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Regular Article

Amino acid residues of bitter taste receptor TAS2R16 that determine sensitivity in primates to β-glycosides

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In mammals, bitter taste is mediated by TAS2Rs, which belong to the family of seven transmembrane G protein-coupled receptors. Since TAS2Rs are directly involved in the interaction between mammals and their dietary sources, it is likely that these genes evolved to reflect species-specific diets during mammalian evolution. Here, we analyzed the amino acids responsible for the difference in sensitivities of TAS2R16s of various primates using a cultured cell expression system. We found that the sensitivity of TAS2R16 varied due to several amino acid residues. Mutation of amino acid residues at E86T, L247M, and V260F in human and langur TAS2R16 for mimicking the macaque TAS2R16 decreased the sensitivity of the receptor in an additive manner, which suggests its contribution to the potency of salicin, possibly via direct interaction. However, mutation of amino acid residues 125 and 133 in human TAS2R16, which are situated in helix 4, to the macaque sequence increased the sensitivity of the receptor. These results suggest the possibility that bitter taste sensitivities evolved independently by replacing specific amino acid residues of TAS2Rs in different primate species to adapt to species-specific food.

Key words: cell signaling, molecular evolution, G protein-coupled receptor, receptor regulation, primates, bitter taste

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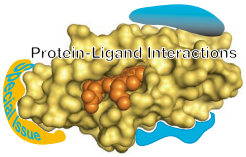
Mammals can perceive and distinguish five basic taste qualities: sweet, bitter, sour, salty, and umami (the taste of glutamate) [1]. Among these, bitter sensitivity is particularly important, as can be seen from the fact that many naturally poisonous or bioactive substances taste bitter to humans, and virtually all animals show an aversive response to such tastants. This suggests that bitter transduction might have evolved as a key defense against the ingestion of harmful substances. Bitter taste in mammals is mediated by TAS2Rs (or T2Rs), which are seven transmembrane G protein-coupled receptors that are expressed in specialized taste bud cells [2–7]. Genomic analyses have revealed the repertoires of TAS2R genes, with species-specific and intra-species variations [8,9] that probably reflect dietary changes during mam-



◀ ***Significance*** ▶

TAS2R16 is one of the most investigated bitter taste receptors, TAS2Rs, while the crystal structure of any TAS2Rs is not solved yet. In this study, we examined the effect of amino acid residues different between primate TAS2Rs and found some residues responsible for the function of TAS2R16, binding of ligands and activation of receptors. This kind of evolutionary and mutational approach will be useful for elucidating the structure-function relationship of GPCRs including TAS2Rs in terms of ligand binding and activation of the receptors from the biophysical points of views.

malian evolution.

Of these genes, human TAS2R16 is one of the best studied at the molecular and population levels. For example, many β-glucopyranosides have been identified as TAS2R16 ligands by human behavioral or functional assays using exogenously expressed proteins [5]. Human-specific amino acid substitution at position 172 results in high potency of harmful cyanogenic glycosides, which may have been advantageous for avoidance of toxic compounds in early human diets [10]. Ligand binding sites were identified by mutational studies of human TAS2R16 [11]. Notably, various primates conserve TAS2R16 largely intact with only a few amino acid substitutions. These facts prompted us to examine inter-species variations of TAS2R genes in non-human primates to better understand the biological significance of bitter perception.

