Members of RTP and REEP Gene Families Influence Functional Bitter Taste Receptor Expression*

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Functional characterization of chemosensory receptors is usually achieved by heterologous expression in mammalian cell lines. However, many chemoreceptor genes, including bitter taste receptors (TAS2Rs), show only marginal cell surface expression. Usually, these problems are circumvented by using chimeric receptors consisting of “export tags” and the receptor sequence itself. It seems likely that chemoreceptor cells express factors for cell surface targeting of native receptor molecules in vivo. For TAS2Rs, however, such factors are still unknown. The present study investigates the influence of RTP and REEP proteins on the functional expression of human TAS2Rs in heterologous cells. We expressed hTAS2Rs in HEK 293T cells and observed dramatic differences in responsiveness to agonist stimulation. By immunocytochemistry we show accumulation of the bitter β-glucopyranoside receptor hTAS2R16 in the Golgi compartment. Coexpression of RTP and REEP proteins changed the responses of some hTAS2Rs upon agonist stimulation, which is likely due to efficient cell surface localization as demonstrated by cell surface biotinylation experiments. The communoprecipitation of hTAS2R16 and RTP3 or RTP4 suggests that the mechanism by which these cofactors influence hTAS2R16 function might involve direct protein-protein interaction. Finally, expression analyses demonstrate RTP and REEP gene expression in human circumvallate papillae and testis, both of which are sites of TAS2R gene expression.

Humans can detect and distinguish the five basic taste qualities salty, sour, sweet, umami, and bitter (1). Gustatory stimuli are detected by taste receptor cells, which are organized in groups of 60–100 cells forming a taste bud. One to multiple taste buds are embedded within morphologically different types of taste papillae distributed over the tongue surface. Each taste bud exhibits a single apical porus exposing the microvilli of the receptor cells to the oral cavity (2).

The human TAS2R gene family of bitter receptors belongs to the G protein-coupled receptor superfamily and consists of ~25 members (3). The identification of activating bitter compounds for hTAS2R4, -10, -14, -16, -38, -43, -44, and -47 using functional expression assays revealed that they are tuned to detect many structurally related compounds or even a variety of diverse chemical structures (4–9). This might explain how humans are able to detect thousands of bitter compounds with only ~25 hTAS2Rs.

Because of insufficient cell surface targeting of native TAS2Rs in heterologous cells (7), which applies to other chemoreceptor families as well (odorant receptors (10), pheromone receptors (V2Rs) (11)), chimeric receptors containing the amino termini of either bovine rhodopsin (7) or rat somatostatin receptor subtype 3 (5) were used to improve functional expression. Whereas odorant receptors (ORs)2 in heterologous cells are retained within the ER (12), expression analyses in odora cells, a cell line derived from rat olfactory epithelium (13), indicates a multistep mechanism for plasma membrane targeting (14) probably involving several auxiliary factors. A growing number of diverse proteins involved in cell surface targeting of chemoreceptors is being identified (11, 15–17).

The present study investigates the functional expression of hTAS2Rs in HEK 293T cells by calcium imaging experiments, the subcellular distribution of hTAS2R16, and the influence of RTP (receptor transporting protein) and REEP (receptor expression enhancing protein) (17) coexpression on hTAS2R function and localization. Gene expression of RTP and REEP genes in tissues expressing hTAS2R was analyzed by RT-PCR and in situ hybridization.

EXPERIMENTAL PROCEDURES

Generation of Receptor Constructs—A fusion construct coding for the rat somatostatin receptor subtype 3 amino terminus, the hTAS2R16 open reading frame, and a HSV tag (5) was used to generate the construct sst3-hTAS2R16FLAG-hsv. By PCR-mediated recombination (18) a FLAG epitope was integrated into the second extracellular loop essentially as described before (19). The amino-terminal subfragment was generated using a forward primer corresponding to a sequence of the cytomegalovirus promoter of the vector and oligonucleotide A (‘‘CTTGTCATCGTACATCCTTGTAGTGACTAAGGATGACGATGACAAGTATCAGTTACAGTCTCG-3’’). The carboxyl-terminal subfragment was amplified with a primer specific for the BGH poly(A) site of the vector and oligonucleotide B (‘‘GACTAGCTAGATGACGATGACAAGTATCAGTTACAGTCTCG-3’’). The subfragments were fused by PCR, and the resulting hTAS2R16 sequence showing an exchange of amino acids 168–

