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Identification of a novel member of the T1R family of putative taste receptors

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Abstract

In the gustatory system, the recognition of sugars, amino acids and bitter-tasting compounds is the function of specialized G protein-coupled receptors. Recently, two members of novel subfamily of G protein-coupled receptors were proposed to function as taste receptors based on their specific expression in taste receptor cells. Here, we report the identification of a third member, T1R3, of this family of receptors. *T1R3* maps near the telomere of mouse chromosome 4 rendering it a candidate for the *Sac* locus, a primary determinant of sweet preference in mice. Consistent with its candidacy for the *Sac* locus, *T1R3* displays taste receptor cell-specific expression. In addition, taster and non-taster strains of mouse harbor different alleles of *T1R3*.

Keywords: G protein-coupled receptor, gustation, gustatory system, mouse, *Sac* locus, taste.

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Mammals have the ability to taste a large variety of chemicals including simple ions, such as sodium and protons, as well as organic molecules, such as sugars, amino acids and numerous bitter-tasting compounds. Despite the structural diversity of tastants, the gustatory system discriminates among only a small number of modalities including sour, salt, bitter, sweet and umami, the taste elicited by glutamate. Studies of the various signal transduction pathways in taste receptor cells indicate that protons and sodium ions depolarize taste receptor cells by interacting directly with ion channels ultimately leading to sour or salty perception. Glutamate and molecules perceived as bitter and sweet, however, are thought to activate G protein-coupled receptors (GPCRs) (Kinnamon and Margolskee 1996; Herness and Gilbertson 1999; Gilbertson *et al*. 2000).

The initial step in tastant recognition occurs on the apical surfaces of taste receptor cells that are clustered within taste buds of the tongue and palate. Individual taste buds contain 50–150 taste receptor cells. Taste buds on the tongue are organized into three types of spatially segregated papillae: the circumvallate, fungiform and foliate papillae. Circumvallate papillae are located in the medial posterior region of the tongue, foliate papillae in the lateral regions of the tongue and fungiform papillae in the anterior two-thirds of the tongue. Dependent on their positions, taste buds are innervated by different cranial nerves; those in the posterior region of the tongue are primarily innervated by the glossopharyngeal nerve, whereas those in the anterior portion of the tongue are innervated by the chorda tympani branch of the facial nerve.

The functional significance of the different types of papillae and their differential innervation and localization on the tongue is unclear. Furthermore, the selectivity and response profiles of taste receptor cells are not well characterized. For example, taste receptor cells, as appears to be the case for those expressing bitter receptors (Adler *et al*. 2000; Matsunami *et al*. 2000), may express multiple taste receptors and be broadly tuned responding to many tastants. Conversely, analogous to mammalian olfactory sensory neurons, a taste receptor cell may express a single receptor and exhibit high selectivity for a particular subset of structurally similar tastants. The identification of taste receptors and the determination of their selectivities and cellular and spatial distributions are

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Abbreviations used: GPCR, G protein-coupled receptor.

critical to understanding the molecular mechanisms that detect and process gustatory information both in the periphery and in the brain.

Recent molecular studies have led to the identification of a large family of GPCRs, two members of which have been shown to function as bitter taste receptors (Adler *et al*. 2000; Chandrashekar *et al*. 2000; Matsunami *et al*. 2000). In addition, a taste cell-derived variant of the mGluR4 receptor, taste-mGluR4, has been proposed to function as a umami taste receptor (Chaudhari *et al*. 2000). The identities of sweet receptors, however, have remained elusive. Possible candidates for such receptors include two taste receptor cell-specific receptors, T1R1 and T1R2 (Hoon *et al*. 1999). These receptors are differentially and selectively expressed on the apical surfaces of subsets of taste receptor cells. To date, however, T1R1 and T1R2 have not been functionally characterized. In addition, the *Sac* (saccharin preferring) locus, which maps near the telomere of mouse chromosome 4, has been shown to be a major genetic determinant of sweet preference in mice (Fuller 1974; Lush 1989; Lush *et al*. 1995; Bachmanov *et al*. 1997; Blizard *et al*. 1999). However, the gene responsible for the *Sac* phenotype has not been cloned (Reed 2000).

