Structural and functional analysis of the canine histamine H2 receptor by site-directed mutagenesis: N-glycosylation is not vital for its action

Yasushi FUKUSHIMA,*§ Yoshitomo OKA,* Toshihito SAITOH,* Hideki KATAGIRI,* Tomoichiro ASANO,* Nobuyuki MATSUHASHI,* Kuniaki TAKATA,‡ Eric VAN BREDA,* Yoshio YAZAKI* and Kentaro SUGANO†

*Third Department of Internal Medicine, Faculty of Medicine, and †Health Service Center, University of Tokyo, 7-3-1 Hongo, Bunkyo-ky, Tokyo 113, Japan, and ‡Laboratory of Molecular and Cellular Morphology, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Gunma 371, Japan

G-protein-coupled receptors generally share a similar structure containing seven membrane-spanning domains and extracellular site(s) for N-glycosylation. The histamine H2 receptor is a member of the family of G-protein-coupled receptors, and has three extracellular potential sites for N-glycosylation (Asn-4, Asn-162 and Asn-168). To date, however, no information has been presented regarding N-glycosylation of the H2 receptor. To investigate the presence, location and functional roles of N-glycosylation of the H2 receptor, site-directed mutagenesis was performed to eliminate the potential site(s) for N-glycosylation singly and collectively. The wild-type and mutated H2 receptors were expressed stably in Chinese hamster ovary (CHO) cells or transiently in COS7 cells. Immunoblotting of the wild-type and mutated H2 receptors with an antiserum directed against the C-terminus of the H2 receptor showed that mutation at Asn-162, but not at Asn-168, resulted in a substantial decrease in the molecular mass. A mutation at Asn-4 led to a further decrease in

the molecular mass. Tunicamycin treatment of the transfected cells yielded a sharp band with a molecular mass identical to that of the mutant devoid of all three potential sites for N-glycosylation. These findings indicate that the H2 receptor is N-glycosylated, and that N-glycosylation takes place mainly at two sites, Asn-4 and Asn-162. Neither the affinity for tiotidine nor that for histamine was affected by the mutagenesis. Immunocytochemistry and tiotidine binding showed that the mutated receptors were exclusively distributed on the cell surface in a fashion similar to that of the wild-type. In addition, the glycosylation-defective receptor was capable of activating adenylate cyclase and elevating the intracellular Ca^{2+} concentration in response to histamine in stable CHO cell lines. Thus N-glycosylation of the H2 receptor is not required for cell surface localization, ligand binding or functional coupling to G-protein(s).

INTRODUCTION

It is widely accepted that histamine exerts its physiological stimulatory action on gastric parietal cells [1], haematopoietic cells [2] and the central nervous system [3] through the histamine H2 receptor. Histamine-mediated activation of the H2 receptor, which is coupled to a G-protein(s), leads to the activation of adenylate cyclase and phospholipase C [2,4]. G-protein-coupled receptors generally share a similar structure containing seven transmembrane domains and potential site(s) for N-glycosylation. The functional role of N-glycosylation of G-protein-coupled receptors varies among receptors [5–10]. For example, the β_2 adrenergic receptor contains two N-linked carbohydrates in the N-terminus which are involved in correct trafficking and G-protein coupling of the receptor [6]. On the other hand, N-glycosylation of the m2 muscarinic acetylcholine receptor, which occurs in the N-terminus, is not essential for the structure and function of the receptor [7].

The deduced amino acid sequence of the histamine H2 receptor contains three extracellular potential sites for N-glycosylation (Asn-Xaa-Thr/Ser) [11–13]. One of these is located at the N-terminus, whereas the other two are located in the third extracellular region. As we have reported previously [14], immunoblotting of the canine H2 receptor expressed in Chinese hamster ovary (CHO) cells showed a broad band with an apparently higher molecular mass than expected from the deduced amino acid sequence. Although the broadness and the larger molecular mass of the observed band suggested a glycoprotein nature, no direct information has been presented, to date, concerning the

characteristics and physiological effects of N-glycosylation of this receptor. In the present study we have performed site-directed mutagenesis to determine the presence, localization and functional properties of N-glycosylation of the H2 receptor. We have demonstrated that the H2 receptor is N-glycosylated mainly at two sites (Asn-4, Asn-162), and that N-glycosylation at these sites is not essential for the function of the receptor as indicated by ligand binding, cyclic AMP (cAMP) production and intracellular Ca^{2+} accumulation.

