Oligomer formation of histamine H2 receptors expressed in Sf9 and COS7 cells

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Abstract A histamine H2 receptor, which had been mutated at its glycosylation site and tagged at its N-terminus with an HA tag (HA-H2 receptor), was expressed in Sf9 cells and COS7 cells. Immunoprecipitation and immunoblotting of HA–H2 receptors with αHA antibody revealed four bands of 31.5 ± 2.5 kDa, 59.0 ± 6.0 kDa, 80.5 ± 4.5 kDa and 120 kDa. These bands were also detected by immunoblot using anti-H2 receptor serum (C-terminus). In addition, H2 receptors without the HA-tag co-immunoprecipitated with HA-tagged H2 receptors devoid of the 51 C-terminal amino acids, via immunoprecipitation with αHA antibody, when the two receptors were coexpressed. These results suggest that H2 receptors are present as receptor oligomers and that the C-terminal portion is not involved in the formation of these oligomers.

Key words: Histamine H2 receptor; Oligomerization

1. Introduction

The histamine H2 receptor belongs to the family of G-protein-coupled receptors and is involved in a variety of cellular processes [1–5]. However, studies of the H2 receptor have been hampered by the lack of an antibody which can immunopurify the receptor protein. In this study, we expressed hemagglutinin (HA) epitope-tagged H2 receptors using an insect *Sporodoptera frugiperda* (Sf9) cell/baculovirus expression system and in COS7 cells. These receptors were functionally equivalent to wild-type receptors. Interestingly, the H2 receptors expressed in Sf9 and COS7 cells were revealed to be present as oligomers.

There is evidence suggesting oligomerization of G-protein-coupled receptors [6–10]. More recent reports have further clarified the oligomerization of β2-adrenergic, dopamine D2 and olfactory receptors [11–13]. G-protein-coupled receptors have many features in common. Thus, oligomerization may be a newly recognized feature common to members of the G-protein-coupled receptor family.

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Abbreviations: HA, hemagglutinin; PBS, phosphate-buffered saline; Sf9, *Sporodoptera frugiperda*; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide electrophoresis; ECL, enhanced chemiluminescence

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2. Materials

Anti-hemagglutinin (HA) monoclonal antibody, 12CA5, was purchased from Boehringer-Mannheim, pBacPAK9 from Clontech, protein G–Sepharose from Pharmacia Biotech, and horseradish peroxidase-labeled anti-rabbit and anti-mouse IgG from Amersham.

2.1. Cell culture

Sf9 cells were grown in monolayer or suspension cultures in TC100 medium containing 10% fetal calf serum and 50 μg/ml kanamycin at 27°C. COS7 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 U/ml penicillin G, and 0.1 mg/ml streptomycin sulfate in 5% CO_2.

2.2. Construction of HA-tagged glycosylation-defective histamine H2 receptor

Construction of a cDNA of the N-glycosylation-defective histamine H2 receptor, of which the three potential N-glycosylation sites had been mutated, was described previously [14]. A cDNA for a mutant histamine H2 receptor with an additional mutation involving C-terminal truncation (51 amino acids) was constructed by polymerase chain reaction (PCR) using the primer 3′-ACCCTCGAGTCA-CACCTGGAGCTTC-5′. The HA epitope (YPYDVPDYA) was inserted into the receptor N-termini. After confirming the sequences, the cDNAs were subcloned into pBacPAK9, a baculovirus vector, and pCAGGS, a mammalian expression vector. The baculoviruses were produced according to the manufacturer's instructions. Sf9 cells were infected with the appropriate virus at a multiplicity of infection of 2–5 and harvested at 48 h post-infection. Transfection of COS7 cells was performed by the DEAE-dextran method, as described previously [14].

2.3. Immunoprecipitation of HA-tagged H2 receptors

Sf9 or COS7 cells expressing receptors were lysed in 1% Triton-X/phosphate-buffered saline (PBS) with 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0.1 mg/ml bacitracin, 0.1 μg/ml pepstatin A, 10 μg/ml soybean trypsin inhibitor and 100 U/ml aprotinin. The samples were centrifuged at 15000 rpm at 4°C for 10 min, the supernatants were incubated with 5 μg of anti-HA monoclonal antibody, 12CA5, conjugated to protein G–Sepharose at 4°C for 2 h. The immunocomplexes recovered by centrifugation were washed 5 times with PBS containing 1% Triton X-100, then boiled in Laemmli sample buffer. The samples were subjected to sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS-PAGE) and then transferred to nitrocellulose filters. The filters were incubated with 12CA5 or antibody against the 14 C-terminal amino acids of the H2 receptor ($\alpha H2R^{CT}$ antibody) [15]. The proteins were visualized by enhanced chemoluminescence (ECL) using horseradish peroxidase-labeled anti-rabbit DO or anti-mouse IgG.