Here we report the identification of a novel candidate taste receptor gene, *T1R3*, which maps to the distal region of mouse chromosome 4 within the genetically defined interval for the *Sac* locus. Requisite for T1R3 functioning as a taste receptor, we show that *T1R3* is selectively expressed in taste receptor cells. Furthermore, we identify several polymorphisms between the *T1R3* genes of sweet-tasting and non-tasting strains of mice; six of which are predicted to result in amino acid substitutions.

Materials and methods

Isolation of genomic and cDNA clones of *T1R3*

Based on human database sequences, degenerate oligonucleotides were designed to PCR amplify a segment of the mouse *T1R3* gene from genomic DNA. The 5′ primer was 5′-AYTGGGTIGCIGCIY-TIGGIWSIGAYGA-3′ and the 3′ primer was 5′-TIATRCARTCR-CAYTGIGGRCAICKYTG-3′. The resulting fragment of ≈ 500 bp (corresponding to amino acids 206–376 in T1R3) was subcloned into vector pCR2.1 (Invitrogen) and used to generate a probe for screening a mouse genomic library. A similar strategy was used to isolate the corresponding fragment from the human genomic DNA. DNA sequence analysis in this region indicated that the mouse and human genes were 72% homologous, verifying that the mouse and human genes were orthologs. The 500-bp fragment was radio-labeled and used to screen at high stringency a lambda 129/Sv mouse genomic library (Stratagene) resulting in the isolation of a 13-kb genomic clone containing the *T1R3* gene and surrounding sequences. To verify the internal intron/exon boundaries of *T1R3* a 3.2-kb fragment of genomic DNA, which contained the six coding exons and intervening introns of *T1R3*, was subcloned into pcDNA3.1 (Invitrogen) and stably transfected into HEK 293 cells. Primers were designed to sequences in the 5′- and 3′-UTRs of *T1R3* to amplify it from first strand cDNA synthesized from two independent, expressing cell lines. RT-PCR products derived from both cell lines displayed the predicted gene structure, although clones that were either incompletely processed or alternatively spliced were also identified. The 5′ and 3′ primers were 5′-TC-AGATTCGTGCTGGAGACTTCTACCTAC-3′ and 5′-AAGGTC-AGTGGAGTCCAAGTCTATAG-3′, respectively.

Radiation hybrid mapping

Radiation hybrid mapping was performed using a mouse/hamster radiation hybrid panel (Research Genetics). Results were submitted to the Jackson Laboratory Mouse Radiation Hybrid Database (http://www.jax.org/resourses/documents/cmdata) for analysis.

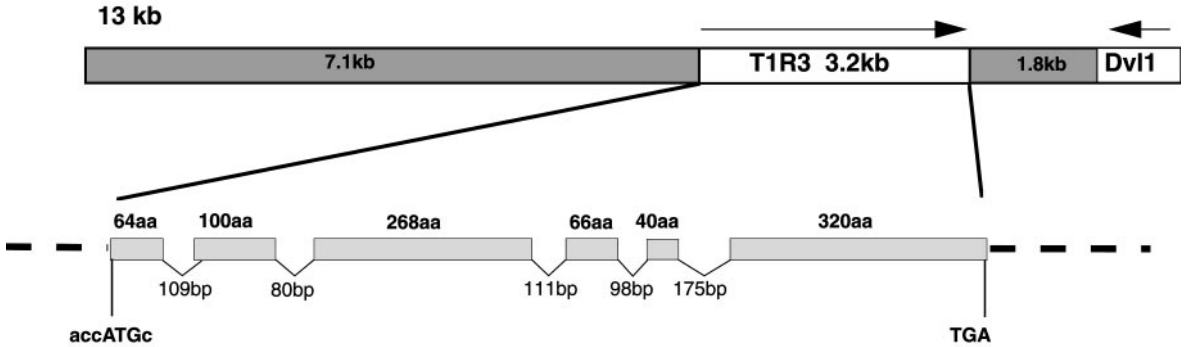


Fig. 1 Genomic organization of the *T1R3* gene. The genomic structure and six predicted coding exons of *T1R3* are shown. The gene structure was determined using gene prediction programs and by comparison of genomic and cDNA sequences. The intron−exon structure is conserved with that of the calcium sensing receptor (Pollak *et al*. 1993). All splice junctions are flanked by GT at the 5′ donor site and by AG at the 3′ acceptor site. The putative initiating methionine, surrounding Kozak sequence (Kozak 1987), and termination signal are indicated. Arrows represent relative directions of transcription of *T1R3* and the 3′-most exon of neighboring gene, *Dvl1*, a homolog of the *Drosophila* segment polarity gene *Dsh* (Klingensmith *et al*. 1994; Sussman *et al*. 1994). The GenBank Accession no. for the *T1R3* gene is AY026318.