MATERIALS AND METHODS

Cell culture

Chinese hamster ovary (CHO) cells were grown in Ham's F-12 medium containing 10% (v/v) fetal calf serum, 100 units/ml penicillin G and 0.1 mg/ml streptomycin sulphate, and were maintained in 5% CO_2 . COS7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum, 100 units/ml penicillin G and 0.1 mg/ml streptomycin sulphate in 5% CO_2 .

Cloning and site-directed mutagenesis

Cloning and sequencing of the canine histamine H2 receptor have been described in detail previously [14]. Substitution mutations were introduced into the gene encoding the canine histamine H2 receptor by oligonucleotide-directed mutagenesis. Three oligonucleotides used to alter the three potential N-

Abbreviations used: CHO cells, Chinese hamster ovary cells; cAMP, cyclic AMP; p[NH]ppG, guanosine 5'-[β -imido]triphosphate; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

§ To whom correspondence should be addressed.

glycosylation sites at Asn-4, Asn-162 and Asn-168 [11] were designed to replace the codon for Asn with that for Gln at amino acid positions 4, 162 and 168 respectively: Asn-4, 5' CGGGG-TCCCAGGATGATATCTCAAGGCACAGGCTCTT 3'; Asn-162, 5' GAAACTGCTGGTCTCTGCCTGCTGTTTCA 3'; Asn-168, 5' GAGACCAGCAGTTTCCAACACACCA-TTCCC 3'.

Substitution of Gln for Asn at position 168, at both positions 162 and 168, and at all three positions resulted in mutations designated Q168, Q162-Q168 and Q4-Q162-Q168 respectively. After sequencing, no changes other than the desired nucleotide substitutions in the mutated cDNAs were observed [15]. The wild-type and mutated H2 receptor cDNAs were then cloned into an expression vector, pMTHneo, and subsequently transfected into CHO cells by use of the calcium phosphate precipitation method [16]. Clones resistant to 600 µg/ml neomycin derivative G418 (Gibco-BRL, Gaithersburg, MD, U.S.A.) were isolated and used in the following experiments. For transfection into COS7 cells, cDNAs were first ligated into vector CDM8 (Invitrogen, San Diego, CA, U.S.A.), and subsequently transiently expressed in COS7 cells by the DEAE-dextran method. Tunicamycin treatment of transfected COS7 and CHO cells was performed as described in the legend of Figure 2.

Membrane preparation

CHO cells were homogenized in 10 mM Tris, 1 mM EDTA, 100 kallikrein inhibitory units/ml aprotinin and 250 mM sucrose (pH 7.4) at 4 °C. To precipitate the fractions containing nuclei, homogenates were centrifuged at 900 *g* for 10 min. The resulting supernatant was centrifuged at 170000 *g* for 90 min at 4 °C and the pellet obtained was subjected to immunoblotting and radioligand binding.

Immunoblot analysis

Membranes from CHO or COS7 cells were subjected to SDS/polyacrylamide (10%) gel electrophoresis and transferred to nitrocellulose filters. H2 receptor proteins were detected by binding of an appropriate anti-peptide antiserum directed against residues 346–359 of the canine H2 receptor followed by ¹²⁵I-Protein A and autoradiography [14]. It should be noted that the affinity of the antibody for the histamine H2 receptor is not altered by mutagenesis, since the antibody is C-terminus-specific.

Immunocytochemical localization of the H2 receptor in COS7 cells

For immunofluorescence microscopy, COS7 cells were fixed in 3% (v/v) formaldehyde/PBS, scraped off the dish with a rubber blade, and embedded in 10% (w/v) gelatin/PBS. Semi-thin frozen sections (1 µm thick) were cut and incubated with the anti-peptide antiserum against the canine H2 receptor. These sections were then incubated with rhodamine-labelled affinity-purified goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, U.S.A.) [17].

