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Two novel genes, *Gpr113*, which encodes a family 2 G-protein-coupled receptor, and *Trcg1*, are selectively expressed in taste receptor cells☆

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Abstract

To identify genes important for taste receptor cell function, we analyzed the sequences and expression patterns of clones isolated from a mouse taste receptor cell-enriched cDNA library. Here, we report the analyses of two novel genes, *Gpr113* and *Trcg1*. *Gpr113* encodes a G-protein-coupled receptor belonging to family 2B, members of which are characterized by having long N-terminal, extracellular domains. The predicted N-terminal extracellular domain of GPR113 contains 696 amino acids with two functional domains, a peptide hormone-binding domain and a G-protein-coupled receptor proteolytic site. Expression analyses indicate that *Gpr113* expression is highly restricted to a subset of taste receptor cells. TRCG1 is also selectively expressed in a subset of taste receptor cells. *Trcg1* is alternatively spliced and encodes *Trcg1* isoforms of 209 and 825 amino acids. BLAST searches of genomic sequences indicate that a putative homolog of *Trcg1* resides on human chromosome 15q22.
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Taste receptor cells (TRCs) are a highly specialized and molecularly diverse population of sensory cells. TRCs are clustered within taste buds in the epithelia of the palate, pharynx, and tongue and are exposed to the oral cavity via apical processes that extend through taste pores. Incoming tastants interact with either ion channels or G-protein-coupled receptors (GPCRs) present on the microvilli of TRCs to initiate downstream signaling pathways [1]. The proposed pathways activated are numerous, with different taste modalities activating different pathways. Salty and sour tastes are evoked by ions including sodium ions and protons, respectively, that pass through or modulate ion channels, whereas sweet- and bitter-tasting compounds, as well as glutamate and other amino acids, activate GPCRs.

Recent molecular studies have begun to identify the components of the taste signal transduction pathways. In mammals, several families of ion channels have been implicated in sour and salty taste based on their functional properties and expression in TRCs. The ion channels proposed to play a role in sour and salty taste transduction include members of the acid-sensing ion channels [2], members of the hyperpolarization-activated ion channel family [3], and the epithelial sodium channel [4,5]. In addition, two families of TRC-specific GPCRs, the T1Rs and the T2Rs, have been identified and functionally shown to be activated by tastants. The T1R family consists of three members of the family 3 GPCRs, which have been shown to function as heterodimers to recognize sweet-tasting compounds and amino acids [6–13]. T2Rs, on the other hand, are family 1 GPCRs and function as receptors for bitter-tasting compounds [14–18]. In addition, several downstream components in the bitter and/or sweet signaling transduction pathway, including PLC-β2 [19,20], TrpM5 [20,21], and the G proteins α-gustducin [22,23], Gβ_3, and Gγ_{13}[24,25], have been identified. Although many of the

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molecules involved have been determined, there are still many gaps in our understanding of the molecular processes underlying TRC function.

Since sensory system transduction pathways are highly specialized, we reasoned that many of the genes involved in taste signal transduction might be specifically expressed in TRCs. Therefore, to identify additional taste signal transduction components, we constructed a cDNA library enriched in genes expressed in TRCs using tissue microdissection followed by a PCR-based suppressive subtractive technique [26]. This technique has the advantage of normalizing gene expression and enriching for differentially expressed genes, allowing for the detection of low-abundant, tissue-specific transcripts. In this report, we describe the isolation and characterization of two novel genes, *Gpr113* and *Trcg1*, that were present in the subtracted library and that are selectively expressed in subsets of TRCs. Mammalian GPCRs are generally classified into three major subfamilies (1, 2, and 3) based on sequence homology [27]. Unlike the known taste receptors that are either family 1 or family 3 GPCRs, GPR113 is a family 2 GPCR and is the first family 2B GPCR demonstrated to be selectively expressed in TRCs.

Results

Strategy to identify genes expressed in TRCs

To identify molecules involved in TRC function, we constructed a normalized, subtracted cDNA library enriched in clones expressed in taste buds of the circumvallate papillae [26]. To construct the mouse library, circumvallate papillae-derived cDNA was subtracted from cDNA isolated from lingual epithelium devoid of taste buds. The sequences of 20,000 clones from the TRC-enriched library were determined. Sequence analyses of clones from this library indicated that the library was enriched in TRC-expressed genes with several of the identified components of the taste signal transduction pathways represented in the library. The library contained multiple independent clones encoding PLC-β2; TrpM5; the G proteins α-gustducin, $G\beta_3$, and $G\gamma_{13}$; and members of the T1R and T2R families of taste receptors. In addition to the unique sequences showing substantial homology (>70%) to known genes, approximately 13% (n = 650) of the unique sequences displayed no significant homology to entries in the GenBank nonredundant and EST databases.

We reasoned that many of the genes involved in taste signal transduction would be expressed specifically in TRCs and not represented in the current EST databases. Therefore, in situ hybridization analyses on tissue sections through circumvallate papillae were performed with approximately 200 of the novel ESTs to determine if they are expressed in TRCs. Several of the clones examined were selectively expressed in TRCs and not in surrounding lingual epithelial cells. Here, we present the genomic organization and expression patterns of two of these clones, 130c02 encoding *Gpr113* and 64c12 encoding *Trcg1*.

Cloning of Gpr113, a TRC-expressed gene encoding a family 2B GPCR

The cDNA clones resulting from the suppression subtractive hybridization cloning procedures are *Rsa*I restriction fragments and therefore are generally partial cDNAs with an average length of 500 bp. The original clone 130c02 was 125 bp in length. To isolate additional sequences, primers were designed to perform 5′ and 3′ rapid amplification of cDNA ends (RACE) reactions. The longest 5′ RACE product was 1.6 kb in length, while the longest 3′ RACE product isolated was 1.4 kb. Based on the sequences of these RACE products, primers were designed to isolate a full-length clone. RT-PCRs using RNA derived from circumvallate papillae yielded a 3.0-kb cDNA predicted to encode a protein of 991 amino acids (Fig. 1A). The coding region is predicted to begin at the first methionine in the context of an adequate Kozak sequence (GCCATGT) after an upstream in-frame termination codon [28]. The cDNA terminated with a poly(A) tail that was present 17 nucleotides downstream of a canonical polyadenylation signal (AATAAA).

A BLAST search of mouse genomic sequences with our full-length sequence indicated that 130c02 encodes a novel GPCR that localizes to mouse chromosome 5 and consists of 14 exons spanning 11 kb of genomic sequence (Fig. 1B). Since our identification and preliminary characterization of this clone, Bjarnadottir et al. [29] reported this gene as *Gpr113* (mCP26973.1), based on database searches of genomic sequences for GPCR-related sequences. Our isolated cDNA differs from their predicted gene by the inclusion of five additional coding exons, increasing the deduced amino acid sequence of GPR113 by 104 amino acids and changing both its amino and its carboxyl termini.

Analysis of the topology of GPR113 using TMHMM 2.0 [30] indicates that it has a large extracellular N-terminal domain followed by seven transmembrane-spanning helices and a short intracellular carboxyl domain of 51 amino acids. In addition, an unambiguous signal sequence is present, with cleavage predicted to occur between amino acids 20 and 21 (SignalP [31]). BLAST analysis with the seven-transmembrane domain of GPR113 indicates that GPR113 is most homologous to family 2B GPCRs, displaying 33–36% amino acid identity to GPR110, GPR111, GPR115, and Ig-hepta/GPR116.