In situ hybridization

In situ hybridizations were performed using a ≈ 500 bp fragment of *T1R3*, corresponding to sequences that encode amino acids 206–376, cloned into pCR4 (Invitrogen). cRNA probes were prepared by *in vitro* transcription of linearized plasmid DNA using T3 or T7 RNA polymerase for the generation of the sense or antisense RNA probe, respectively. For the ^{35}S-labeled probes, templates were transcribed in the presence of 4 μCi/μL [^{35}S]UTP (> 1000 Ci/mmol, NEN). For digoxigenin-labeled probes, templates were transcribed in the presence of 35 mM digoxigenin-11-UTP (Roche Molecular Biochemicals).

For *in situ* hybridization with radiolabeled probes, tissue was dissected and fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS). Following embedding in paraffin, serial cross-sections (10 μm, proceeding posteriorly to anteriorly) of the tongue were collected on gelatin (0.2%)-treated slides and prepared for *in situ* hybridization as described previously (Sassoon *et al*. 1988; Ressler *et al*. 1993). 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 35 000–50 000 c.p.m./μL ^{35}S-labeled RNA probe. Following hybridization, coverslips were removed, and the slides were washed in 50% formamide, 2× NaCl/Cit, 0.1 M dithiothreitol 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 RNaseA (20 μg/mL; Sigma) for 30 min at 37°C, and washed once in each 2 × NaCl/Cit and 0.1 × NaCl/Cit at 37°C for 15 min. Slides were processed for standard autoradiography using NTB-2 Kodak emulsion and then exposed for ≈ 14 days at 4°C. To test for tissue specificity, sagittal sections of E16.5 embryos, and coronal sections of adult olfactory epithelium and vomeronasal tissue were also hybridized with the *T1R3* probe. No specific hybridization signals were observed in non-taste tissues.

For *in situ* hybridization using digoxigenin-labeled cRNA probes, tissue was dissected and rapidly frozen in OCT compound (Tissue-Tek). Cross-sections (12 μm) of the tongue were collected on Superfrost slides, air dried at room temperature and fixed in 4% paraformaldehyde in PBS. Slides were prepared for hybridization as described previously (Schaeren-Wiemers and Gerfin-Moser 1993). Sections were hybridized overnight at 72°C in hybridization buffer (50% formamide, 5× NaCl/Cit, 5× Denhardt's, 250 μg/mL yeast RNA, 500 μg/mL herring sperm DNA containing 200 ng/mL digoxigenin labeled probe) and washed at high stringency (0.2 × NaCl/Cit, 72°C). Immunological detection and visualization of the digoxigenin-labeled hybrids were performed using an anti-digoxigenin alkaline phosphatase-conjugated antibody and standard chromogenic reagents following the manufacturer's recommendations (Roche Molecular Biochemicals). Mice used in these studies were sacrificed according to NIH approved guidelines.

Results

T1R3 is a novel member of the T1R subfamily of putative taste receptors

With the aim of identifying novel chemosensory receptors, database searches of draft human genomic sequences were performed querying with GPCR sequences. One contig (GenBank Accession no. AL139287) derived from human chromosome 1p36 displayed several stretches of sequence with significant homology (> 30%) to members of family 3 GPCRs. This chromosomal region was of particular interest and further pursued due to its conserved synteny with the distal region of mouse chromosome 4 which includes the *Sac* locus, a primary contributor to sweet preference in mice.

Based on human sequences, a segment of the mouse gene was obtained and used to screen a 129/Sv genomic library resulting in the isolation a 13-kb genomic clone containing the murine ortholog. Analysis of ORFs within this genomic sequence identified a novel putative GPCR (T1R3). Comparisons of genomic and cDNA sequences indicate that the *T1R3* gene spans 3.2 kb of genomic DNA and consists of six exons encoding a predicted 858 amino acid