Ligand binding assay on intact cells

CHO cells, grown in 24-well plates, were assayed at a density of 1×10^5 cells/well. The cells were incubated for 2 h at 37 °C in 200 µl of Hepes/Tyrodé's buffer containing 1 nM [³H]tiotidine and increasing concentrations of unlabelled tiotidine [18]. All samples were analysed in triplicate. After incubation, cells were washed three times with ice-cold PBS, removed from the wells in 0.1% SDS, and radioactivity was determined by liquid scintillation counting. Specific binding was calculated by subtracting

the non-specific binding, which was determined in the presence of either 0.1 mM cimetidine (SmithKline Beecham) or 10 µM unlabelled tiotidine (I.C.I.). Since no significant differences in non-specific binding were observed with 0.1 mM cimetidine or 10 µM unlabelled tiotidine in cells expressing either the wild-type or the mutated histamine H2 receptor, it is reasonable to conclude that all expressed receptors are located at the cell surface. No specific binding was observed in either parental CHO cells or CHO cells transfected with the expression vector alone.

Adenylate cyclase activities

CHO cells, grown in 24-well plates, were assayed at a density of 1×10^5 cells/well. The cells were incubated for 30 min at 37 °C in 450 µl of Hepes/Tyrodé's buffer containing 0.1% BSA and 0.1 mM 3-isobutyl-1-methylxanthine; 50 µl of histamine (final concn. 0 , 10^{-8} or 10^{-4} M) was added to initiate the reaction. After 15 min of incubation, the reaction was terminated by adding 500 µl of 12% (w/v) trichloroacetic acid. Next, the samples were centrifuged for 5 min at 3000 *g* and after extracting the supernatants three times with diethyl ether, cAMP contents in the samples were measured using a commercially available radioimmunoassay (Yamasa, Choshi, Japan) [19].

Measurement of fura-2 Ca²⁺ signals

CHO cells were suspended at a density of 1×10^7 cells/ml in Hepes/Tyrodé's buffer containing 0.1% BSA. Next, the cells were loaded in the dark for 60 min at 37 °C with the acetoxy-methyl ester of fura-2 (3 µM). After washing with Hepes/Tyrodé's buffer containing 0.1% BSA, the cells were suspended in the same buffer at a density of 2×10^6 cells/ml. Intracellular fura-2 signals were measured at 340 nm and 380 nm excitation and 510 nm emission with a CAF-100 fluorimeter (Jasco Co., Tokyo, Japan). The values of the intracellular Ca²⁺ concentration ($[Ca^{2+}]_i$) were calibrated by adding 0.01 vol. of 10% (v/v) Triton X-100 (F_{max}) followed by 0.01 vol. of 500 mM EGTA (F_{min}). $[Ca^{2+}]_i$ values were calculated using the following equation: $[Ca^{2+}]_i$ (nM) = $K_d(F - F_{min}) / (F_{max} - F)$, where K_d = 224 nM [20].

RESULTS AND DISCUSSION

Occurrence and location of N-glycosylation

In the present study, in order to investigate the potential site(s) for N-glycosylation in the histamine H2 receptor, we have replaced one, two and all three of the Asn residues (Asn-4, Asn-162, and Asn-168) with Gln using site-directed mutagenesis (Figure 1). The mutated cDNAs were transfected either transiently into COS7 cells or permanently into CHO cells. Immunoblotting with an antiserum directed against the C-terminus of the receptor showed that expression of the wild-type receptor in CHO cells yielded a broad band with a calculated molecular mass of approx. 75 kDa (Figure 2a, lane 1). The mutant receptor Q168, which lacks Asn-168, showed an identical molecular mass to that of the wild-type receptor (Figure 2a, lane 2), whereas the H2 receptor lacking both Asn-162 and Asn-168 (Q162-Q168) showed a broad band with a lower molecular mass of approx. 40 kDa (Figure 2a, lane 3). Furthermore the H2 receptor lacking all three potential glycosylation sites, Q4-Q162-Q168, migrated as a sharp band with a substantial decrease in molecular mass (Figure 2a, lane 4). These results lead us to conclude that the H2 receptor is N-glycosylated at Asn-4 and Asn-162, but the lack of a significant change in mobility would suggest no or only low-molecular-mass carbohydrate addition at Asn-168.