Family 2B GPCRs, a subgroup of family 2 GPCRs, are distinct from the prototypic hormone-binding family 2 members, now referred as family 2A GPCRs, in that they have very long extracellular N-terminal domains. These extracellular domains are composed of one or several

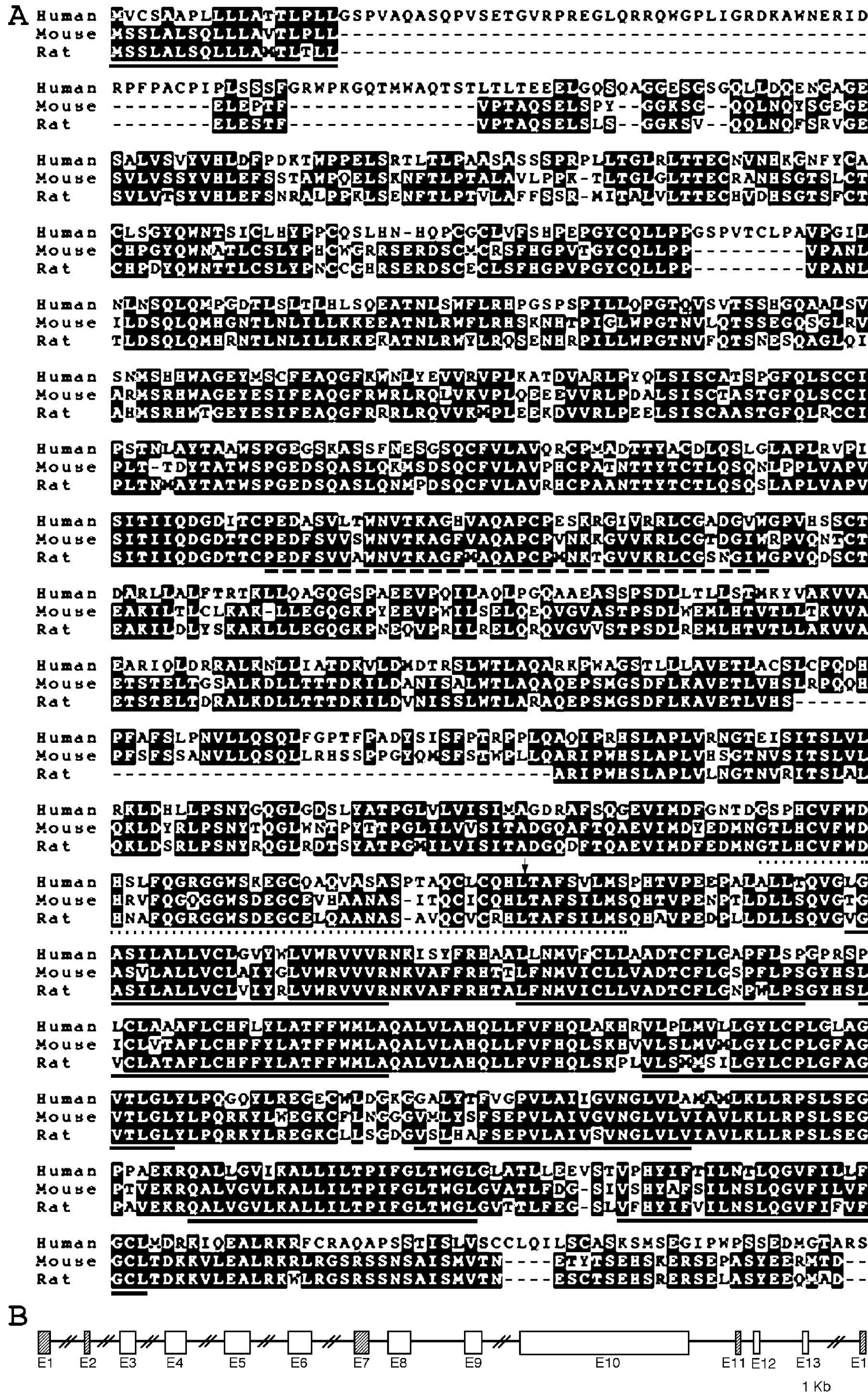

Fig. 1. Predicted amino acid sequence and genomic organization of *Gpr113*. (A) An alignment of the deduced sequences of mouse, human, and rat GPR113 proteins. Highlighted amino acids are conserved in all three proteins. Putative transmembrane domains and signal sequence are underlined with solid lines, putative hormone-binding domain is underlined with a dashed line, and the GPS domain is indicated by a dotted line. The arrow indicates the putative cleavage site. (B) Schematic organization of the genomic structure of *Gpr113*. Coding exons (E1 to 14) are shown as boxes and introns by lines. The five exons not previously reported are hatched. The GenBank accession numbers for the mouse, human, and rat sequences are AY701869, AAN46669, and XP233935, respectively.

functional motifs such as EGF modules, cadherin domains, and immunoglobulin domains. The N-terminal extracellular domain of GPR113 is 696 amino acids long and contains two well-conserved functional domains, a hormone-binding domain (amino acids 353–392), and a GPCR proteolytic site (GPS), which is located just proximal to the first transmembrane domain (Fig. 1A). Hormone-binding domains are a common feature of the family 2A GPCRs and are found in

approximately one-third of the family 2B members. In contrast, the GPS motif, which is present in all of the family 2B members, is not found in other GPCRs. The presence of these domains suggests that GPR113 exists as a heterodimer with a seven-transmembrane domain noncovalently associated with a peptide-binding extracellular domain (see Discussion).

Gene prediction programs on the human and rat genomes indicate that *Gpr113* is conserved in both rat and human [32]. The rat homolog (XM_233935.2), assigned to chromosome 6, is predicted to encode a protein of 952 amino acids displaying 75% amino acid identity to the mouse GPR113. The predicted human homolog (NM_153835) encodes a protein of 1079 amino acids that displays 62% amino acid identity to the mouse GPR113. GPR113 is located on human chromosome 2p24.1. An alignment of the predicted mouse, human, and rat amino acid sequences is shown in Fig. 1A.

Cloning of Trcg1, a novel TRC expressed gene

The second clone of the library to be analyzed, designated 64c12, was 545 bp in length. BLAST analysis with this sequence on an earlier genome build (May 2002) of mouse genomic sequences indicated that it showed identity to a mouse genomic segment on chromosome 9 in a region with a predicted hypothetical protein (XP_165093) of 520 amino acids. However, in the most recent genome build (May 2004) this prediction was removed.

To characterize the structure of this gene better, 64c12 was used to screen an oligo(dT)-primed cDNA library generated from mouse circumvallate papillae mRNA. The largest clone isolated was 1.6 kb and encoded a predicted open reading frame of 474 amino acids followed by a termination codon and poly(A) tail 18 bp downstream of an alternative, but acceptable, polyadenylation signal sequence (CAUAAA). The 5′ end of the cDNA was cloned by 5′ RACE, with the longest clone being 1.5 kb.