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2The abbreviations used are: OR, odorant receptor; RT, reverse transcription; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; HA, hemagglutinin; ER, endoplasmic reticulum.
175 (DKLENFHQ to DYDDDDK) was cloned into the vector pcDNA5FRT (Invitrogen). Subsequently, the rat sst3 amino terminus was removed by PCR with oligonucleotide C (5'-GGCCCAATGGGAATTCGCCACCATGGTGTCCTGGCTTATACGGAGCTACGGAAAGAGAACTGCAGG; RTP1for, CAATGAATTCGCCGCCACCATGTGTAAAAGCCTTATACGGAGCTACGGAAAGAGAACTGCAGG; RTP2rev, GACTAGCGGCCGCTCATTCTGATGATCTCCAGAG; RTP3for, CAATGAATTCGCCGCCACCATGTGTACCAGCTTGCTTATACGGAGCTACGGAAAGAGAACTGCAGG; RTP4rev, GACTAGCGGCCGCTTAAAAAGGTAAATGAACAA; RTP5for, CAATGAATTCCACCATGGTGTCATGGATCATCTCCAGAG; RTP6rev, GACTAGCGGCCGCTAGGCGGTGCCTGACCACTTGTGAGTGGAAG; RTP7for, CAATCAATTGGCCGCCACCATGGCTGGGGACACAGCCGCCTAAAAGAAGGCAGGACTGAGGAAGGAG; RTP8for, CAATGAATTCGCCGCCACCATGTGTAAAAGCCGCCTGAGGCAGCGCGTG; and REEP6rev, GACTAGCGGCCGCTTAAAAAGGTAAATGAACAA). The resulting PCR products were subcloned into the pcDNA5FRT vector.

**cDNA Cloning of Human RTP1–4 and REEP1–6**—The coding sequences of human RTP and REEP cDNAs were cloned by RT-PCR from RNA of brain (RTP1, REEP1–3), fungiform papillae (REEP4–5), spleen (RTP4), and testis (RTP2–3 and REEP6). RNA from fungiform papillae was prepared from biopsy material using TRIzol reagent (Invitrogen); the other RNAs were purchased from BD Biosciences (Heidelberg, Germany). Random primed cDNA synthesis was done as before (20). Primer sequences (5′ to 3′) are given below:

- RTP1for, CAATGAATTCGCCACCACATGTGGAAGG; RTP1rev, GACTAGCGGCCCTTATACGGAGCTACGGAAAGAGAACTGCAGG; RTP2for, CAATGAATTCGCCACCACATGTGGAAGG; RTP3for, CAATGAATTCGCCACCACATGTGGAAGG; RTP4for, CAATGAATTCGCCACCACATGTGGAAGG; RTP5for, CAATGAATTCGCCACCACATGTGGAAGG; RTP6for, CAATGAATTCGCCACCACATGTGGAAGG; RTP7for, CAATGAATTCGCCACCACATGTGGAAGG; RTP8for, CAATGAATTCGCCACCACATGTGGAAGG.

**Calcium Imaging**—Functional calcium imaging experiments were done as before (4, 6, 8, 21). Briefly, HEK 293T cells stably expressing the G protein chimera Go16gust44 (22) were cotransfected with taste receptors and RTP/REEP constructs and changes in fluorescence were monitored. Dose-response curves of hTAS2R16FLAG-hsv coexpressed with RTP3/4, a single experiment was performed in triplicate. The cells were stimulated with salicin, helicin, and arbutin (5). Because of artifacts observed in cells stimulated with higher than 10 mM helicin and arbutin, respectively, these responses were excluded.