Previously, we reported the analysis of the function of TAS2R16 of some representative primate species: human, chimpanzee, macaque, and langur [12]. TAS2R16 function was assayed with respect to increases in intracellular Ca levels induced by ligand binding after transient expression of TAS2R16. Variation in protein expression levels affected the maximal response; accordingly, we compared half maximal effective concentration (EC50), which refers to the concentration of bitter ligands that induces a response halfway between the baseline and maximum. We found that TAS2R16s of these species show different patterns of sensitivities for bitter compound repertoires. Particularly, when we compared the responses of TAS2R16 of various primates to salicin, human TAS2R16 was the most sensitive to salicin, while among the responsive TAS2R16s, macaque TAS2R16 showed a remarkably reduced response to salicin. While the differences in EC50 values were about 15-fold, mutation of one residue to a macaque-specific amino acid (E86T) decreased the sensitivity of human (EC50=0.48±0.09 to 2.74±0.60) and langur (2.3±0.20 to 3.9±0.21) TAS2R16s to salicin several-fold to a level similar to that of the macaque TAS2R16 EC50. Inversely, T86E mutation of the macaque TAS2R16 increased the potency of salicin (7.5±2.2 to 3.5±0.63), although full conversion was not achieved. These observations indicate that the species differences in the orthologues between the closely related species are the result of amino acid replacements during the course of evolution. Interestingly, the sensitivities for other ligands were not very different between the species, especially for the potentially poisonous cyanogenic glycoside amygdalin. In this study, we analyzed the mechanism underlying the differences in β-glycoside potency observed between the primate TAS2Rs, particularly human and macaque, by using chimeric and site-directed mutants. The results show that different regions contribute to the down-regulation and up-regulation of the receptor function, which harmonically tune the sensitivities of the receptors.

Materials and Methods

Salicin and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Genomic DNA of chimpanzee, macaque, and marmoset was isolated from blood using a DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany). Genomic DNA of white-headed langur was isolated from feces using a QiaAmp Stool Kit (Qiagen).

Standard PCR was used to amplify the TAS2R16 gene from genomic DNA (10 ng per 25 uL reaction). Products were sub-cloned into the EcoRV site of expression vector pEAK 10 with sequences encoding the first 45 amino acids of rat somatostatin receptor type 3 and the last 8 amino acids of bovine rhodopsin at the N- and C-termini, respectively. All sequences of PCR products were confirmed using standard Big Dye Terminator chemistry (Applied Biosystems, Foster City, California, USA). Chimeric vectors were constructed by ligating the fragments of the NcoI and the NotI sites situated in the middle and 3'-end of the TAS2R16 genes, respectively (Supplementary Fig. S1). Site-directed mutant vectors were constructed using Quickchange (Agilent Technology, Santa Clara, CA, USA) as described previously [12].

Cell culture, transfection, and cell-based assays were performed as described previously [11–13]. To calculate EC50 values, plots of amplitude (F) or ΔF/F value [ΔF/F = (F − F0)/F0], which is a normalized peak response relative to background fluorescence (F0), versus concentration were prepared in Igor Pro (Wavemetrics, Portland, OR, USA). Nonlinear regression of the plots produced the function $f(x) = I\ min + (I\ max - I\ min)/(1+(x/EC50)h)$, where x is the ligand concentration and h is the Hill coefficient, which was used to calculate the EC50 values for ligand-receptor interactions. TAS2R16 expression levels were examined by western blotting using a rho1D4-antibody, which recognizes the C-terminal 8 amino acids of bovine rhodopsin tagged to TAS2R16. Protein expression levels varied among experiments and affected the maximal response; accordingly, the half maximal effective concentrations (EC50) were compared, similar to a previous paper [14].

Results

First, we estimated the region responsible for the difference between macaque and human TAS2R16 using chimeric proteins (Fig. 1). HEK 293T cells transiently expressing TAS2R16 and G16gust44 were assayed using an intracellular calcium indicator, Calcium-4. After adding various concentrations of salicin to cells, the increases in fluorescence were monitored. The time course of fluorescence increase in the cells expressing both chimeric receptors, macaque(1–4)/human(5–7) (M/H) and human(1–4)/macaque(5–7) (H/M), showed normal responses to salicin (Fig. 1A and 1B, respectively), with sensitivities slightly lower than that of human TAS2R16 and slightly higher than that of macaque TAS2R16.