To examine whether the observed sharp bands correspond to a completely deglycosylated protein, we treated COS7 cells

Figure 1 Mutated canine histamine H2 receptors lacking extracellular sites for N-glycosylation

The upper part of the figure shows a schematic representation of the histamine H2 receptor. In the bottom part of the figure each vertical line represents an N-glycosylation site. WT, wild-type canine histamine H2 receptor (H2R); Q168, H2R lacking the third N-glycosylation site; Q162-Q168, H2R lacking the second and third N-glycosylation sites; Q4-Q162-Q168, H2R lacking all three N-glycosylation sites.

Figure 2 Immunoblotting of the wild-type and mutated histamine H2 receptors and effects of tunicamycin on N-glycosylation

(a) CHO cell lines stably expressing wild-type or mutated histamine H2 receptors were obtained. Membranes prepared from the cells were subjected to SDS/PAGE and immunoblotting was performed. Lane 1, wild-type H2 receptor; lane 2, Q168 receptor; lane 3, Q162-Q168 receptor; lane 4, Q4-Q162-Q168 receptor. (b) The wild-type and mutated histamine H2 receptor cDNAs were transfected into COS7 cells using the DEAE-dextran method. The cells were incubated with or without 1 μ g/ml tunicamycin from 24 h to 48 h post-transfection, and harvested at 48 h. Lane 1, parental COS7 cells; lane 2, wild-type histamine H2 receptor treated with tunicamycin; lane 3, Q168 mutant receptor treated with tunicamycin; lane 4, Q162-Q168 mutant receptor treated with tunicamycin; lane 5, Q4-Q162-Q168 mutant receptor without tunicamycin treatment.

expressing the wild-type or mutated receptors with tunicamycin. This treatment resulted in a sharp band with a molecular mass identical to that of the Q4-Q162-Q168 mutant (Figure 2b). Similar results were observed with the wild-type and mutated receptors stably expressed in CHO cells (results not shown). The apparent molecular mass of the glycosylation-defective mutant Q4-Q162-Q168 and of the H2 receptor from tunicamycin-treated cells is smaller than the 39.7 kDa that is predicted from the deduced amino acid sequence of the wild-type H2 receptor. Since the mutated receptor, Q4-Q162-Q168, and receptors from

Table 1 Equilibrium dissociation constant (K_d) for the radioligand [3 H]tiotidine on intact CHO cells expressing wild-type or mutated H2 receptors

H2 receptor	K_d (nM)
Wild-type	19.1 ± 2.2
Q168	25.8 ± 3.2
Q162-Q168	21.8 ± 4.1
Q4-Q162-Q168	22.7 ± 3.9

tunicamycin-treated cells showed the same molecular mass, proteolysis seems unlikely. Although an explanation for these findings is not easy to provide, we expect the discrepancy to be due solely to limitations of the electrophoretic technique, which does not always provide the actual molecular mass of the protein. Together with the sharpness of the band, our results suggest a completely deglycosylated protein. These findings indicate that two of the three potential sites for N-glycosylation, Asn-4 and Asn-162, are primarily utilized in the H2 receptor.

Agonist and antagonist binding to wild-type and glycosylation-defective histamine H2 receptors

To examine the role of N-glycosylation in antagonist binding, we investigated the binding of [3 H]tiotidine, the specific histamine H2 receptor antagonist, on intact CHO cells expressing a wild-type or mutated H2 receptor. A linear relationship was observed on Scatchard plot analysis of the binding data for both the wild-type and the mutated H2 receptors (results not shown). This is consistent with a single class of binding sites in both the wild-type and the mutated H2 receptors. The equilibrium dissociation constants (K_d) for [3 H]tiotidine are shown in Table 1. No significant differences in the K_d of [3 H]tiotidine binding were observed among the wild-type and mutated H2 receptors. Gantz and co-workers [21] have shown that the third and fifth trans-membrane regions of the H2 receptor are involved in antagonist binding. However, our results suggest that N-glycosylation at the Asn-162 site does not affect antagonist binding despite its proximity to the binding site.

