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IDENTIFICATION OF NOVEL GUANYLYL CYCLASES FROM CHEMOSENSORY TISSUES OF RAT AND CATTLE

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SUMMARY: A number of studies have shown that cGMP may play some roles in chemosensory transduction. To identify the structure of guanylyl cyclase in chemosensory tissues, cDNA fragments encoding guanylyl cyclase catalytic domain were amplified from rat and bovine olfactory and tongue epithelium using degenerate oligonucleotide primers and reverse transcription-polymerase chain reaction (RT-PCR). Three novel clones, two membrane type guanylyl cyclases (RAT GC-1, BOV GC-3) and one soluble type guanylyl cyclase (RAT GC-2) were identified. RAT GC-1 was distributed over various rat tissues in addition to these chemosensory organs. BOV GC-3 was similar to but distinct from recent cloned olfactory-specific guanylyl cyclase. RAT GC-2 was identified as rat homologue of $\alpha 2$ subunit of the soluble guanylyl cyclase.

Recent advances in the biochemical and molecular analysis of the chemosensory tissues revealed important roles of second messengers in chemosensory transduction(1-3). The cAMP and IP_3 transduction pathways, in particular, are best understood in vertebrate chemosensory system. Various odor molecules activate either adenylyl cyclase or phospholipase C (or both) to produce cAMP or IP_3 through G-protein coupled receptors. cAMP and IP_3 thus produced directly or indirectly induce conductance changes in receptor cell. For example, cAMP activates a cyclic nucleotide-gated cation channel specifically expressed in olfactory receptor neuron(4,5).

On the other hand, the existence of other second messengers such as cGMP in chemosensory transduction remain ambiguous. Nagahama *et al.* (6) showed that incorporation of cGMP into frog taste cells through perfusion of the lingual artery leads to enhancement of the gustatory nerve responses to various stimuli. Direct injection of cGMP into the mouse and frog taste cells depolarized the cells (7-9). Recently, it was shown

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cytochemically that the activity of guanylyl cyclase is localized in microvilli of the rabbit taste cells and this activity was increased upon application of sucrose, a typical sweet substance (10). In olfactory system, it was reported that cGMP concentration was elevated upon application of certain types of odorants to the preparations of vertebrate olfactory cilia (11). It is interesting to note that the cyclic nucleotide-gated cation channel has rather higher affinity for cGMP than the affinity for cAMP (5). These observations all suggest that cGMP plays a role as a second messenger in chemosensory transduction. To clarify the role of cGMP in chemosensory transduction, it is necessary to identify structure, function and subcellular localization of guanylyl cyclase expressed in the chemosensory tissues.

Guanylyl cyclases are classified into two subfamilies termed the membrane type and the soluble type(12,13). Membrane types of guanylyl cyclase are thought to serve as receptors. For example, guanylyl cyclase A and B (GC-A and GC-B) are members of the natriuretic peptide receptor family (14-17). Other recently cloned membrane type guanylyl cyclases remain to be orphan receptors (18,19). Amino acid sequence of guanylyl cyclase catalytic domain is well conserved over the subfamilies (13). Making use of the conserved amino acid sequences of the catalytic domain of the guanylyl cyclase, we have cloned three novel cDNAs encoding guanylyl cyclase catalytic domain from chemosensory tissues of rat and cattle. We analyzed tissue specific expression pattern of one of the novel clones obtained. We discuss the function of the guanylyl cyclase expressed in chemosensory tissues and the roles of cGMP in chemosensory signal transduction.