Based on these sequences, we isolated two cDNAs by RT-PCR using circumvallate papillae mRNA as a template. The two clones were 2.7 and 1.1 kb in length. Comparisons to genomic sequences indicated that the longer cDNA derives from 12 exons spanning 13.3 kb of genomic sequence and is predicted to encode a protein of 825 amino acids. The shorter cDNA appears to be a splice variant that lacks exon 10 and uses an alternative splice site within exon 2. The resultant sequence is predicted to encode a protein of 209 amino acids. The deduced amino acid sequences and genomic structures of the two identified splice variants of *Trcg1* are shown in Fig. 2.

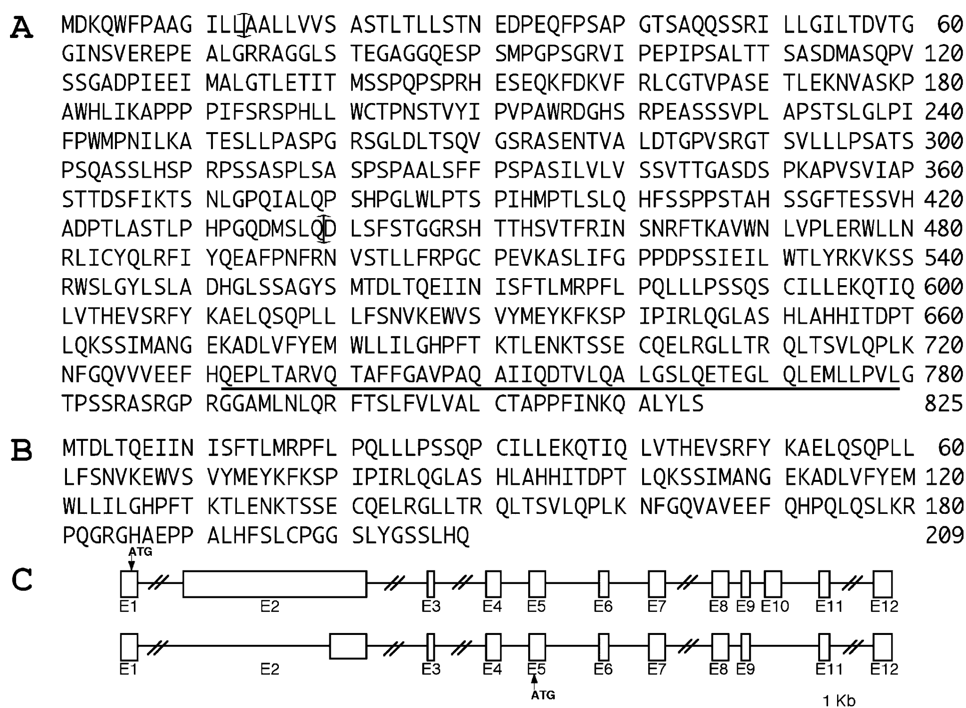

Fig. 2. Predicted amino acid sequence and genomic organization of TRCG1. Deduced protein sequences of *Trcg1* isoform A (A; 825 amino acids) and shorter isoform B (B; 209 amino acids). In the shorter form, exon 1 is spliced to an alternative site within exon 2. Vertical lines (A) indicates exon boundaries between exon 1 and exon 2 long and short forms. Furthermore, exon 10 (underlined in A) is missing from the shorter form, with exon 9 spliced directly to exon 11. In comparisons of the two forms, these alternative-splicing events result in the use of different initiating methionines and different reading frames downstream of exon 9. (C) Schematics of the organization of the genomic structures of two *Trcg1* isoforms are shown. Coding exons (E1 to 12) are shown as boxes and introns by lines. Putative initiating methionines are also indicated. The GenBank accession numbers for these sequences are AY701870 (A isoform) and AY701871 (B isoform).

Extensive BLAST searches with cDNA and translated sequences indicate that *Trcg1* displays no significant homology to any known gene, although putative rat and human homologs were identified. In addition, no known functional motifs are present within the predicted protein sequence. With no insights into its function based on sequence information, we tentatively and unofficially designate this gene as *Trcg1*, for taste receptor cell gene 1.

Trcg1 is highly conserved in rat. BLAST searches against rat high-throughput genomic sequences indicated that a rat homolog is localized on chromosome 8 and displays ~90% nucleotide homology to the mouse *Trcg1*. BLAST searches against human genome sequences indicated that *Trcg1* displayed homology to a human BAC clone (AC113208.5) that maps to chromosome 15q22. Since no other significant hits were found in human genome sequences, we propose that this region encodes the human homolog of *Trcg1*. In support of this proposition, the human and mouse regions of homology map to the same block of conserved synteny. To characterize further the putative human homolog, we used the Web-based GeneMachine program to predict genes within the homologous human BAC clone (http://genome.nhgri.nih.gov/genemachine/). A novel gene consisting of 14 exons and spanning 10.2 kb of genomic DNA was identified. This predicted human gene encodes a putative protein of 745 amino acids displaying 33% amino acid identity and 42% amino acid similarity to the mouse TRCG1 was identified.

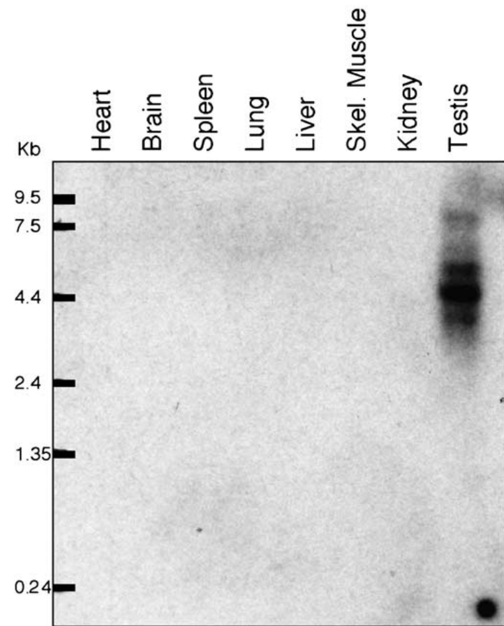

Fig. 3. Northern analysis of *Gpr113* expression. Clontech MTN (Multitissue Northern) blots were hybridized with a probe specific for *Gpr113*. Transcripts for *Gpr113* were detected only in the testis. Size markers are indicated on the left side. Control experiments using a probe for the ubiquitously expressed gene β-actin indicated that lanes were equivalently loaded and that the RNA was not degraded (data not shown).

Gpr113 and Trcg1 are selectively expressed in taste tissue

The expression patterns of *Trcg1* and *Gpr113* were investigated by Northern blot analyses of poly(A)$^+$ mRNAs obtained from a variety of mouse tissues. On the Northern blots, *Trcg1* expression was evaluated using a 2.2-kb probe corresponding to exons 1 to 8, and *Gpr113* expression was evaluated using a 1.4-kb probe corresponding to exon 10.

The *Gpr113* probe revealed transcripts that were highly expressed in testis but not in the other tissues tested, including heart, brain, spleen, lung, liver, skeletal muscle, and kidney (Fig. 3). Four predominant *Gpr113* transcripts of 3.6, 4.5, 5.6, and 8.5 kb were observed in the testis tissue indicative of alternative splice forms. The longest *Gpr113* cDNA clone isolated from taste tissue together with its poly(A) tail was 3.0 kb, suggesting that additional sequences, and possibly tissue-specific splice variants, remain to be isolated.