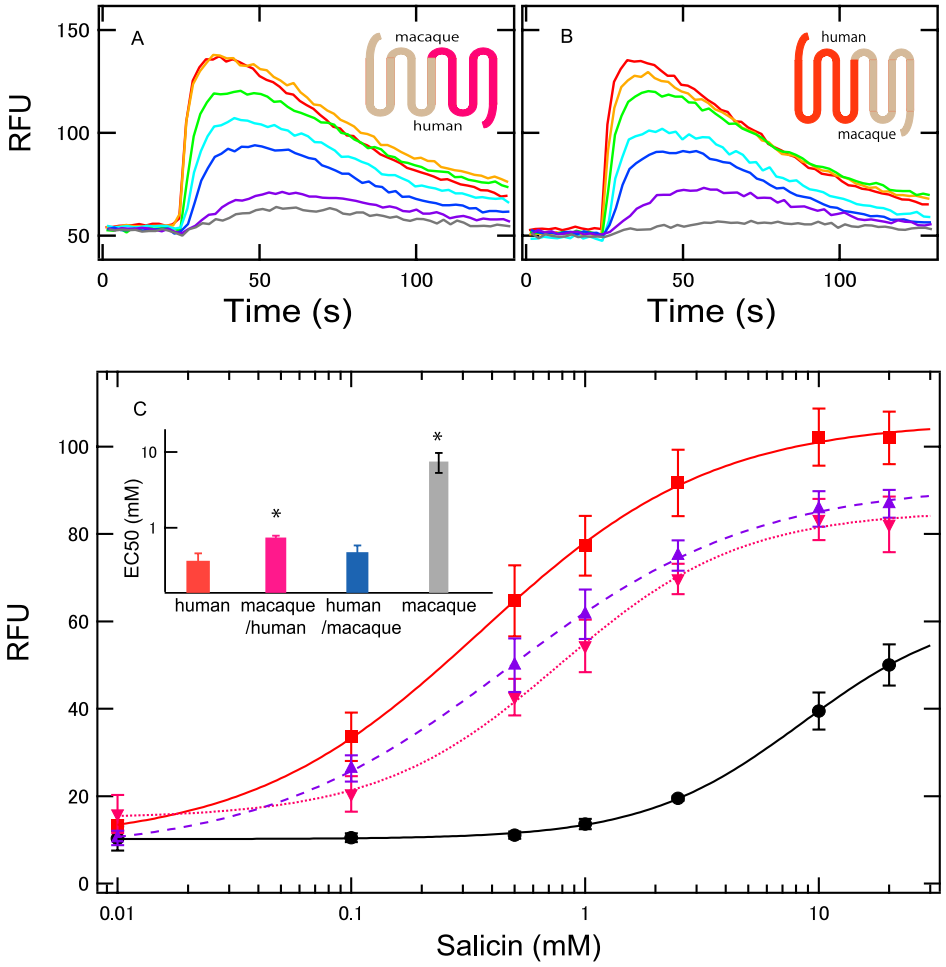


Figure 1 The responses of human, macaque, and chimeric TAS2R16 proteins to salicin.
HEK 293T cells transiently expressing TAS2R16 and G16gust44 were assayed using an intracellular calcium indicator, Calcium-4. After adding various concentrations of salicin to cells, changes in fluorescence were monitored. (A) Time course of fluorescence increase in the cells expressing macaque (1–4)/human (5–7) TAS2R16. (B) Time course of fluorescence increase in the cells expressing human (1–4)/macaque (5–7) TAS2R16. After addition of various concentrations (0, 0.1, 0.5, 1, 2.5, 10, and 20 mM) of salicin, increases in fluorescence were observed. (C) Maximal responses for each time course are plotted versus the concentration of salicin (n=4). Inset: EC50 values calculated by fitting the response curves using the Hill equation. Chimeric proteins macaque (1–4)/human (5–7) (purple reversed triangles and dotted line) and human (1–4)/macaque (5–7) (blue triangles and broken line), showed responses that were intermediate between human (red squares and smooth line), and macaque (black circles and smooth line) TAS2Rs. The EC50 value for macaque TAS2R16 was extrapolated by curve fitting; saturation was not observed, even at 20 mM. (*p<0.01, *t*-test. compared to the wild type)

Maximal responses for each time course are plotted versus salicin concentration (Fig. 1C, n = 4); EC50 values were calculated by fitting the response curves using the Hill equation. Expression of the chimeric proteins M/H (reversed triangles and dotted line) and H/M (triangles and broken line) showed responses that were intermediate between human (squares and smooth line) and macaque (circles and smooth line) TAS2Rs as indicated by the intermediate EC50 values (Fig. 1C, inset). This observation suggests that, at least, there are several amino acid residues responsible for the differences between human and macaque TAS2R16s, and are distributed in both the N-terminal half and C-terminal half of the protein.