MATERIALS AND METHODS

Isolation of guanylyl cyclase catalytic domain from chemosensory tissues

Total RNA was prepared from various tissues of rat and cattle according to the method of Chomczynski and Sacchi (20). The total RNA (1 μg) was treated with DNase I (TaKaRa) and single-strand cDNA was synthesized with random hexamer and M-MLV reverse transcriptase (Gibco BRL). Then, the cDNA was subjected to series of PCR experiments. Degenerate oligonucleotide primers corresponding to the conserved amino acid sequences of guanylyl cyclase catalytic domain of the both soluble and membrane types were synthesized as follows:

GC51, 5'-GA(T/C)ATIGTIGGITT(T/C)ACI(G/A)C-3';
GC52, 5'-GTIGTIACI(T/C)TI(T/C)TIAA(T/C)GA(T/C)(T/C)TITA(T/C)AC-3';
GC53, 5'-GTIGA(A/G)ACIATIGGIGA(T/C)G(T/C)ITA(T/C)ATG-3';
GC31, 5'-GT(A/G)TCICC(A/G)AAIA(A/G)(A/G)CA(A/G)TAIC(G/T)IGGCAT-3';
GC32, 5'-CCATIC(T/G)I(G/C)(A/T)IGCIGT(A/G)TTIACIGT(A/G)TCICC-3'.

PCR was performed according to the following schedule for 45 cycles: 94 °C 1 min (3 min for the first cycle), 45 °C 2 min, 72 °C 3 min (10 min for the last cycle). Amplified products were analyzed by electrophoresis on 1.5 % agarose gel, and bands of interest were extracted (GENE CLEAN II, BIO 101) and cloned into plasmid vector (pCR II, INVITROGEN). cDNA clones were sequenced by dideoxy method (Sequenase, Amersham or Taq Dye Terminator Cycle Sequencing Kit, ABI).

Distribution of RAT GC-1 on various rat tissues

Oligonucleotide primers corresponding to sequence of a novel guanylyl cyclase clone, RAT GC-1 were designed as follows:

rGC1-5', 5'-ATGAGATTGCCACCATGTCTTTGC-3';

rGC1-3', 5'-TGATTCCCACCACACCAGCC-3'.

To identify the tissue distribution of the transcripts of RAT GC-1, 0.5 μg each of total RNA prepared from various tissues was subjected to RT-PCR analysis. PCR was performed according to the following schedule for 40 cycles: 94 °C 1 min (3 min for the first cycle), 55 °C 1 min, 72 °C 1 min (10 min for the last cycle). Amplified products of each PCR sample were analyzed by electrophoresis on 3 % agarose (Agarose X, Nippon Gene) gel.

RESULTS AND DISCUSSION

To identify novel guanylyl cyclase expressed in mammalian chemosensory tissues, RT-PCR was performed with total RNA prepared from olfactory and tongue epithelium of rat and cattle. PCR was carried out under low stringent condition (45 °C for annealing) with various degenerate primers corresponding to the guanylyl cyclase catalytic domain of known soluble and membrane guanylyl cyclases. Consequently, amplified products of expected size were obtained with all sets of primer pairs used. The DNA sequencing of the subcloned PCR products identified the presence of several sequences of the known guanylyl cyclase as well as three novel clones (RAT GC-1, RAT GC-2, BOV GC-3) that were homologous to but distinct from known ones. From the deduced amino acid sequence of these three novel clones, it was revealed that conserved amino acid residues in the known guanylyl cyclases were also conserved in these clones (Fig. 1). Sequences of the three novel clones and the known guanylyl cyclases were subjected to alignment analysis and a dendrogram was made (Fig. 2). The three clones were located in the branches of either the membrane or soluble type guanylyl cyclase and not in the independent branch. This result indicates that these three clones belong to the family of guanylyl cyclase.

The clone RAT GC-1 was cloned from the cDNA of both olfactory and tongue epithelia of rat, but not from bovine tissues. On the other hand, RAT GC-2 was amplified from

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CONSENSUS  SGLP      HA  IA MAL                      IG H G   AGVVG
-------------------------------------------------------------------
RAT GC-1   ASGLPIRNGAQHADEIATMSLHLLSVTTNFQIGHMPEERLKLRIGLHTGPVVAGVVGIT    59
BOV GC-3   ASGVPRHNGSWHAAEMANMALDILSSVGDFRMRHVPTVPIRIRAGLHSGPCVAGVMGLT    59
RAT GC-2   ASGLH-RKSLCHAKPIALMALKMMELSEE--VLTPDGRPIQMRIGIHSGSVLAGVVGVR    56
           ***'' ''' **  '* *'*'''' ''  '   ' ''''* *'*'*' '***'*'
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Fig. 1. Predicted amino acid sequences of the three novel guanylyl cyclases obtained from rat and bovine chemosensory tissues. Sequences derived from PCR primers are eliminated. Amino acid residues conserved in all of the three clones are indicated by asterisks (*) and residues varied within those of similar physiochemical properties are indicated by periods (.). Consensus amino acids among the known guanylyl cyclase catalytic domains are also indicated.