Northern analyses of *Trcg1* expression were also attempted. However, despite using blots with mRNAs from 15 tissues, including testis, heart, brain, spleen, lung, liver, skeletal muscle, kidney, uterus, large intestine, prostate, stomach, thyroid, thymus, and salivary gland, no *Trcg1* transcripts were observed even after long exposures. In contrast, hybridization with a control β-actin probe on the same blots revealed positive transcripts of the predicted size in all tissues tested, indicating that the mRNA being evaluated was intact and the hybridization conditions were favorable (data not shown). We interpret these results as indicating that *Trcg1* is either not expressed or expressed at undetectable levels in the tested tissues. Due to the inaccessibility of circumvallate papillae tissue, it is not practical to obtain sufficient amounts of circumvallate papillae-derived RNA necessary for Northern analysis. Therefore, we cannot rule out the possibility that there was a technical problem with the *Trcg1* probe.

To extend the expression analysis of *Gpr113* and to verify the expression of *Trcg1*, we performed PCR on an array of first-strand cDNAs prepared from RNA isolated from 21 different adult tissues or embryos of different developmental stages (Fig. 4). For analysis of *Gpr113* expression, we used gene-specific primers spanning exons 10 to 14 that were expected to amplify a DNA fragment of 417 bp. Consistent with Northern blot analysis, *Gpr113* expression is very restricted, with expression evident in only the testis and circumvallate papillae.

For analysis of *Trcg1* expression, we used gene-specific primers spanning exons 8 to 12 that were expected to generate amplicons of either 499 or 353 bp, dependent on the splice variant (with or without exon 10) being detected. Strong expression of *Trcg1* was observed only in circumvallate papillae cDNA, whereas weaker expression was detected in the uterus. Sequence analyses of the amplicons indicated that the weak band of 353 bp amplified from the

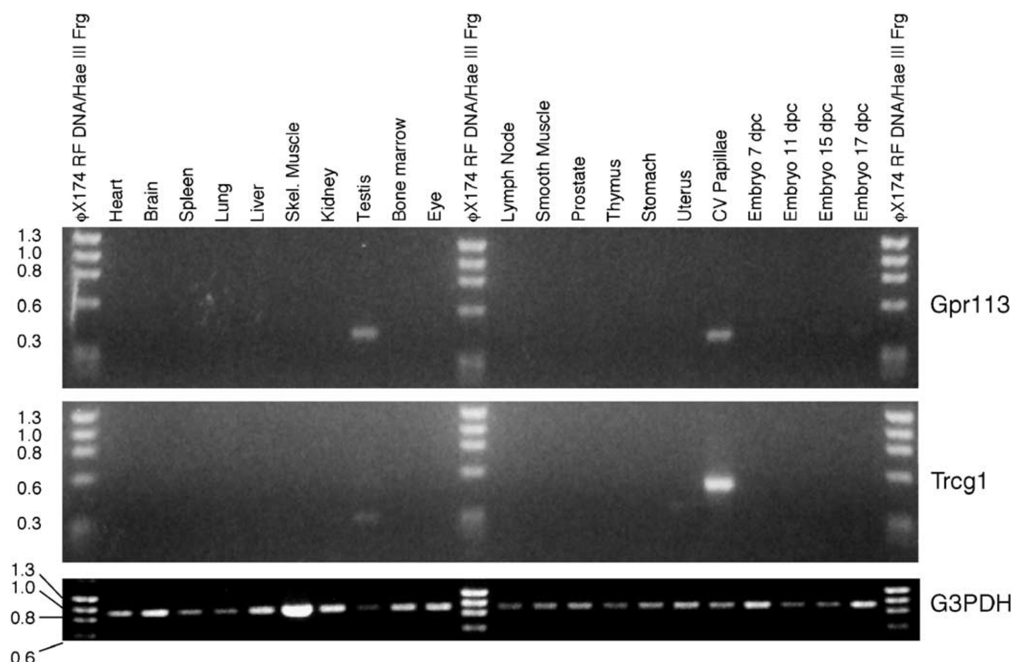

Fig. 4. Expression of *Gpr113* and *Trcg1* in mouse tissues. PCRs were performed using commercial multiple tissue cDNA panels and RT-PCR on RNA isolated from circumvallate papillae. Gene-specific primers were designed to amplify either *Gpr113* (top) or *Trcg1* (middle). For *Gpr113*, an amplified product of the expected size (417 bp) was observed only in cDNAs derived from the testis and circumvallate papillae. The *Trcg1* gene-specific primers were designed to amplify a region from exon 8 to 12 and, depending on the splice variant (with or without exon 10), amplicons of either 499 or 353 bp were expected. The *Trcg1* primers amplified a 499-bp product when circumvallate papillae-derived cDNA was used as a template. In addition, a faint band of 353 bp was observed when uterus cDNA was used as a template. The PCR product observed in the testis lane was determined by sequence analysis not to be derived from *Trcg1*. Primers for G3PDH were used as a control (bottom).

uterus cDNA corresponds to a splice variant of *Trcg1* that lacks exon 10, while the predominant band of 499 bp amplified from circumvallate papillae cDNA corresponds to a splice variant that includes exon 10. Under the cycling conditions used for these experiments, the shorter 353-bp fragment was not detected in the circumvallate papillae cDNA. Sequencing of the amplified product of 275 bp observed when testis cDNA was used as the template indicated that it was unrelated in sequence to *Trcg1*. These results suggest that alternative forms of *Trcg1* may be differentially expressed in the uterus and circumvallate papillae.

Since our original identification of *Trcg1* and *Gpr113* as novel ESTs in our TRC-enriched library, three ESTs from the rat homolog of *Trcg1* and four ESTs from the mouse *Gpr113* have appeared in the databases. The three *Trcg1* ESTs were identified in a rat heart library, while the four *Gpr113* ESTs were identified in libraries derived from either eight-cell or whole embryos. In addition, Bjarnadottir et al. [29] reported the presence of three testis-derived GPR113 ESTs in human.

Gpr113 and Trcg1 are expressed in a subset of TRCs

To examine in detail the cellular expression of *Trcg1* and *Gpr113* in circumvallate papillae, in situ hybridization experiments with antisense cRNA probes were performed. Both *Trcg1* and *Gpr113* are expressed in a subset of TRCs but absent from surrounding lingual epithelium (Fig. 5). We compared the pattern of expression of *Gpr113* with that of *T1R3*, which encodes another GPCR known to function as a taste receptor and that is expressed in a subset (~20%) of TRCs. On comparable sections hybridized under the same conditions, the

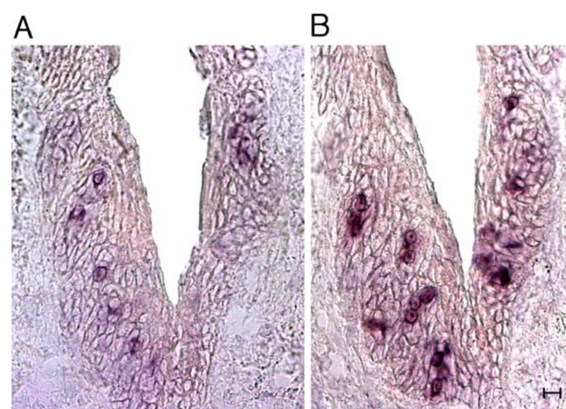

Fig. 5. *Gpr113* and *Trcg1* are expressed in a subset of TRCs. Representative sections of circumvallate papillae were hybridized with digoxigenin-labeled (A) *Grp113* or (B) *Trcg1* cRNA probes. Sections hybridized with sense probes showed no specific hybridization signal (data not shown). Scale bar, 10 μm.