2.4. cAMP production in membranes and in whole cells

Membranes were prepared from Sf9 cells, and cAMP productions in membranes in response to histamine and forskolin were measured as previously described [15]. Transfected COS7 cells were split onto 24-well plates at a density of 10^5 cells/well 24 h post-transfection, and cAMP productions in response to histamine in whole cells were measured 48 h post-transfection [14].

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2.5. Tiotidine binding to membranes from Sf9 cells

Tiotidine bindings to membrane fractions from Sf9 cells were measured as described previously [15].

3. Results

We constructed a cDNA coding for the histamine H2 receptor which had been mutated so as to be N-glycosylation defective, and tagged this receptor at the N-terminus with an HA epitope (HA–H2 receptor). This cDNA was subcloned into pBacPAK9 and the recombinant baculovirus was prepared. Infection with the recombinant baculovirus resulted in expression of HA–H2 receptors in Sf9 cells, as demonstrated by specific binding of [3H]tiotidine, an H2 receptor antagonist, to membrane fractions, while no specific binding was observed in Sf9 cells infected with control baculovirus. The affinity of the HA-tagged H2 receptors, expressed in Sf9 cells, for tiotidine was comparable to that of wild-type H2 receptors expressed in CHO cells (Table 1). In addition, membranes from Sf9 cells infected with the H2 receptor virus produced cAMP in response to histamine (Table 1). Histamine-dependent cAMP production was inhibited by the H2 receptor antagonist cimetidine, but not by the H1 receptor antagonist diphenhydramine (Table 1). These results indicated that the HA–H2 receptor functioned like the wild-type H2 receptor.

Immunoprecipitation and subsequent immunoblotting of cell lysates from Sf9 cells expressing HA–H2 receptors with αHA antibody revealed four bands with molecular masses of 31.5 ± 2.5 kDa, 59.0 ± 6.0 kDa, 80.5 ± 4.5 kDa, and approximately 120 kDa (Fig. 1, right panel, lane 2). These bands were also detected with the antibody against the 14 C-terminal amino acids of the H2 receptor (αH2R^{CT} antibody), indicating that all of the bands corresponded to intact HA–H2 receptors but not to proteolytic fragments (Fig. 1A, left panel, lane 2). Molecular masses of the higher bands corresponded to approximately double, triple and quadruple that of the 31.5 kDa band, while the molecular mass of the canine histamine H2 receptor predicted from its deduced amino acid sequence is 39.7 kDa. Thus, it is likely that the 59.0 kDa band corresponded to the dimer, the 80.5 kDa band to the trimer and the 120 kDa band to the tetramer of the histamine H2 receptor. Addition of dithiothreitol and β-mercaptoethanol to the SDS-PAGE sample buffer did not affect the above findings (data not shown).

Similar results were obtained in COS7 cells expressing HA–H2 receptors (Fig. 1B), indicating that oligomerization of the H2 receptor is not unique to insect cells. In COS7 cells, the