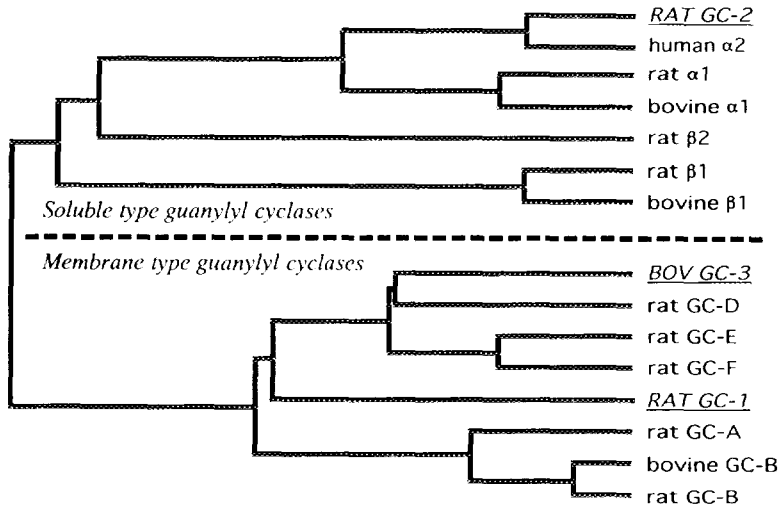

Fig. 2. Dendrogram of the amino acid sequence of guanylyl cyclase. Amino acid sequences of catalytic domains of GC-A (rat), GC-B (rat, bovine), GC-D (rat), GC-E (rat), GC-F (rat), β1 subunit (rat, bovine), α1 subunit (rat, bovine), β2 subunit(rat) and α2 subunit (human) together with the three novel clones (indicated by *italic* and underline) were compared using CLUSTAL software. Branch order is a function of structural similarity, and branch length reflects sequence identity. Dashed line indicates the border between the soluble and membrane type guanylyl cyclase subfamilies.

olfactory epithelium of only rat and not of cattle, and BOV GC-3 was obtained from olfactory epithelium of only cattle and not of rat. At amino acid level RAT GC-1 and BOV GC-3 were 55-77 % identical to the known membrane guanylyl cyclases (GC-A, GC-B, GC-D, GC-E, and GC-F) (14-19), while they were 30-39 % identical to the known soluble guanylyl cyclase (α1 and β1 subunits) (21-24). Therefore, it is concluded that both RAT GC-1 and BOV GC-3 belong to the subfamily of membrane guanylyl cyclase. RAT GC-1 has similar degrees of homology to both the natriuretic peptide receptor family (GC-A and GC-B) and the recently identified sensory membrane guanylyl cyclase family (GC-D, GC-E and GC-F), but it is obviously distinct from both subfamilies. BOV GC-3 is most similar to GC-D among sequences of previously identified guanylyl cyclases (77 % identity at amino acid level). One may consider the identity of BOV GC-3 as a bovine homologue of rat GC-D. However, each guanylyl cyclase subtype is highly conserved (≥ 95 % identity) over species, and then one should regard BOV GC-3 as a novel guanylyl cyclase subtype expressed in bovine tissues. On the other hand, RAT GC-2 is 73 % identical to α1 subunit of rat soluble guanylyl cyclase and has less than 50 % identity with other guanylyl cyclases. Therefore, RAT GC-2 is classified into the soluble guanylyl cyclase subfamily. A sequence homology search on SWISS-PROT database indicated that RAT GC-2 was more similar to human α2 subunit

(98 % identity) (25) than to rat α1 subunit (73 % identity) or to human α3 subunit (73 % identity) (26). Since rat α2 subunit has not been identified yet, RAT GC-2 should be considered as a rat α2 subunit.