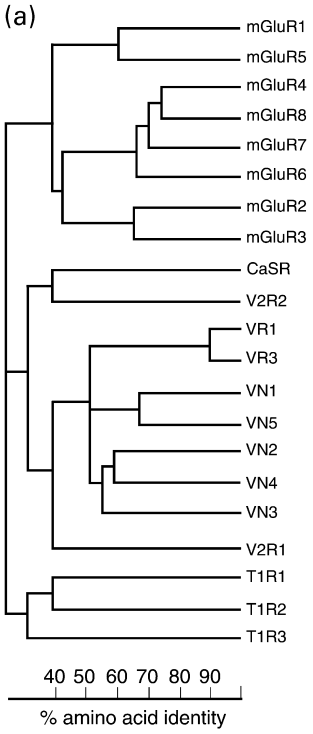


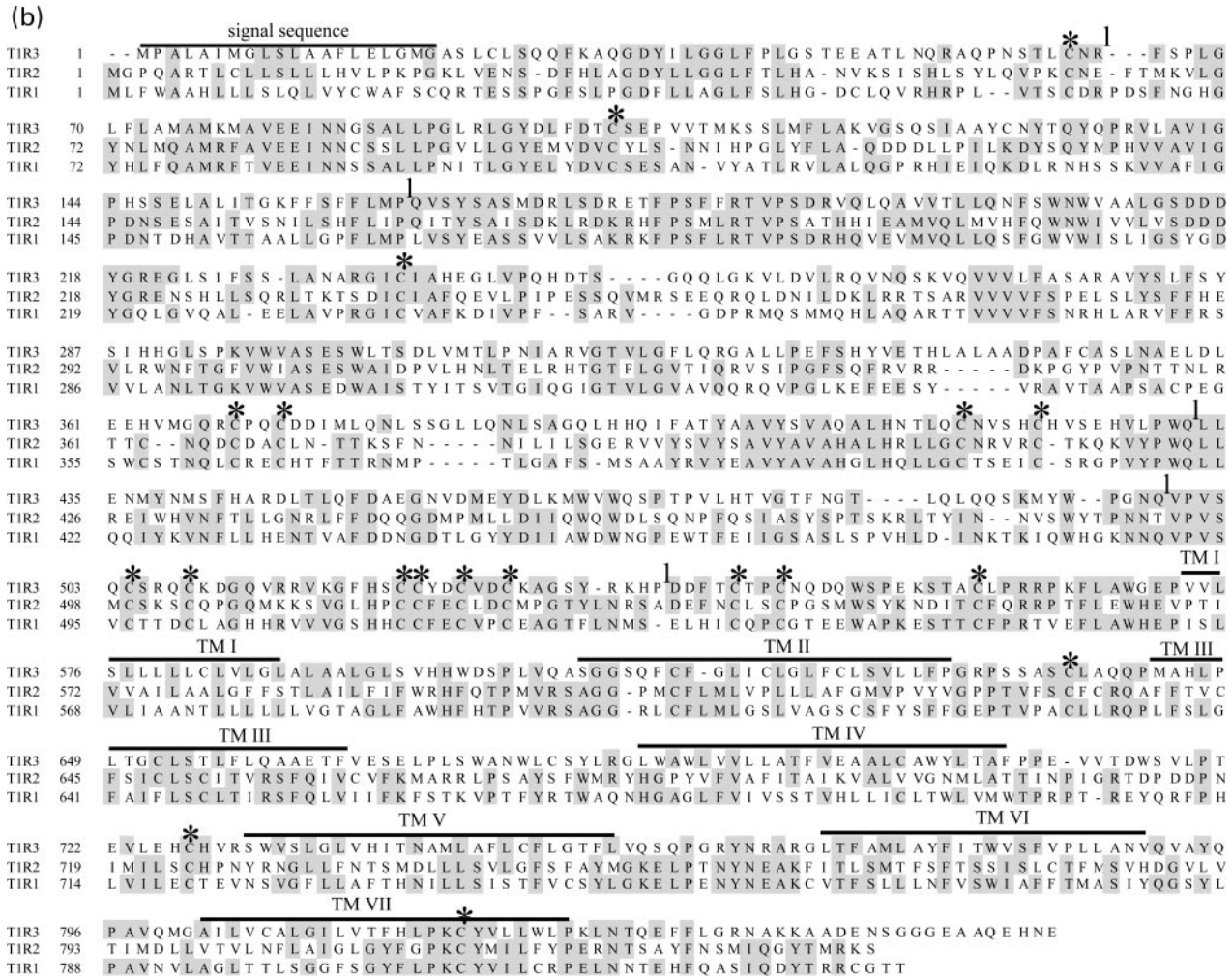
Fig. 2 T1R3 is a member of the family 3 G protein-coupled receptors (GPCRs) (a) The sequence relationships between selected members of family 3 GPCRs are presented as a dendrogram. Sequences include the rat (T1R1 and T1R2) and mouse (T1R3) putative taste receptors, rat metabotropic glutamate receptors (mGluRs), mouse (VRs and V2Rs) and rat (VNs) vomeronasal receptors, and the mouse calcium sensing receptor (CaSR). (b) The predicted protein sequences of members of the T1R family are aligned. Amino acid residues present in two or more members are highlighted. Putative transmembrane domains (I−VII) and a predicted signal sequence are indicated with lines. Intron−exon boundaries determined for T1R3 are marked with bars, and cysteines conserved among family 3 GPCRs are indicated with asterisks.