Next, we made site-directed mutants of human TAS2R16 to identify the amino acids responsible for the differences between human and macaque TAS2R16. Because we previously showed that chimpanzee and langur proteins have higher sensitivity to bitter compounds than macaque [12], we focused on the 6 macaque-specific amino acid residues (Supplemental Fig. S1) that were revealed in the comparison between the human, chimpanzee, and langur TAS2R16 sequences (Fig. 2A). When we substituted these residues one by one for the human residues, one of the six (E86T) changed the EC50 (Fig. 2B) [12], although a complete change to the macaque EC50 was not achieved. Because the differences in affinity for each ligand may be due to differences in several amino acid residues, we constructed a series of site-directed mutants based on the residues that are unique to

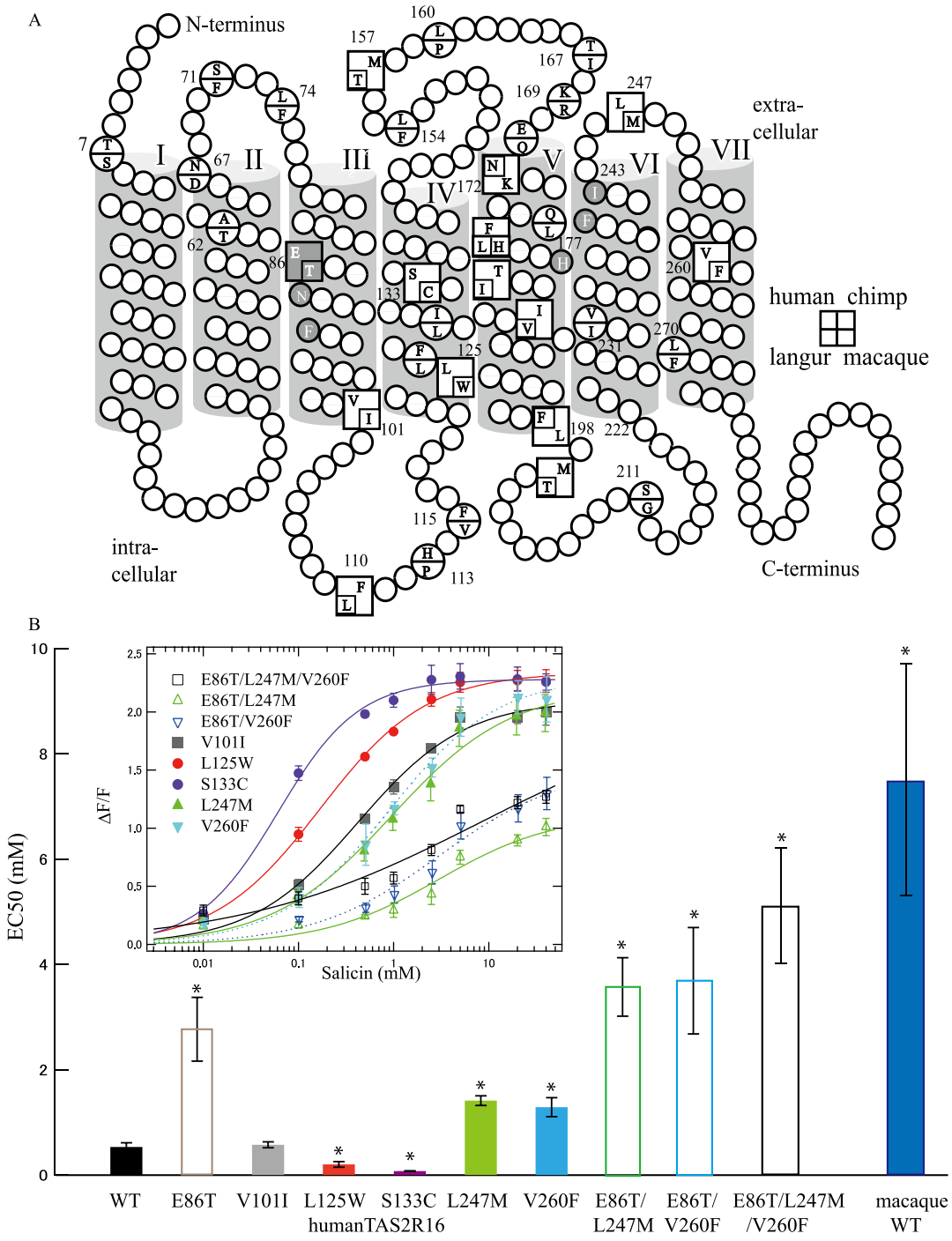


Figure 2 The sensitivities of human TAS2R16s and their mutants to salicin.
(A) Schematic representation of the membrane topology and amino acid differences between TAS2R16s from humans, chimpanzees, langurs, and macaques. The transmembrane topology is based on the structure of bovine rhodopsin. Circles show identical residues between four species and squares show mutated residues. Grey circles and squares represent the putative binding site for salicin [11]. (B) EC50 values of site-directed mutants of human TAS2R16 are plotted with wild-type human and macaque TAS2R16s. The values are derived from at least three independent experiments (*p<0.01, *t*-test. compared to the wild type). (Inset) ΔF/F value [ΔF/F=(F−F0)/F0], which was a