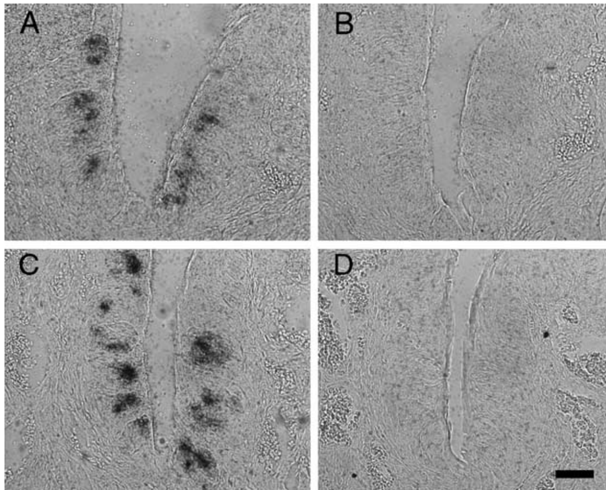

Fig. 6. Comparison of *Gpr113* and *T1R3* expression. Comparable sections were hybridized with ^{35}S-UTP-labeled (A) *Gpr113* antisense, (B) *Gpr113* sense, (C) *T1R3* antisense, or (D) *T1R3* sense cRNA probes. The pattern of expression of *Gpr113* resembles that of *T1R3*, known to label ~20% of TRCs.

patterns of expression of *Gpr113* and *T1R3* appear similar (Fig. 6).

Discussion

Trcg1 is a novel TRC-expressed gene

Other than the existence of a few partial rat ESTs, we provide the first experimental evidence that *Trcg1* is an expressed gene and present its genomic structure. We identified two alternate transcripts of *Trcg1*, one encoding a predicted protein of 825 amino acids and a second encoding a predicted protein of 209 amino acids. The expression of *Trcg1* is highly restricted, with strong expression detected only in the circumvallate papillae, and weaker expression of an alternative splice variant evident in the uterus. Within the circumvallate papillae, *Trcg1* expression is detected only in a subset of TRCs. We also report a novel human gene that resides on chromosome 15q22 and encodes a predicted protein that displays 33% amino acid identity to the mouse TRCG1. Despite its low sequence similarity, this gene is likely the human homolog of *Trcg1*, since it lies within a block of conserved synteny between the human and the mouse genomes, and no other candidate homologs were identified. TRCG1 displays no significant homology to characterized proteins. Therefore, the role of *Trcg1* in the subset of TRCs that express it is not known.

Gpr113 encodes a family 2B GPCR expressed in a subset of TRCs

The cDNA encoding *Gpr113*, which we isolated from circumvallate papillae tissue, is predicted to encode a GPCR. Our cDNA corresponds to *Gpr113*, which was recently identified in searches of the mouse genome as the homolog of the previously predicted human gene, *GPR113*[29,32]. Although we note several differences between our reported sequence of 991 amino acids and the predicted sequence of 887 amino acids, overall the sequences are in good agreement.

GPR113 belongs to the 2B subgroup of family 2 GPCRs [33] and displays no sequence similarity to members of the two known families of GPCR taste receptors, namely the T1Rs and T2Rs. T1Rs are family 3 GPCRs that recognize sweet-tasting compounds and amino acids, whereas the T2Rs are family 1 GPCRs that recognize bitter-tasting compounds. *Gpr113* encodes the first member of the family 2B GPCRs demonstrated to be selectively expressed in a subset of TRCs.

Members of the family 2B GPCRs are also referred to as EGF-TM7 receptors, to denote that the founding members contained epidermal growth factor modules [34]; LNB-TM7 receptors, for long N-termini seven-transmembrane domains [35]; and adhesion receptors, to denote the presence of extracellular adhesion-like functional domains [36]. As their various names indicate, members of the family are characterized by having large extracellular domains often composed of one or several functional domains. Recent searches of the mouse and human genomic sequences have led to the identification of over 30 members of the family [29,32,37]. These molecules display sequence similarity within their transmembrane regions both to each other (25–70% identity) and to family 2A GPCRs (25–30% identity) [35]. The functional domains found within the extracellular domains of members of this family are diverse and include EGF modules, hormone-binding domains, GPSs, laminin repeats, mucin-like domains, thrombospondin repeats, immunoglobulin domains, and cadherin repeats, among others. The extracellular domain of GPR113 is 696 amino acids and has two functional domains, a GPS domain and a hormone-binding domain.

The GPS domain is a common feature of the members of the family 2B GPCRs, not found in other GPCRs [38–40]. GPS domains are highly structured and characterized by a 50-amino-acid stretch with two invariant tryptophan residues and four conserved cysteines postulated to form two disulfide bridges. Comparisons of the known cleavage sites of members of the mammalian family 2B GPCRs yield a consensus sequence in the vicinity of the cleavage site of TXCXCXHL(S/T)XF, with cleavage occurring between the leucine and the serine/threonine residues [38,41–47]. In family 2B GPCRs, the GPS motif is located immediately before the first transmembrane domain. Following cleavage, the extracellular N-terminus of the receptor remains non-covalently associated with the seven-transmembrane core [39,41,45]. Lin et al. [48] demonstrated that the cleavage is a self-mediated hydrolysis of an internal peptide bond. Given the highly ordered structure and conservation of the GPS motif, they hypothesized that all proteins with a GPS

will undergo autoproteolysis by the same mechanism. Therefore, we propose that GPR113 exists as a heterodimer with a seven-transmembrane subunit associated with an extracellular hormone-binding subunit.

Hormone-binding domains are a common feature of the classic family 2A GPCRs that bind hormone peptides such as glucagon, secretin, vasoactive intestinal peptide, parathyroid hormone, thyrotropin-releasing hormone, and growth-hormone-releasing hormone [49]. All of these ligands are peptides that range in size from 3 to 84 amino acids. The hormone-binding site consists of three or four cysteine residues as well as two conserved tryptophan residues and an aspartate [33]. The GPR113 hormone-binding domain conforms to the consensus, suggesting that, like family 2A GPCRs, one ligand for GPR113 may be a peptide. However, we note that given its size, it is probable that the extracellular domain of GPR113 is involved in additional interactions. For example, the extracellular domains of other members of the 2B family act as adhesion molecules, interacting with extracellular matrix components or participating in cell–cell interactions via the binding of cell-surface ligands [35,44,50–52].

In addition to being innervated by sensory nerve fibers and in contact with other taste buds cells, TRCs are exposed to the oral cavity through their apical projections through taste pores. Therefore, candidate peptide ligands for GPR113 include both endogenous and environmentally encountered peptides. GPR113 may function to recognize one of the known neuropeptides that have been localized to the nerve fibers and cells that are associated with taste buds [53]. Such neuropeptides have been proposed to play roles in either the modulation of taste sensitivities or the maintenance and regeneration of TRCs. Alternatively, GPR113 may function as a receptor for an ingested peptide(s), many of which are known to elicit taste sensations. Knockout mouse models of both *Gpr113* and *Trcg1* are currently being developed to address their specific roles in TRCs.