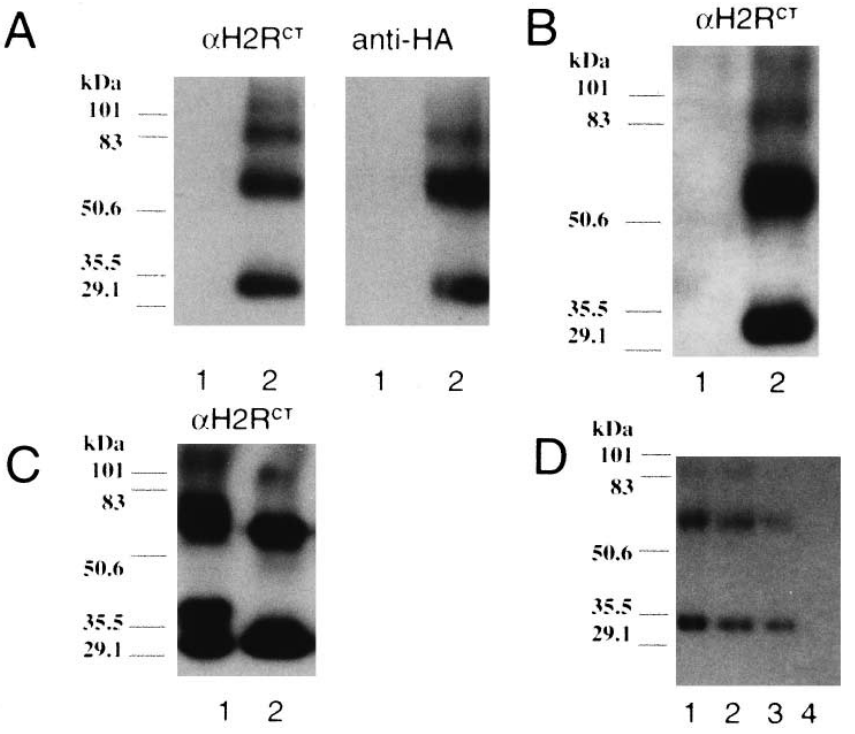

Fig. 1. A: At 48 h postinfection, Sf9 cells infected with control baculovirus or HA–H2 receptor recombinant baculoviruses were solubilized in 1% Triton-X/PBS. Then, the samples were immunoprecipitated with 5 μg of αHA antibody conjugated to protein G-Sepharose, subjected to SDS-PAGE and immunoblotting with αHA antibody (right panel) or αH2R^{CT} antibody (left panel). The proteins were visualized by ECL using horseradish peroxidase-labeled anti-rabbit or anti-mouse IgG. Lane 1, Sf9 cells infected with control baculovirus; lane 2, Sf9 cells infected with HA–H2 receptor virus. B: COS7 cells were transfected with HA–H2 receptor cDNA plasmid. At 48 h post-transfection the cells were lysed and the samples were subjected to immunoprecipitation with 5 μg of 12CA5, SDS-PAGE, and immunoblotting with αH2R^{CT} antibody. Lane 1, control; lane 2, HA–H2 receptors. C: COS7 cells were transfected with either the HA–H2 receptor (without glycosylation) or the HA–WT receptor (with glycosylation) cDNA plasmids. At 48 h post-transfection the cells were lysed and the samples were subjected to immunoprecipitation with 5 μg of 12CA5, SDS-PAGE, and immunoblotting with αH2R^{CT} antibody. Lane 1, HA–WT receptors; lane 2, HA–H2 receptors. D: Sf9 cells were infected with smaller amounts of HA–H2 receptor recombinant baculoviruses (lane 1, 1/10 of the amounts used for the experiments in (A); lane 2, 1/100; lane 3, 1/1000, lane 4, 1/10 000). At 48 h post-infection, the cells were solubilized in 1% Triton-X/PBS. Then, the samples were immunoprecipitated with 5 μg of αHA antibody and subjected to SDS-PAGE and immunoblotting with αH2R^{CT} antibody (left panel). The proteins were visualized by ECL using horseradish peroxidase-labeled anti-rabbit IgG.