normalized peak response relative to background fluorescence (F0), is plotted versus the concentration of salicin. EC50 values were calculated by fitting the response curves using the Hill equation for each experiment. The structure of salicin is also described.

A

TAS2R16	86	101	125	133	247	260
Human	E	V	L	S	L	V
Chimpanzee	E	V	L	S	L	V
Gorilla	E	V	L	S	L	V
Orangutan	E	V	W	S	L	V
Langur	E	V	L	S	L	V
Baboon	T	V	W	C	M	V
Macaque	T	I	W	C	M	F

B

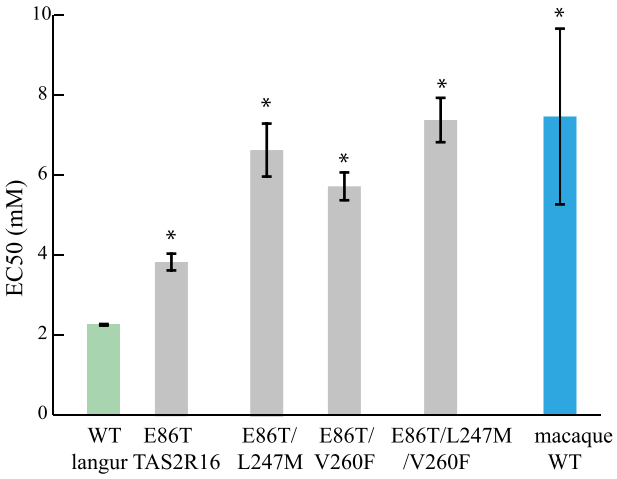


Figure 3 The amino acid residues of primate TAS2R16s responsible for the regulation of sensitivity to salicin.
(A) Differences in primate TAS2R16 amino acid residues relevant to this study. Sequences are highlighted from Figure 1. (B) EC50 values of site-directed mutants of langur TAS2R16s plotted with wild-type langur and macaque TAS2R16s. The values are derived from at least three independent experiments (*p<0.01, *t*-test. compared to the wild type).

macaque TAS2R relative to the human, chimpanzee, and langur TAS2R16 sequences (Fig. 2B). In addition to E86T, mutations L247M and V260F slightly increased the EC50 values (1.40±0.09 and 1.28±0.18, respectively). The double mutations E86T/L247M and E86T/V260F increased the EC50 to values (3.54±0.55 and 3.65±1.00, respectively) that were higher than those of the single mutations E86T, L247M, and V260F. The triple mutation consisting of E86T/L247M/V260F showed a further increase in EC50 to an average value (5.09±1.10) that is not significantly different from that of macaque wild type. Interestingly, mutations L125W and S133C decreased the EC50 values (0.20±0.05 and 0.074±0.006, respectively).