Materials and methods

Isolation of circumvallate papillae and nontaste lingual epithelium

Tongues were isolated from 500 freshly sacrificed C57B6 mice, and circumvallate papillae and nontaste lingual epithelia were dissected. The dissections were performed following separation of the lingual epithelium from underlying muscle and connective tissue using a published protocol with minor modifications [54]. Briefly, isolated tongues were injected with an enzyme mixture consisting of dispase (5 mg/ml), collagenase B (4 mg/ml), and trypsin inhibitor (2 mg/ml) in 1× PBS. This mixture was injected beneath the lingual epithelium surrounding the circumvallate papillae, and the injected tongues were incubated for 10 min in 1× PBS at room temperature. Following the enzymatic treatment, the lingual epithelium was gently peeled away from the underlying tissue. The circumvallate papillae were then removed. In addition, to serve as the source of driver cDNA in subtractive procedures, patches of nontaste lingual epithelium devoid of taste buds and located just anterior to the circumvallate papillae were isolated. Dissected tissues were snap frozen in liquid nitrogen. Total RNA was isolated using TRIZOL reagent (Life Technologies) and then polyadenylated RNA was extracted using the Micro-FasTrack 2.0 kit (Invitrogen). Poly(A)$^+$ circumvallate papillae-derived RNA (0.4 μg) was obtained from 500 mice. To obtain sufficient amounts of double-stranded cDNA for a subtraction, both circumvallate papillae and nontaste lingual epithelium-derived poly(A)$^+$ RNAs went through a preamplification step using the Smart PCR cDNA synthesis kit (BD Biosciences). Circumvallate papillae cDNA was subtracted against nontaste lingual epithelium cDNA using the PCR-Select cDNA subtraction kit (BD Biosciences) according to the manufacturer's protocol [26]. Subsequently, a subtracted TRC-specific cDNA library was generated in the T/A cloning vector pCRII (Invitrogen). Approximately 20,000 clones were sequenced. The BLAST program (http://www.ncbi.nlm.nih.gov/blast/) was used for the comparisons of the cloned sequences with existing public databases at NCBI [55].

Isolation of full-length cDNAs

Because we used a PCR-based suppression subtractive hybridization procedure for the construction of the TRC-enriched library, the ESTs obtained were generally partial and averaged about 500 bp in length. To obtain a longer clone for *Trcg1*, an oligo(dT)-primed, full-length circumvallate papillae cDNA library was constructed in the UniZAP XR vector (Stratagene) and screened with a 545-bp [^{32}P]dCTP-labeled *Trcg1* probe. The labeling reaction was carried out with a Prime-It II random primer labeling kit (Stratagene). Hybridization was performed at 68°C in 0.5 M phosphate buffer containing 1% BSA and 4% SDS, and washes were performed using high-stringency conditions. Two partial 1.6-kb cDNA clones of *Trcg1*,which contained the 3′ end of the gene, were obtained and sequenced.

Based on sequences within the original clones and isolated cDNAs, the 5′ end of *Trcg1* and the 5′ and 3′ ends of *Gpr113* were obtained by RACE reactions (SMART RACE cDNA amplification kit; BD Biosciences). For *Trcg1*, the primer used for the 5′ RACE reaction was 5′-GTGAGGGAGGGTAGAGGCCAG-3′. For *Gpr113*, the primers used for the 5′ and 3′ RACE reactions were 5′-TGTGAACCAAGGTCTCCACAGCCTTCA-3′ and 5′-CGGTGTCAACGGGCTGGTCCTT-3′.

Gene-specific primers complementary to sequences in the 5′ and 3′ untranslated regions of each gene were designed, and intact full-length clones of both genes were obtained by RT-PCRs using circumvallate papillae mRNA. cDNA clones (2.7 and 1 kb) of *Trcg1* were obtained using

forward, 5′-GACACAAGAAGACCGTGCACAACA3-′, and reverse, 5′-GCGGCAAGGAAGTGGGGTGAAGC-3′, primers. A 3.0-kb *Gpr113* cDNA clone was isolated using forward, 5′-GACTGCTGCGTTGAGCCATGTCCTCT-3′, and reverse, 5′-GGTTCCTGTCAATCAGTCATCCT-3′, primers.

Northern analyses

Commercial mouse multiple tissue Northern blots (MTN and MTN II; BD Biosciences) were hybridized under the conditions recommended by the manufacturer. For *Gpr113*, a [^{32}P]dCTP-labeled 630-bp probe corresponding to exon 10 was used. For *Trcg1*, a [^{32}P]dCTP-labeled 2.2-kb probe corresponding to exons 1 through 8 was used. A human β-actin cDNA, which detects a transcript of 2.0 kb, was used as a control probe. Hybridization with this probe indicated that an equivalent amount of RNA was loaded in each lane (data not shown).

PCR analyses of Trcg1 and Gpr113 expression

Mouse PCR-ready single-stranded cDNA was either purchased (Mouse MTC Panels I and II; BD Biosciences) or isolated from tongue tissue (Advantage RT-for-PCR kit; BD Biosciences). Briefly, mRNA was isolated from circumvallate papillae and reverse transcribed with an oligo(dT)$_{18}$ primer utilizing MMLV reverse transcriptase under the conditions recommended. Using the circumvallate papillae cDNA and the MTC panel cDNAs, a 0.4-kb fragment from *Gpr113* or a 0.49-kb fragment from *Trcg1* was amplified by PCR using gene-specific forward (5′-CGGTGTCAACGGGCTGGTCCTT-3′ and 5′-GATGTGGCTCTTGATCCTGG-3′, *Gpr113* and *Trcg1*, respectively) and reverse (5′-GGTTCCTGTCAATCAGT-CATCCT-3′ and 5′-GCGGCAAGGAAGTGGGGTGAA-GC-3′, *Gpr113* and *Trcg1*, respectively) primers under nonsaturating conditions. The cycling parameters were as follows: 95°C for 2 min; 30 cycles of 95°C for 10 s, 56°C for 10 s, and 72°C for 20 s; followed by a final extension at 72°C for 10 min. Thermal cycling reactions were carried out using the Applied Biosystems GeneAmp PCR System 9700. Amplicons were analyzed by electrophoresis on agarose gels to determine size and sequenced to verify identity. Each of the cDNAs was also used as a PCR template with control G3PDH primers under nonsaturating conditions as recommended by the manufacturer. Each reaction with the G3PDH primers yielded a single band of the expected size (983 bp).

In situ hybridization

In situ hybridizations were performed using a 545-bp fragment of *Trcg1* corresponding to sequences within exons 5 to 9, a 630-bp fragment of *Gpr113* from exon 10, or a 500-bp fragment of *T1R3* from exon 3. ^{35}S-labeled or digoxigenin-labeled antisense cRNA probes were prepared by in vitro transcription of linearized plasmid DNA using T3 (*Gpr113*) or T7 polymerases (*Trcg1* and *T1R3*). Sense probes were also generated from the same templates and tested for hybridization; no signals were observed. Mice used in these studies were sacrificed according to NIH-approved guidelines.

