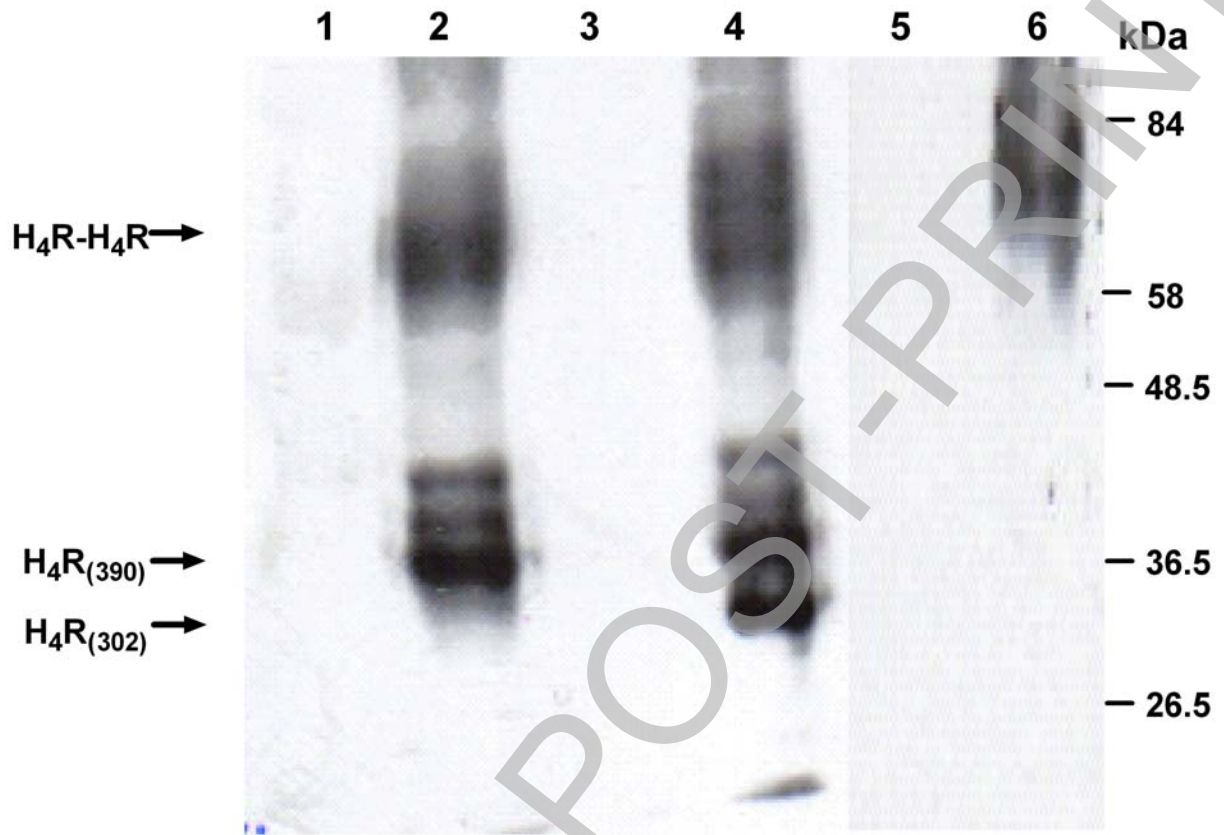


Figure 6



IP	FLAG		FLAG		FLAG	
	-	H ₄ R	-	H ₄ R	-	H ₄ R
WB	H ₄ R	H ₄ R	H ₄ R	H ₄ R	H ₄ R	H ₄ R
	FLAG-H ₄ R ₍₃₉₀₎ + H ₄ R ₍₃₉₀₎		FLAG-H ₄ R ₍₃₀₂₎ + H ₄ R ₍₃₉₀₎		FLAG-H ₄ R ₍₆₇₎ + H ₄ R ₍₃₉₀₎	

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Stage 2.0