We have also examined whether N-glycosylation contributes to agonist binding. Since no appropriate labelled agonist is available at present, we compared the effect of histamine on [3 H]tiotidine binding to the wild-type and mutated receptors. Membrane fractions from CHO cells expressing either the wild-type H2 receptor or the Q4-Q162-Q168 receptor were incubated in the presence of [3 H]tiotidine and increasing concentrations of histamine. No significant differences in the degree of inhibition of [3 H]tiotidine binding by histamine were observed between the wild-type and the mutant Q4-Q162-Q168 receptors (Figure 3). Addition of the non-hydrolysable GTP analogue guanosine 5'-[β - γ -imido]triphosphate (p[NH]ppG), which uncouples G-proteins from their receptors, resulted in a rightward shift of the histamine inhibition curve of [3 H]tiotidine binding for both the wild-type and the Q4-Q162-Q168 receptors (Figure 3). Thus N-glycosylation of the histamine H2 receptor is also not essential for agonist binding.

Subcellular localization of wild-type and glycosylation-defective histamine H2 receptors

Expression of the wild-type and mutated H2 receptors on the plasma membranes of COS7 cells was assessed by indirect

Figure 3 Inhibition of [^3H]tiotidine binding by histamine to wild-type and Q4-Q162-Q168 H2 receptors

Membranes from CHO cells expressing wild-type (a) or Q4-Q162-Q168 (b) H2 receptors were incubated with [^3H]tiotidine (2 nM) and increasing concentrations of histamine in the absence (○) or presence (●) of 100 μM p[NH]ppG. Each curve is representative of five independent experiments. Each point is the mean $\pm$ S.E.M. of duplicate measurements.

immunostaining using an antiserum directed against the C-terminus of the receptor. The wild-type and mutated H2 receptors were localized mainly at the plasma membrane (Figure 4),

suggesting that N-glycosylation is not involved in intracellular targeting of the H2 receptor. The amounts of the wild-type and mutated H2 receptors in the stably transfected CHO cells were too small to be detected by immunostaining with the antiserum. Therefore, to examine the distribution of receptors, [^3H]tiotidine binding experiments were performed on CHO cells. Tiotidine is hydrophobic and membrane-permeable at 37 $^{\circ}\text{C}$, whereas cimetidine is hydrophilic and membrane-impermeable and does not inhibit binding of [^3H]tiotidine to receptors which are located intracellularly at the same temperature. [^3H]Tiotidine binding in the presence of an excess concentration of cimetidine was equivalent to that in the presence of an excess concentration of unlabelled tiotidine in both CHO cell lines expressing the wild-type receptor and those expressing the mutated histamine H2 receptor (results not shown). These findings suggest that the receptors expressed in CHO cells are exclusively distributed on the cell surface and that N-glycosylation is not involved in the subcellular distribution of the H2 receptor.

Adenylate cyclase coupling and intracellular Ca^{2+} accumulation: effects of the glycosylation-defective histamine H2 receptor

Histamine stimulation of the H2 receptor leads to accumulation of cAMP and intracellular Ca^{2+} via a G-protein-coupled process [1,2,4]. To determine the physiological significance of N-glycosylation in the function of the H2 receptor, CHO cell lines stably expressing the wild-type or glycosylation-defective (Q4-Q162-Q168) receptor were studied. Two clones each were selected for the wild-type (W-A, W-B) and glycosylation-defective (QT-A and QT-B) histamine H2 receptor. Since the wild-type and glycosylation-defective H2 receptors are located exclusively on the cell surface, as described above, their functions can be assessed when the amount of expressed receptors is equivalent. Clones WT-A and WT-B expressed 55 and 47 fmol of the wild-type receptor and clones QT-A and QT-B expressed 49 and 47 fmol of the glycosylation-defective H2 receptor per 10^5 cells, as estimated from a [^3H]tiotidine binding assay (results not shown). Expression of the H2 receptor in CHO cells results in functional coupling with the endogenous hamster adenylate cyclases through endogenous G-proteins [14]. Therefore if the amount of the expressed receptors is too large compared with the amounts of adenylate cyclase and G-proteins, the stimulus

Figure 4 Immunocytochemical localization of H2 receptor in COS7 cells

(a) Wild-type H2 receptor; (b) Q168 receptor; (c) Q162-Q168 receptor; (d) Q4-Q162-Q168 receptor. The bar represents 10 μm .