For the in situ hybridizations performed with digoxigenin-labeled probes on fresh frozen sections, adult female C57B6/J mice were killed in a closed chamber containing CO_2, followed by cervical dislocation. The tissue was dissected and rapidly frozen in OCT compound (Tissue-Tek). Cross sections (8 μm) of the tongue were collected on Superfrost slides and fixed in 4% paraformaldehyde in cold PBS. Sections were hybridized overnight at 68°C in hybridization buffer (50% formamide, 5× SSC, 5× Denhardt's, 10 mg/ml herring sperm DNA containing 100 ng/ml digoxigenin-labeled probe) and washed at high stringency (0.2× SSC, 68°C). Immunological detection and visualization of the digoxigenin-labeled hybrids were performed using an alkaline phosphatase-conjugated antibody and standard chromogenic reagents following the manufacturer's recommendations (DIG Nucleic Acid Detection Kit; Roche Molecular Biochemicals).

In situ hybridizations performed with ^{35}S-UTP-labeled probes were performed on 10-μm paraffin sections through the circumvallate papillae. Hybridizations were carried out at 52°C for 16 h in 50% deionized formamide, 0.3 M NaCl, 20 mM Tris–HCl (pH 7.4), 5 mM EDTA, 10 mM $NaPO_4$ (pH 8), 10% dextran sulfate, 1× Denhardt's solution, 50 μg/ml yeast RNA with 50,000 cpm/μl ^{35}S-labeled RNA probe. Following hybridization, coverslips were removed, and the slides were washed in 50% formamide, 2× SSC, 0.1 M DTT at 65°C. Slides were then rinsed in 0.4 M NaCl, 0.1 M Tris (pH 7.5), 0.05 M EDTA, treated with RNase A (20 μg/ml; Sigma) for 30 min at 37°C, and washed once each in 2× SSC and 0.1× SSC at 37°C for 15 min. Slides were processed for standard autoradiography using NTB-2 Kodak emulsion and then exposed for approximately 21 days at 4°C.

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References

[1] B. Lindemann, Receptors and transduction in taste, Nature 413 (2001) 219–225.

[2] S. Ugawa, Y. Minami, W. Guo, Y. Saishin, K. Takatsuji, T. Yamamoto, et al., Receptor that leaves a sour taste in the mouth, Nature 395 (1998) 555–556.
[3] D.R. Stevens, R. Seifert, B. Bufe, F. Muller, E. Kremmer, R. Gauss, et al., Hyperpolarization-activated channels HCN1 and HCN4 mediate responses to sour stimuli, Nature 413 (2001) 631–635.
[4] O. Kretz, P. Barbry, R. Bock, B. Lindemann, Differential expression of RNA and protein of the three pore-forming subunits of the amiloride-sensitive epithelial sodium channel in taste buds of the rat, J. Histochem. Cytochem. 47 (1999) 51–64.
[5] W. Lin, T.E. Finger, B.C. Rossier, S.C. Kinnamon, Epithelial Na^+ channel subunits in rat taste cells: localization and regulation by aldosterone, J. Comp. Neurol. 405 (1999) 406–420.
[6] J.P. Montmayeur, S.D. Liberles, H. Matsunami, L.B. Buck, A candidate taste receptor gene near a sweet taste locus, Nat. Neurosci. 4 (2001) 492–498.
[7] M.A. Hoon, E. Adler, J. Lindemeier, J.F. Battey, N.J. Ryba, C.S. Zuker, Putative mammalian taste receptors: a class of taste-specific GPCRs with distinct topographic selectivity, Cell 96 (1999) 541–551.
[8] G. Nelson, J. Chandrashekar, M.A. Hoon, L. Feng, G. Zhao, N.J. Ryba, et al., An amino-acid taste receptor, Nature 416 (2002) 199–202.
[9] E. Sainz, J.N. Korley, J.F. Battey, S.L. Sullivan, Identification of a novel member of the T1R family of putative taste receptors, J. Neurochem. 77 (2001) 896–903.
[10] M. Kitagawa, Y. Kusakabe, H. Miura, Y. Ninomiya, A. Hino, Molecular genetic identification of a candidate receptor gene for sweet taste, Biochem. Biophys. Res. Commun. 283 (2001) 236–242.
[11] G. Nelson, M.A. Hoon, J. Chandrashekar, Y. Zhang, N.J. Ryba, C.S. Zuker, Mammalian sweet taste receptors, Cell 106 (2001) 381–390.
[12] G.Q. Zhao, Y. Zhang, M.A. Hoon, J. Chandrashekar, I. Erlenbach, N.J. Ryba, et al., The receptors for mammalian sweet and umami taste, Cell 115 (2003) 255–266.
[13] X. Li, L. Staszewski, H. Xu, K. Durick, M. Zoller, E. Adler, Human receptors for sweet and umami taste, Proc. Natl. Acad. Sci. USA 99 (2002) 4692–4696.
[14] E. Adler, M.A. Hoon, K.L. Mueller, J. Chandrashekar, N.J. Ryba, C.S. Zuker, A novel family of mammalian taste receptors, Cell 100 (2000) 693–702.
[15] H. Matsunami, J.P. Montmayeur, L.B. Buck, A family of candidate taste receptors in human and mouse, Nature 404 (2000) 601–604.
[16] J. Chandrashekar, K.L. Mueller, M.A. Hoon, E. Adler, L. Feng, W. Guo, et al., T2Rs function as bitter taste receptors, Cell 100 (2000) 703–711.
[17] M. Behrens, A. Brockhoff, C. Kuhn, B. Bufe, M. Winnig, W. Meyerhof, The human taste receptor hTAS2R14 responds to a variety of different bitter compounds, Biochem. Biophys. Res. Commun. 319 (2004) 479–485.
[18] B. Bufe, T. Hofmann, D. Krautwurst, J.D. Raguse, W. Meyerhof, The human TAS2R16 receptor mediates bitter taste in response to beta-glucopyranosides, Nat. Genet. 32 (2002) 397–401.
[19] P. Rossler, C. Kroner, J. Freitag, J. Noe, H. Breer, Identification of a phospholipase C beta subtype in rat taste cells, Eur. J. Cell Biol. 77 (1998) 253–261.
[20] Y. Zhang, M.A. Hoon, J. Chandrashekar, K.L. Mueller, B. Cook, D. Wu, et al., Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signaling pathways, Cell 112 (2003) 293–301.
[21] C.A. Perez, L. Huang, M. Rong, J.A. Kozak, A.K. Preuss, H. Zhang, et al., A transient receptor potential channel expressed in taste receptor cells, Nat. Neurosci. 5 (2002) 1169–1176.
[22] G.T. Wong, K.S. Gannon, R.F. Margolskee, Transduction of bitter and sweet taste by gustducin, Nature 381 (1996) 796–800.
[23] S.K. McLaughlin, P.J. McKinnon, R.F. Margolskee, Gustducin is a taste-cell-specific G protein closely related to the transducins, Nature 357 (1992) 563–569.
[24] P. Rossler, I. Boekhoff, E. Tareilus, S. Beck, H. Breer, J. Freitag, G protein betagamma complexes in circumvallate taste cells involved in bitter transduction, Chem. Senses 25 (2000) 413–421.
[25] L. Huang, Y.G. Shanker, J. Dubauskaite, J.Z. Zheng, W. Yan, S. Rosenzweig, et al., Ggamma13 colocalizes with gustducin in taste receptor cells and mediates IP3 responses to bitter denatonium, Nat. Neurosci. 2 (1999) 1055–1062.
[26] D.V. Rebrikov, S.M. Desai, P.D. Siebert, S.A. Lukyanov, Suppression subtractive hybridization, Methods Mol. Biol. 258 (2004) 107–134.
[27] L.F. Kolakowski Jr., GCRDb: a G-protein-coupled receptor database, Receptors Channels 2 (1994) 1–7.
[28] M. Kozak, Interpreting cDNA sequences: some insights from studies on translation, Mamm. Genome 7 (1996) 563–574.
[29] T.K. Bjarnadottir, R. Fredriksson, P.J. Hoglund, D.E. Gloriam, M.C. Lagerstrom, H.B. Schioth, The human and mouse repertoire of the adhesion family of G-protein-coupled receptors, Genomics 84 (2004) 23–33.
[30] E.L. Sonnhammer, G. von Heijne, A. Krogh, A hidden Markov model for predicting transmembrane helices in protein sequences, Proc. Int. Conf. Intell. Syst. Mol. Biol. 6 (1998) 175–182.
[31] H. Nielsen, S. Brunak, G. von Heijne, Machine learning approaches for the prediction of signal peptides and other protein sorting signals, Protein Eng. 12 (1999) 3–9.
[32] R. Fredriksson, M.C. Lagerstrom, P.J. Hoglund, H.B. Schioth, Novel human G protein-coupled receptors with long N-terminals containing GPS domains and Ser/Thr-rich regions, FEBS Lett. 531 (2002) 407–414.
[33] A.J. Harmar, Family-B G-protein-coupled receptors, Genome Biol. 2 (2001) 1–13.
[34] A.J. Mcknight, S. Gordon, EGF-TM7: a novel subfamily of seven-transmembrane-region leukocyte cell-surface molecules, Immunol. Today 17 (1996) 283–287.
[35] M. Stacey, H.H. Lin, S. Gordon, A.J. McKnight, LNB-TM7, a group of seven-transmembrane proteins related to family-B G-protein-coupled receptors, Trends Biochem. Sci. 25 (2000) 284–289.
[36] R. Fredriksson, M.C. Lagerstrom, L.G. Lundin, H.B. Schioth, The G-protein-coupled receptors in the human genome form five main families: phylogenetic analysis, paralogon groups, and fingerprints, Mol. Pharmacol. 63 (2003) 1256–1272.
[37] R. Fredriksson, D.E. Gloriam, P.J. Hoglund, M.C. Lagerstrom, H.B. Schioth, There exist at least 30 human G-protein-coupled receptors with long Ser/Thr-rich N-termini, Biochem. Biophys. Res. Commun. 301 (2003) 725–734.
[38] V. Krasnoperov, M.A. Bittner, R.W. Holz, O. Chepurny, A.G. Petrenko, Structural requirements for alpha-latrotoxin binding and alpha-latrotoxin-stimulated secretion: a study with calcium-independent receptor of alpha-latrotoxin (CIRL) deletion mutants, J. Biol. Chem. 274 (1999) 3590–3596.
[39] V.G. Krasnoperov, M.A. Bittner, R. Beavis, Y. Kuang, K.V. Salnikow, O.G. Chepurny, et al., Alpha-Latrotoxin stimulates exocytosis by the interaction with a neuronal G-protein-coupled receptor, Neuron 18 (1997) 925–937.
[40] S. Sugita, K. Ichtchenko, M. Khvotchev, T.C. Sudhof, Alpha-Latrotoxin receptor CIRL/latrophilin 1 (CL1) defines an unusual family of ubiquitous G-protein-linked receptors: G-protein coupling not required for triggering exocytosis, J. Biol. Chem. 273 (1998) 32715–32724.
[41] T. Nechiporuk, L.D. Urness, M.T. Keating, ETL, a novel seven-transmembrane receptor that is developmentally regulated in the heart: ETL is a member of the secretin family and belongs to the epidermal growth factor-seven-transmembrane subfamily, J. Biol. Chem. 276 (2001) 4150–4157.
[42] J. Abe, T. Fukuzawa, S. Hirose, Cleavage of Ig-Hepta at a "SEA" module and at a conserved G protein-coupled receptor proteolytic site, J. Biol. Chem. 277 (2002) 23391–23398.
[43] G.W. Chang, M. Stacey, M.J. Kwakkenbos, J. Hamann, S. Gordon, H.H. Lin, Proteolytic cleavage of the EMR2 receptor requires both