RAT GC-1 was expressed in both olfactory and tongue epithelium. Then, we asked whether this clone is expressed in tissues other than these tissues. To determine a tissue specific expression pattern of RAT GC-1 clone, we performed a RT-PCR analysis on RNAs from various rat tissues using primer pairs specific for RAT GC-1. We found that RAT GC-1 was expressed in all of the tissues we examined (Fig. 3). Control RT-PCR experiment carried out without M-MLV reverse transcriptase showed no amplified products from contamination of either genomic DNA or RAT GC-1 cDNA (Fig. 3 lower panel). We confirmed that the total RNAs were evenly recovered by the strength of amplification products of β-actin (data not shown). Because the slight variation in the degree of amplification among RNAs from different tissues is inevitable in RT-PCR, the expression levels in each tissue must be carefully compared. As shown in the upper panel of Fig. 3, relatively high levels of expression of RAT GC-1 were observed in kidney, skeletal muscle, and lung. On the contrary, testis and brain gave weaker signals.

In this study, we obtained novel guanylyl cyclase clones expressed in various tissues including chemosensory tissues. As the expression of RAT GC-1 is observed in various tissues like that of natriuretic peptide receptor subfamily (15,17), RAT GC-1 may play a role similar to that of the peptide hormone reception in these tissues. According to the

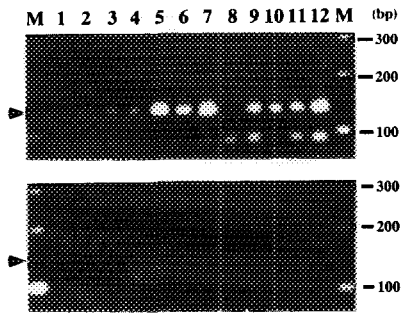

Fig. 3. Expression of RAT GC-1 in various rat tissues. (Upper panel) Total RNA from the indicated tissues was subjected to RT-PCR. Amplified products were electrophoresed on 3 % agarose gel and stained with ethidium bromide. Lanes: 1, testis; 2, eye; 3, spleen; 4, liver; 5, kidney; 6, skeletal muscle; 7, lung; 8, brain; 9, heart; 10, olfactory epithelium; 11, tongue epithelium containing circumvallate papillae; 12, tongue epithelium containing fungiform papillae. The position of the specifically amplified product (134 bp) is indicated by arrowhead. (Lower panel) Control experiment performed without M-MLV reverse transcriptase indicated no detectable products.

cytochemical study of rabbit taste buds, guanylyl cyclase located in the microvilli of the taste cells is membrane-bound (10). Hence, RAT GC-1 is a candidate for guanylyl cyclase located in microvilli which serves as a novel type of taste receptor. It is necessary to obtain a full length RAT GC-1 cDNA to characterize further its ligands and functions. RAT GC-2 is similar to the human $\alpha 2$ subunit of the soluble type guanylyl cyclase. Vertebrate olfactory epithelium contains NO synthase which is known to activate the soluble type guanylyl cyclase (11,27). Therefore, one may speculate that odorant-induced cGMP formation is catalyzed by soluble guanylyl cyclase such as RAT GC-2. On the other hand, BOV GC-3 is most homologous to GC-D (77 % identity) whose expression is restricted to a subpopulation of olfactory neurons randomly dispersed within a particular topographic zone on whole olfactory epithelium(19). Although the authentic ligand and function in olfactory tissue of GC-D is not known, there is a possibility that BOV GC-3 and GC-D form a subfamily of the membrane type of guanylyl cyclase which serves as a novel class of odorant receptor other than the seven transmembrane-domain olfactory receptor family(28). Of course, further study is needed to prove this hypothesis.

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