Because the amino acid sequence of the langur TAS2R16 is more similar to that of the macaque than the human, we expect that mutation of the langur protein to the corresponding macaque sequence would exhibit a response that more closely resembles that of the macaque protein (Fig. 3). In fact, wild-type protein showed a slightly higher EC50 value than human wild type. The E86T, E86T/L247M (6.67±0.67), and E86T/V260F (5.74±0.35) mutants displayed EC50 values that were higher than langur wild type, and the E86T/L247M/V260F (7.4±0.56) triple mutant resulted in an EC50 value that was virtually identical to that of macaque wild type. These results suggest that the sensitivity of TAS2R16 to salicin is regulated by several amino acid residues in a combinatorial manner.

The effects of amino acid replacements in human and langur TAS2R16s were largely similar for other glycoside ligands, like arbutin (Supplemental Fig. S2) and amygdalin (Supplemental Fig. S3). That is, E86T, L247M, V260F and their combination mutants of human TAS2R16 showed less sensitivity than the wild type, while L125W and S133C showed more sensitivity than the wild type. However, for some amino acid residues, the effect of replacement differed between salicin and other ligands. For example, the additive effect of E86T, L247M, and V260F was weaker for arbutin than salicin (Supplemental Fig. S2). For the response to amygdalin, E86T/L247M/V260F of human (2.00±0.08) and langur (1.94±0.36) TAS2R16 showed sensitivities close to that of macaque TAS2R16 (2.03±0.34) (Supplemental Fig. S3). However, the effects of L125W (0.80±0.07) and S133C (0.66±0.49) in human TAS2R16 (wild-type: 0.76±0.40) were smaller for amygdalin (Supplemental Fig. S3) than salicin (Fig. 2B). These results suggest that the effects of amino acid replacements are dependent on the glycoside ligands, whose chemical structures and contents in plants vary depending on ecological context.

Discussion

A previous study indicated that mutation of E86 to Q or D in the human TAS2R16 strongly reduces the response to salicin, suggesting that it is located in the binding site for salicin [11]. Therefore, one of the likely mechanisms of the lower potency of salicin in the context of the macaque TAS2R16 compared to TAS2R16s of other species involves the change of this residue to T in the macaque lineage (Fig. 2) [12], which presumably alters the binding site structure. In addition, in this study we identified amino acids 247 and 260 as being responsible for the relatively low sensitivity of the macaque TAS2R16. That is, the triple mutation of E86, L247, and V260 in human and langur TAS2R16 to the macaque residues resulted in a sensitivity of the receptor to salicin that was indistinguishable from that of macaque TAS2R16. That langur TAS2R16 exhibited lower sensitivity to salicin than the human orthologue suggests that additional residues must contribute to the higher sensitivity of human TAS2R16. For salicin, mutation of these three residues additively changed the sensitivity of TAS2R16. In the related species, E86 and L247 are changed in baboon and macaque, while V260 is changed only in macaque (Fig. 3A); therefore, the macaque TAS2R16 was specifically insensitive to salicin