the extracellular stalk and the GPS motif, FEBS Lett. 547 (2003) 145–150.

[44] M. Stacey, G.W. Chang, S.L. Sanos, L.R. Chittenden, L. Stubbs, S. Gordon, et al., EMR4, a novel epidermal growth factor (EGF)-TM7 molecule up-regulated in activated mouse macrophages, binds to a putative cellular ligand on B lymphoma cell line A20, J. Biol. Chem. 277 (2002) 29283–29293.

[45] J. Gray, M. Haino, M. Roth, J. Maguire, P. Jensen, A. Yarme, et al., CD97 is a processed, seven-transmembrane, heterodimeric receptor associated with inflammation, J. Immunol. 157 (1996) 5438–5447.

[46] H. Obermann, A. Samalecos, C. Osterhoff, B. Schroder, R. Heller, C. Kirchhoff, HE6, a two-subunit heptahelical receptor associated with apical membranes of efferent and epididymal duct epithelia, Mol. Reprod. Dev. 64 (2003) 13–26.

[47] T. Moriguchi, K. Haraguchi, N. Ueda, M. Okada, T. Furuya, T. Akiyama, DREG, a developmentally regulated G protein-coupled receptor containing two conserved proteolytic cleavage sites, Genes Cells 9 (2004) 549–560.

[48] H.H. Lin, G.W. Chang, J.Q. Davies, M. Stacey, J. Harris, S. Gordon, Autocatalytic cleavage of the EMR2 receptor occurs at a conserved G protein-coupled receptor proteolytic site motif, J. Biol. Chem. 279 (2004) 31823–31832.

[49] G.V. Segre, S.R. Goldring, Receptors for secretin, calcitonin, parathyroid hormone (PTH)/PTH-related peptide, vasoactive intestinal peptide, glucagonlike peptide 1, growth hormone-releasing hormone, and glucagon belong to a newly discovered G-protein-linked receptor family, Trends Endocrinol. Metab. 4 (1993) 309–314.

[50] M. Stacey, G.W. Chang, J.Q. Davies, M.J. Kwakkenbos, R.D. Sanderson, J. Hamann, et al., The epidermal growth factor-like domains of the human EMR2 receptor mediate cell attachment through chondroitin sulfate glycosaminoglycans, Blood 102 (2003) 2916–2924.

[51] M. Stacey, H.H. Lin, K.L. Hilyard, S. Gordon, A.J. McKnight, Human epidermal growth factor (EGF) module-containing mucin-like hormone receptor 3 is a new member of the EGF-TM7 family that recognizes a ligand on human macrophages and activated neutrophils, J. Biol. Chem. 276 (2001) 18863–18870.

[52] J. Hamann, B. Vogel, G.M. van Schijndel, R.A. van Lier, The seven-span transmembrane receptor CD97 has a cellular ligand (CD55, DAF), J. Exp. Med. 184 (1996) 1185–1189.

[53] B. Lindemann, Taste reception, Physiol. Rev. 76 (1996) 718–766.

[54] P. Behe, J.A. DeSimone, P. Avenet, B. Lindemann, Membrane currents in taste cells of the rat fungiform papilla: Evidence for two types of Ca currents and inhibition of K currents by saccharin, J. Gen. Physiol. 96 (1990) 1061–1084.

[55] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (1990) 403–410.