Figure 5 Histamine-stimulated cAMP accumulation in CHO cell lines expressing wild-type or Q4-Q162-Q168 H2 receptors

cAMP accumulation in response to histamine was measured in intact CHO cell lines expressing wild-type or Q4-Q162-Q168 H2 receptors. Measurements were performed in triplicate, and the results are expressed as pmol of cAMP produced by 10^5 cells in 15 min (means $\pm$ S.E.M.). Clones: $\circ$, W-A; $\triangle$, W-B; $\bullet$, QT-A; $\blacktriangle$, QT-B. $\blacksquare$, Parental CHO cells.

triggered by the receptors is saturated at relatively low concentrations of the agonist, rendering it very difficult to analyse the functions of the receptor. However, in the clones selected for this assay, histamine stimulation led to the dose-dependent production of cAMP (Figure 5). Thus the selected clones are also suitable for assessing the functional role of N-glycosylation with regard to the expression level. Parental CHO cells or CHO cells transfected with the expression vector alone showed no histamine-dependent cAMP production. As shown in Figure 5, no significant differences in cAMP production were observed among the four independent clones selected. Neither the maximal cAMP production nor the EC_{50} of histamine for cAMP production were altered by mutagenesis. These findings indicated that N-glycosylation does not play a significant role in histamine-mediated cAMP production via the H2 receptor.

Histamine-dependent intracellular Ca^{2+} accumulation in the same clones as described above was measured using the Ca^{2+} -sensitive dye fura-2. No histamine-induced change in $[Ca^{2+}]_i$ was observed in parental CHO cells or in CHO cells transfected with the expression vector alone (results not shown). As shown in Figure 6, in both the W-A and QT-A clones a rapid rise in $[Ca^{2+}]_i$, followed by a gradual decrease to a level higher than the basal level, was observed after stimulation with $10 \mu M$ histamine. The extent and time course of the stimulation with histamine in these two clones were similar. Comparable results were observed with clones W-B and QT-B (results not shown). These results indicate that N-glycosylation is not essential for the histamine-stimulated $[Ca^{2+}]_i$ elevation via the H2 receptor.

Recently it was revealed that stimulation of G_s leads to stimulation of phospholipase $C\beta$ by β and γ subunits of G-proteins [22], which also results in an elevation of $[Ca^{2+}]_i$. Although it has not been determined whether the histamine H2 receptor is coupled to G_s alone or to other G-protein(s) as well as G_s , our results indicate that N-glycosylation of the receptor is not involved in the G-protein coupling of the H2 receptor.

In conclusion, we have demonstrated that the canine histamine H2 receptor is N-glycosylated, and that this glycosylation takes

Figure 6 Histamine-stimulated mobilization of intracellular Ca^{2+} in transfected CHO cells

(a) Fura-2-loaded CHO cells (clone W-A) expressing wild-type H2 receptors were challenged with $10 \mu M$ histamine. Traces shown are representative of five different experiments. (b) As for (a), but cells were transfected with Q4-Q162-Q168 H2 receptors (clone QT-A).

place mainly at two sites, Asn-4 and Asn-162. In addition, N-glycosylation of the H2 receptor is not an essential step for either cell surface localization or agonist and antagonist binding. Furthermore, N-glycosylation of the H2 receptor is not required for functional coupling of the receptor to stimulation of adenylate cyclase and elevation of $[Ca^{2+}]_i$. The role of N-glycosylation of G-protein-coupled receptors varies among receptors, and it remains to be determined whether or not N-glycosylation of a particular receptor is important. Therefore it is essential to determine the roles of N-glycosylation in the H2 receptor. The results observed in the present study are important, since we can utilize the glycosylation-defective H2 receptor, which is much easier to handle than the wild-type, for further characterization of the histamine H2 receptor.

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