protein. The intron−exon structure of the *T1R3* gene is shown (Fig. 1). In addition to *T1R3*, this 13 kb genomic fragment harbors part of the 3′-exon of the *Dvl1* gene, a homolog of the *Drosophila* segment polarity gene *Dsh* (Klingensmith *et al*. 1994; Sussman *et al*. 1994).

Hydropathy analysis of the predicted T1R3 protein identified a 20 amino acid N-terminal segment characteristic of eukaryotic signal sequences and seven potential transmembrane-spanning regions indicative of members of the superfamily of GPCRs (Fig. 2b). T1R3 exhibits significant homology and structural resemblance to family 3 GPCRs which include the calcium-sensing receptor (Brown *et al*. 1993), $GABA_B$ receptor (Kaupmann *et al*. 1997) and metabotropic glutamate receptors (Nakanishi 1992), as well as a number of candidate chemosensory receptors. Chemosensory receptors belonging to this family include T1R1 and T1R2 (Hoon *et al*. 1999), vomeronasal V2R receptor family members (Herrada and Dulac 1997; Matsunami and Buck 1997; Ryba and Tirindelli 1997) and members of a family of fish olfactory receptors (Cao *et al*. 1998; Naito *et al*. 1998; Speca *et al*. 1999). T1R3 is most closely related to T1R1 and T1R2 sharing ≈ 30% amino acid identity with each (Figs 2a and b). Like other members of family 3 GPCRs, T1R3 is predicted to have an unusually large (572 amino acids) N-terminal extracellular domain that forms a ligand-binding domain in structurally related proteins (O'Hara *et al*. 1993; Takahashi *et al*. 1993; Okamoto *et al*. 1998; Parmentier *et al*. 1998; Kunishima *et al*. 2000). T1R3 displays additional structural similarities with family 3 GPCRs, including a cysteine-rich domain preceding the first transmembrane domain and a series of positionally conserved cysteines in the extracellular domain and extracellular loops, presumably important for conformational structure (Fig. 2b).

T1R3 is a candidate gene for the *Sac* locus

Genetic studies employing two bottle preference tests indicate that the *Sac* (saccharin preferring) locus is located near the distal end of mouse chromosome 4 ≈ 84 ± 3.4 cM from the centromere (Fuller 1974; Lush 1989; Lush *et al*.