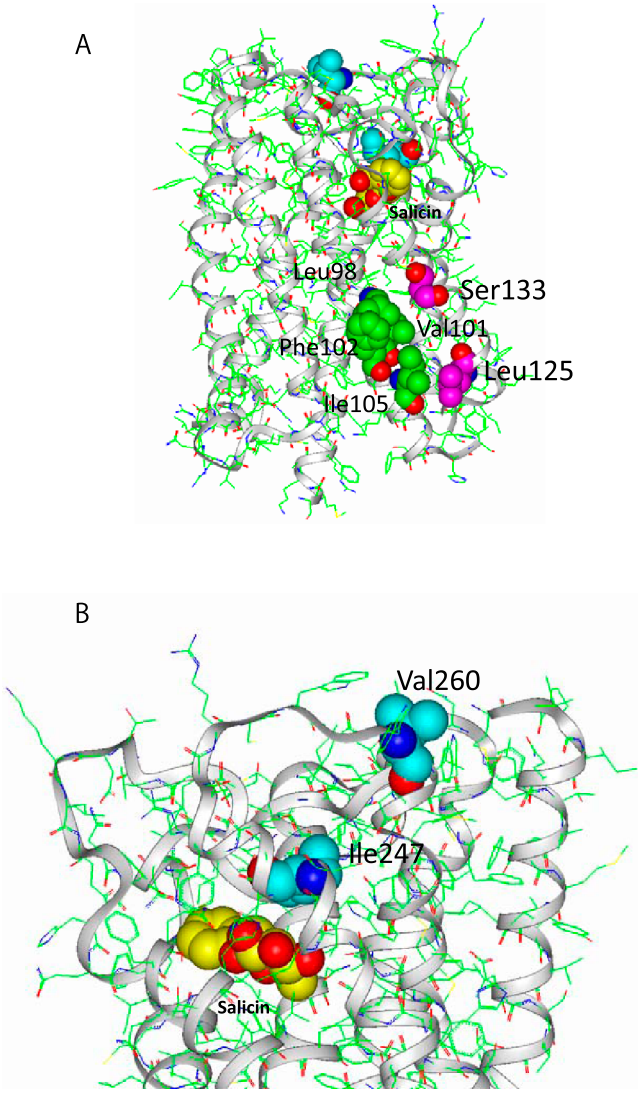


Figure 4 Model of human TAS2R16.
(A) The polypeptide backbone structure is derived from a previously published structure [11]. Amino acid residues of the third (Leu98, Val101, Phe102, and Ile105) and fourth (Lue125, Ser133) transmembrane domains are represented as green and violet space-filling models, respectively. (B) Extracellular domain of TAS2R16. Amino acid residues Ile247 and Val260 are represented as blue space-filling models.

mainly due to these three amino acid residues. This would be advantageous for the feeding behavior of macaques, which eat the salicin-containing bark of willow trees in winter.

It is of interest that the single mutations L125W and S133C resulted in a human TAS2R16 that was more sensitive to salicin than wild type. Because these mutations are included in the N-terminal half of the chimeric proteins, these changes led to negation of the effects due to E86, L247, and V260. In a homology model of TAS2R16 [11], L125 and S133 are situated close to the hydrophobic surface made by L98, V101, F102, and V105 in transmembrane helix 3. If this predicted structure is accurate, mutating these residues would change the hydrophobicity of this region. Based on the activation mechanism of other GPCRs such as rhodopsin, this change could be related to the activation of TAS2R16, as it would likely induce conformational changes in helices 3 and 4. Therefore, these changes likely increase the functional diversity of the protein by providing a different mechanism from the interactive network of amino acids 247, 260, and 86.

Conclusion

There are species-specific differences in the sensitivity of TAS2R16 to salicin and related glycosides, even among primates. Our data suggest that the molecular mechanism underlying the gain/loss in sensitivity of this receptor is distributed among the extracellular, transmembrane, and intracellular domains, which function harmonically in tuning the response of the receptors to the individual species' diets. Although the species difference is less pronounced for amygdalin than for salicin, the effect of replacing each amino acid residue is different between the ligands that bind and activate TAS2R16, the bitter taste receptor for various kinds of glycosides produced by plants.

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Conflict of Interest

H. I, N. S. H, Y. I., T. S., T. M., Y. L., W. P., M. I., K. M., K. A, and H. H. declare that they have no conflict of interest.

Author Contributions

H. I. designed this study, performed the molecular experiment, analyzed the data and drafted the manuscript. N. S. H., Y. I., T. S., and T. M. performed the molecular experiments and drafted the manuscript. Y. L. and W. P. collected the samples on white-headed-langur, M. I. and K. M. performed modelling, and K. A. and H. H. contributed to finalize the manuscript.

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