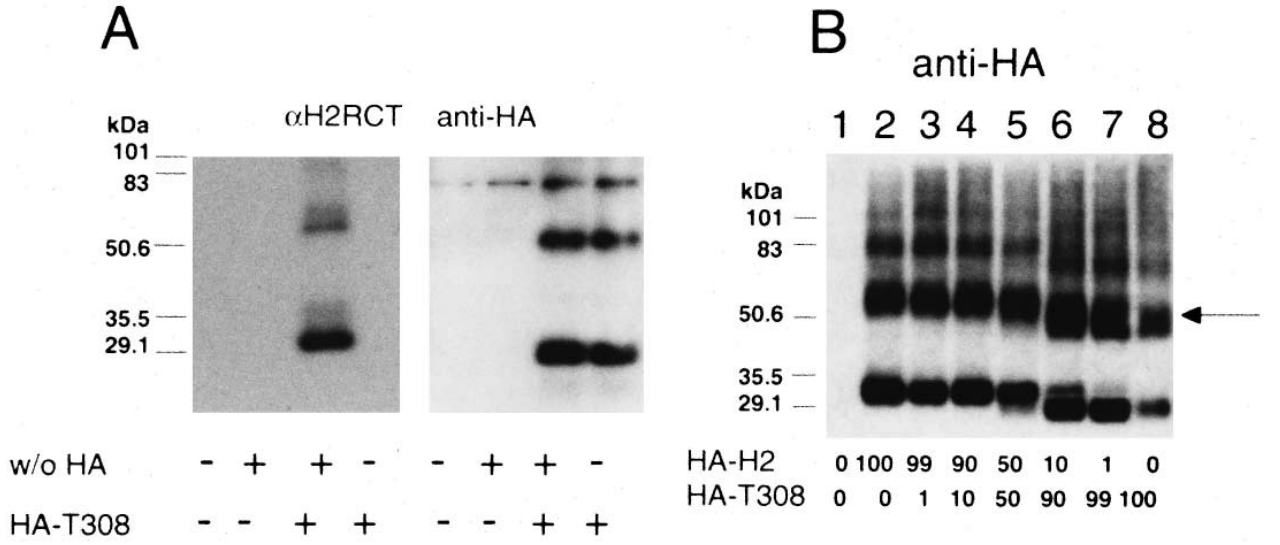

Fig. 2. A: Coexpression of HA–T308 receptors and H2 receptors in COS7 cells. COS7 cells were cotransfected with HA–T308 receptor and H2 receptor cDNAs. At 48 h post-transfection the cells were lysed and the samples subjected to immunoprecipitation with 5 μg of αHA antibody conjugated to protein G-Sepharose, SDS-PAGE, and immunoblotting with $\alpha H2R^{CT}$ antibody (left panel) or αHA antibody (right panel). The proteins were visualized by ECL using horseradish peroxidase-labeled anti-rabbit or mouse IgG. Lane 1, control; lane 2, H2 receptors; lane 3; HA–T308 receptors and H2 receptors; lane 4, HA–T308 receptors. B: Coexpression of HA–H2 and HA–T308 receptors in Sf9 cells. Varying amounts of each virus solution were used to infect Sf9 cells. At 48 h post-transfection the cells were lysed in 1% Triton-X/PBS, and immunoprecipitated with αHA antibody conjugated to protein G-Sepharose. Immunoblotting was performed with αHA antibody. The proteins were visualized by ECL using horseradish peroxidase-labeled anti-mouse IgG.

HA–H2 receptor was functionally equivalent to the WT H2 receptor in terms of stimulation of adenylyl cyclase and tiotidine binding and plasma membrane localization (not shown). HA-tagged receptors with N-glycosylation (HA–WT receptor) expressed in COS7 cells migrated as three bands which were broader and had a higher molecular mass than those of the HA–H2 receptor (Fig. 1C). These findings show that the HA–WT receptors were also present as oligomers. However, to identify the broad bands of the HA–WT receptor as receptor oligomers was impossible without knowing the migration pattern of the HA–H2 receptor. Thus, using a glycosylation-defective H2 receptor was very helpful and important in this study. The number of expressed receptors in COS7 cells, as determined by tiotidine binding, ranged from 50 000 to 300 000 per cell and were thus apparently more numerous than endogenous receptors. Therefore, we infected Sf9 cells with increasingly smaller amounts of recombinant baculovirus and checked oligomer formation. As shown in Fig. 1D, when the amount of baculovirus used for infection was decreased, the dimer and monomer bands disappeared simultaneously. Similar results were obtained with COS7 cells expressing HA–H2 receptor. This demonstrates that, although a high number of receptors was expressed in this study, oligomer formation of the histamine H2 receptor is not a function of overexpression.

Table 1
Expression of histamine H2 receptor in Sf9 cells

cAMP production (pmol/min/mg)	
Basal	32 ± 3
10^{-5} M histamine	135 ± 5
10^{-5} M histamine + 10^{-4} M cimetidine	40 ± 5
10^{-5} M histamine + 10^{-4} M diphenhydramine	120 ± 12
10^{-4} M forskolin	176 ± 25
Affinity for tiotidine (K_d)	
HA–H2 receptor in Sf9 cells	22.5 ± 3.2 nM
Wild-type H2 receptor in CHO cells [14]	19.1 ± 2.2 nM

Data are means ± SEM of three separate experiments.