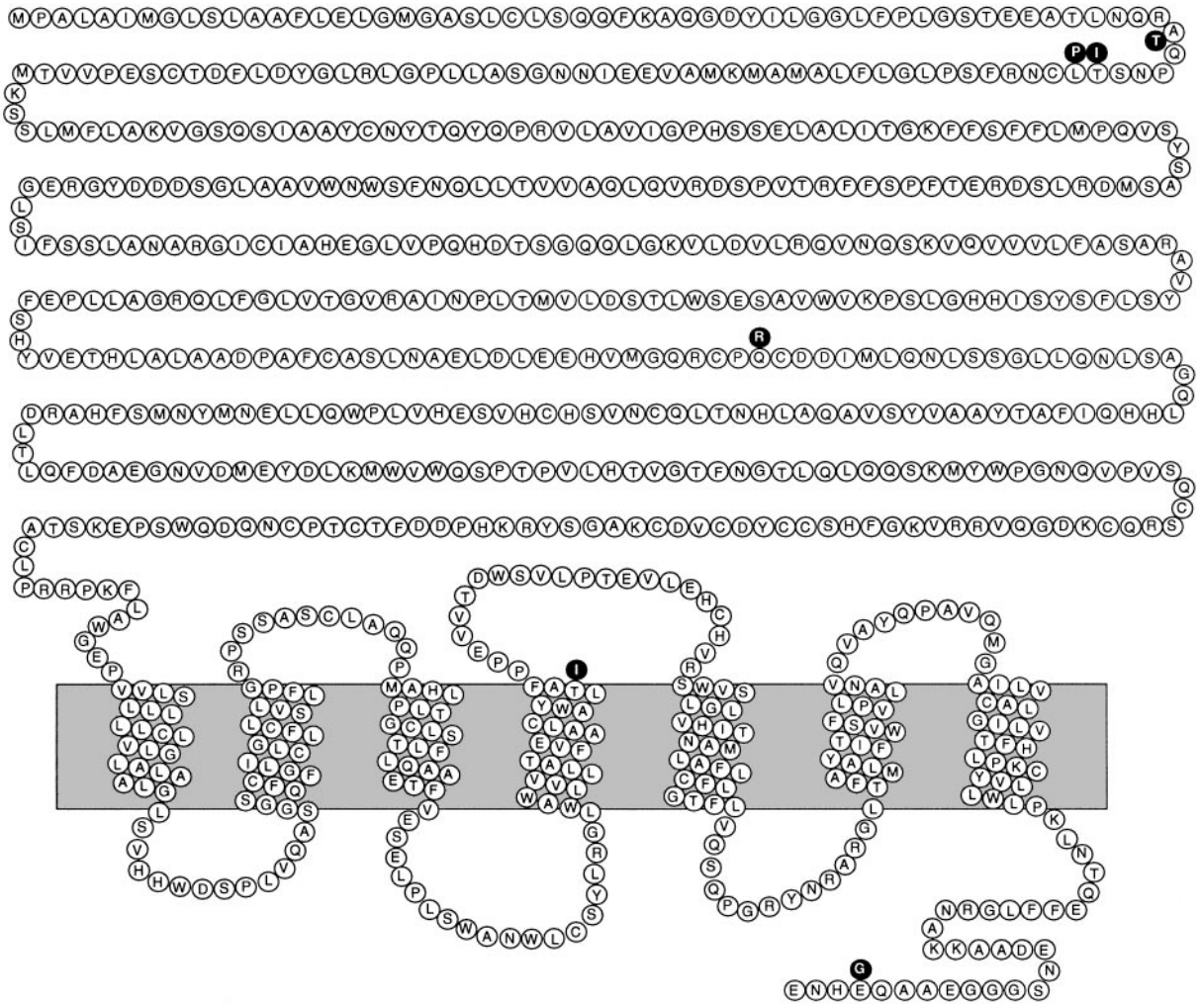


Fig. 3 Several polymorphisms resulting in amino acid substitutions in T1R3 are observed between sweet non-taster and taster strains of mice. The predicted topology of T1R3 is shown. Amino acid differences observed between C57Bl/6 J (taster) and 129/Sv (non-taster) strains of mice are indicated by black balls that represent amino acids found in the *C57Bl/6* J allele.

1995; Bachmanov *et al*. 1997; Blizard *et al*. 1999). In humans, *T1R3* maps to 1p36, a region syntenic to the distal end of mouse chromosome 4, rendering *T1R3* a candidate gene for the *Sac* locus. To address this possibility, we mapped the chromosomal position of *T1R3* using mouse/hamster radiation hybrid panels. Results indicated that *T1R3* maps between the anchor locus *D4Mit59* [78.9 cM from the centromere, Mouse Genome Database (Blake *et al*. 2000)] and the telomere of chromosome 4 within the region defined for the *Sac* locus (Lush *et al*. 1995; Bachmanov *et al*. 1997). Linkage between the *Sac* locus and *T1R3* was further substantiated by the fact that *T1R3* is physically linked to the *Dvl1* gene that maps at 82 cM from the centromere near the distal end of chromosome 4 [Mouse Genome Database (Blake *et al*. 2000)].

We reasoned that if *T1R3* is responsible for the *Sac* phenotype, inbred strains of mice with different sensitivities to sweet compounds are likely to exhibit polymorphisms within the *T1R3* gene. To test this hypothesis, we compared the genomic sequences of the *T1R3* genes isolated from well-studied taster (C57BL/6 J) and non-taster (129/Sv) strains of mice. Thirteen polymorphisms were detected in coding sequences. Six of these resulted in amino acid substitutions: four in the extracellular domain, one is in putative transmembrane domain IV and one in the C-terminal intracellular domain. (Fig. 3). Interestingly, three of the substitutions in the extracellular domain are clustered within a region shown to form a dimeric interface in the structurally related mGluR1 receptor (Kunishima *et al*. 2000).

T1R3 is selectively expressed in a subset of taste receptor cells

In the tongue, taste receptor cells are found clustered in the taste buds of three types of spatially segregated papillae: circumvallate papillae located in the medial posterior region

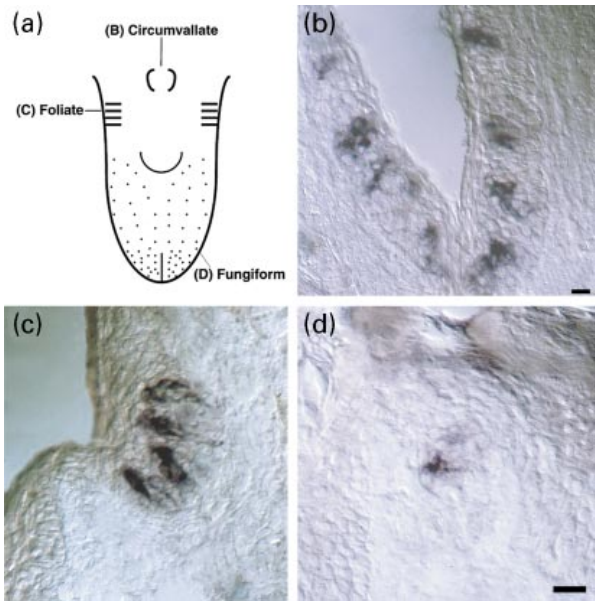


Fig. 4 *T1R3* is expressed in a subset of taste receptor cells. (a) Schematic diagram of the tongue indicating positions of different types of papillae. Sections of circumvallate (B), foliate (C) or fungiform (D) papillae were hybridized with a digoxigenin labeled *T1R3* cRNA probe. *T1R3* is expressed in a subset (≈20–30%) of taste receptor cells in all taste buds of circumvallate and foliate papillae. In contrast, *T1R3* is expressed in < 4% of the fungiform taste buds. A rare hybridizing fungiform taste bud displaying *T1R3*-expressing cells is shown. A control sense probe displayed no specific hybridization signals. Scale bars: 50 μm. Scale bar in (b) also applies to (c).

of the tongue, foliate papillae located in the lateral posterior regions of the tongue and fungiform papillae distributed over the anterior two-thirds of the tongue (Fig. 4a). To evaluate the potential of T1R3 to function as a chemosensory receptor, we performed *in situ* hybridization analyses with radiolabeled cRNA probes on tissue sections from the circumvallate, fungiform and foliate papillae of the tongue, as well as sections of the olfactory epithelium, the vomeronasal organ and whole embryos. Hybridization was observed in taste tissue, but was not observed in the non-taste sensory and embryonic tissues examined, indicating that *T1R3* is expressed selectively in taste tissue. Furthermore, *T1R3* displayed differential expression among the different papillae and regions of the tongue. *T1R3* transcripts were detected in all of the taste buds of the circumvallate and foliate papillae. In contrast, < 4% of the fungiform papillae contained *T1R3*-expressing cells (Table 1). These results indicate that the vast majority of *T1R3*-expressing taste receptor cells are located in the posterior regions of the tongue.

Table 1 *T1R3* expression in papillae of the tongue

Papillae	% Taste buds with *T1R3*-expressing cells	% *T1R3*-expressing cells per taste bud
Circumvallate	100	23 (± 3)
Foliate	100	26 (± 5)
Fungiform	< 4	< 1*

In situ hybridization, to determine the number of *T1R3*-expressing cells, and histological staining with nuclear dye Hoechst 33258, to determine total cell counts, were performed on 10 μm sections through various taste papillae. Cell counts were derived from the examination of 150 circumvallate and 110 foliate taste bud sections from 3 to 4 animals. *For the fungiform papillae, only six taste buds out of the 178 examined contained *T1R3* expressing cells; in each case the number of expressing cells was < 1%.

To examine the cellular distribution of *T1R3*, additional *in situ* hybridization experiments with digoxigenin-labeled probes were performed. Representative sections are shown in Fig. 4. These experiments indicate that *T1R3* is expressed in a subset of taste receptor cells. From examination of 150 circumvallate and 110 foliate taste bud sections, we estimate that that *T1R3* is expressed in ≈20–30% of the taste receptor cells in these papillae (Table 1).