To confirm that the detected bands corresponded to receptor oligomers, H2 receptors with neither the HA-tag nor N-glycosylation were coexpressed with HA-tagged H2 receptors, the 51 C-terminal amino acids of which had been truncated (HA–T308), in COS7 cells. The H2 receptor reacted with $\alpha H2R^{CT}$ antibody, but not with αHA antibody, whereas the HA–T308 receptor reacted with αHA antibody, but not with $\alpha H2R^{CT}$ antibody. As shown in Fig. 2A, immunoprecipitation with αHA antibody of COS7 cells expressing both HA–T308 and H2 receptors revealed H2 receptors to be co-immunoprecipitated with HA–T308 receptors. This finding indicates that the expressed receptors were present as oligomers and also that neither the HA-tagged nor the C-terminal portion of the H2 receptor is involved in the oligomerization of H2 receptors. Similar results were obtained with COS7 cells expressing both H2 receptors and HA–T308 receptors with N-glycosylation. It is noteworthy that there was no heterodimer between the HA–T308 and the receptor with neither the HA-tag nor N-glycosylation, as shown in Fig. 2A. Although an explanation for these findings is not easy to provide, we speculate that the heterodimers or heterooligomers might not be as stable as the homodimers or homooligomers.

Next, we coexpressed HA–H2 and HA–T308 receptors in Sf9 cells. Expression of HA–T308 receptors yielded three distinct molecular species with smaller molecular masses (28.0 ± 1.0, 51.5 ± 3.5, 69.5 ± 2.5 kDa) than those of the HA–H2 receptor (Fig. 2B, lane 8), suggesting that HA–T308 receptors were also present as oligomers. A faint band with a molecular mass of 90 ± 5 kDa was also observed, which was considered to most likely to represent receptor tetramers. Coinfection of HA–H2 receptors with various concentrations of HA–T308 receptor virus solutions (1:100 to 100:1) yielded

another band with a molecular mass distinct from those of the HA–H2 and HA–T308 receptors (Fig. 2B, lanes 6 and 7, arrow). In addition, the 31.5 kDa band of the HA–H2 receptor disappeared as the amount of HA–T308 receptor expression increased (Fig. 2B, lanes 6, and 7). In contrast, the 28.0 kDa band became increasingly apparent as the amount of HA–T308 receptor expression increased (Fig. 2B, lanes 6, 7). These findings suggest that heterooligomers of HA–H2 and HA–T308 receptors were present in Sf9 cells and that monomers of the receptors, which were present in smaller amounts, were adsorbed with other receptors thereby becoming member(s) of heterooligomers.

4. Discussion

A number of other G-protein coupled receptors have been expressed using the baculovirus/Sf9 expression system, which allows highly efficient expression of receptors. In this study, we first expressed HA-tagged and glycosylation-defective H2 receptors in Sf9 cells with functional coupling to G-protein. HA-tagging and expression in Sf9 cells allowed purification of large amounts of H2 receptor proteins, useful for further characterization of the receptor.

Interestingly, immunoprecipitation with αHA antibody in 1% Triton-X isotonic buffer revealed that the receptors in Sf9 cells migrated as three or four distinct molecular species on SDS-PAGE. The difference in molecular mass is not due to the different extents of N-glycosylation, because the H2 receptor we used was devoid of all potential N-glycosylation sites [14]. Furthermore, tunicamycin treatment of cells affected neither the apparent pattern of migration nor the width of the bands (not shown). In addition, the migration pattern of the HA–H2 receptor was different from that of the receptor with N-glycosylation. Taken together, these results make it clear that the molecular fragments represented receptor oligomers of HA–H2 receptors. Despite the HA–H2 receptor being N-glycosylation-defective, the bands corresponding to the dimer, trimer and tetramer were rather broad. This might be because these molecular species, which are composed of linked monomers, are not completely linear on SDS-PAGE and probably migrated somewhat irregularly.

There is evidence suggesting oligomerization of G-protein-coupled receptors [6–10]. Recently, more direct evidence was presented concerning oligomerization of β2-adrenergic, dopamine D2 and olfactory receptors [11–13]. Since G-protein coupled receptors have many features in common, it is possible that other receptors are present as oligomers. The H2 receptor trimer is consistent with the case of the M2 muscarinic and olfactory receptors [10,13], while in other cases receptor dimers were observed. There may functional and/or differences, which have as yet to be elucidated among these receptor groups. The presence of the H2 receptor tetramer suggests that monomers and dimers may be derived from tetramers and that H2 receptors are present as tetramers in vivo. Further studies are needed to resolve these issues.

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