Discussion

In this study we report the identification of a novel GPCR that is specifically expressed in a subset of taste receptor cells. Based on its cell type-specific expression, we propose that T1R3, like the two other members of the T1R family (Hoon *et al*. 1999), is a putative taste receptor. The T1Rs belong to the family 3 GPCRs and thus are structurally similar to a number of other candidate chemosensory receptors including taste-mGluR4 (Chaudhari *et al*. 2000), mammalian V2R vomeronasal receptors (Herrada and Dulac 1997; Matsunami and Buck 1997; Ryba and Tirindelli 1997) and a family of fish olfactory receptors (Cao *et al*. 1998; Naito *et al*. 1998; Speca *et al*. 1999). However, T1Rs are only distantly related to the recently identified T2R family of bitter taste receptors (Adler *et al*. 2000; Matsunami *et al*. 2000). Thus far, searches of human genome sequences have not revealed additional members of the T1R family. Therefore, in contrast to the T2R family, which is estimated to consist of ≈40–80 members (Adler *et al*. 2000), the T1R family is likely to be relatively small.

What are the ligands for the three identified members of the T1R family of receptors? Given the evidence that both sweet and bitter taste transduction is mediated by G protein-coupled processes, the T1R receptors may be responsible for the recognition of sweet and/or bitter tasting compounds. Based on their topographic patterns of expression, it was previously suggested that T1R1 and T1R2 may be receptors for sweet- and bitter-tasting substances, respectively (Hoon *et al*. 1999). With respect to *T1R3*, its homology to other

chemosensory receptors, expression in taste receptor cells, chromosomal mapping position, and allelic variation between sweet taster and non-taster strains of mice makes it a compelling candidate gene for the *Sac* locus. As such, its list of putative ligands would include those demonstrated to be associated with the Sac phenotype such as sucrose, saccharin, acesulfame and dulcin (Lush 1989).

Of the six predicted amino acid differences identified between the T1R3 receptors expressed by taster and non-taster strains of mice, three are clustered between amino acids 55 and 61 within the large N-terminal extracellular domain. Based on alignments to the structurally related mGluR1 receptor, changes in this region are predicted to have important functional consequences. Crystallization studies of the extracellular domain of mGluR1 indicate that the homologous region in mGluR1 serves as the dimeric interface between mGluR1 protomers which form homodimeric complexes (Kunishima *et al*. 2000). This is significant because in the case of the $GABA_B$ receptor, another member of the family 3 GPCRs, oligomerization is critical to the production of functional receptor (Jones *et al*. 1998; Kaupmann *et al*. 1998; White *et al*. 1998). It is important to note, however, that polymorphisms are common among inbred strains of mice and that functional studies are required to determine whether T1R3 is, in fact, a sweet receptor and whether the observed amino acid substitutions within T1R3 affect receptor function.

T1R3 is expressed in all taste buds of the circumvallate and foliate papillae, but is expressed in $< 4\%$ of fungiform taste buds. Comparisons of the expression patterns of members of the T1R family indicate that *T1R3* shows a similar distribution to that of *T1R2*, which is also expressed in all taste buds of the circumvallate and foliate papillae, while rarely being expressed in fungiform papillae (Hoon *et al*. 1999). In stark contrast to *T1R2* and *T1R3*, *T1R1* is expressed in all the taste buds of the fungiform papillae but $< 10\%$ of taste buds in the circumvallate papillae (Hoon *et al*. 1999). These results indicate that members of the T1R family exhibit differential topographic distributions among the various papillae types and regions of the tongue. Similarly, Adler *et al*. (2000) established that members of the T2R family also display regional topography, being more frequently expressed in the taste buds of circumvallate and foliate papillae. These spatial patterns of expression of candidate taste receptors suggest that the number of cells expressing different receptors varies dramatically between different regions of the tongue.

The continuing efforts to identify taste receptors as well as other components of the taste signal transduction pathways will provide the molecular tools necessary to address many basic questions concerning the strategies used by the gustatory system to detect and encode incoming sensory information.

Note added in proof

Since the submission of this paper, more sensitive *in situ* hybridization experiments have reveealed low levels of *TIR3* expression in a high percentage of fungiform papillae.

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