**Immunocytochemistry**—For immunocytochemistry of hTAS2R16FLAG-hsv and subcellular marker proteins HEK 293T-Ga16gust44 cells were used. 24 h after transfection some wells were treated with 10 μg/ml brefeldin A for 2 h (Sigma) to cause dissociation of Golgi components before fixation. The cells were then rinsed twice with 37 °C warm PBS and fixed for 5 min at room temperature with 3% paraformaldehyde in PBS (pH 7.2). After rinsing with TBS, the cells were permeabilized by 0.25% Triton X-100 in TBS (4 min at room temperature). The cells were then washed with TBS and incubated with blocking solution (5% normal horse serum in TBS) for 30 min at room temperature before primary antibodies (1:1000 mouse anti-FLAG M2, 1:1000 rabbit anti-HA from Sigma; 1:100 mouse anti-EEA1, 1:50 mouse anti-GM130, 1:100 mouse anti-EEA1, 1:100 rabbit anti-HA from Sigma; 1:100 mouse anti-p230 from BD Biosciences; 1:200 rabbit anti-calnexin from Stressgen) were applied in blocking solution and left overnight at 4 °C on the cells. On the next day the cells were washed with TBS, treated with blocking solution, and incubated for 1 h with secondary antibodies (1:4000 sheep anti-mouse Cy3, 1:2500 sheep anti-mouse Alexa488, 1:1000 mouse anti-FLAG M2, 1:100 mouse anti-HA from Sigma; 1:1000 goat anti-mouse Alexa488, 1:1000 goat anti-rabbit Alexa 488 from Invitrogen) diluted in blocking solution. Finally, washes with TBS and a brief rinse with H2O were performed before the glass coverslips were mounted onto slides using a fluorescence stabilizing mounting medium (DakoCytomation, Hamburg, Germany). Confocal images were obtained using a Leica TCS-SP2 system (Leica, Mannheim, Germany). The settings for secondary antibodies coupled to the fluorophore Alexa488 were: laser emission wavelength, 488 nm; detection wavelength, 500–530 nm; and for antibodies coupled to Cy3: laser emission wavelength, 543 nm; detection wavelength, 580–640 nm. For cellular cotransfection of hTAS2R16FLAG-hsv with RTP3 and RTP4, the cDNAs of RTP3 and RTP4 were fused with amino-terminal hemagglutinin (HA) tags by PCR using the following oligonucleotides: RTP3, 5′-TCTAAGCTTCCACCATTGTACCATTGAATCCATGGCTGTGGAAGG; and RTP4, 5′-TCTAAGCTTCCACCATTGAATCCATGGCTGTGGAAGG.

**Auxiliary Factors for hTAS2R Trafficking**—The codons for the amino terminus of the vector pcDNA5FRT were removed by PCR with oligonucleotide C (5′-GGCCCAATGGGAATTCGCCACCATGGTGTCCTGGCTTATACGGAGCTACGGAAAGAGAACTGCAGG; RTP1for, CAATGAATTCGCCACCACATGTGGAAGG; RTP1rev, GACTAGCGGCCCTTATACGGAGCTACGGAAAGAGAACTGCAGG; RTP2for, CAATGAATTCGCCACCACATGTGGAAGG; RTP3for, CAATGAATTCGCCACCACATGTGGAAGG; RTP4for, CAATGAATTCGCCACCACATGTGGAAGG; RTP5for, CAATGAATTCGCCACCACATGTGGAAGG; RTP6for, CAATGAATTCGCCACCACATGTGGAAGG; RTP7for, CAATGAATTCGCCACCACATGTGGAAGG; RTP8for, CAATGAATTCGCCACCACATGTGGAAGG.

The resulting PCR products were subcloned into the pcDNA5FRT vector.
Auxiliary Factors for hTAS2R Trafficking

Transfection and immunostaining of HEK 293T-Ga16gust44 cells were mainly done as before. Briefly, the cells were fixed, permeabilized, blocked, and incubated with primary antibodies overnight at +4 °C (1:2000 mouse anti-FLAG M2; 1:500 rabbit anti-HA). Treatment with secondary antibodies (1:2000 anti-rabbit Alexa488; 1:4000 anti-mouse Cy3) was done as before.

Bioluminescence of Cell Surface Proteins—To quantify the proportion of receptor proteins present at the plasma membrane in the presence or absence of RTP proteins, bioluminescence experiments were performed. HEK 293T cells were either transfected with hTAS2R16-FLAG-FLAG alone or in combination with RTP3 and RTP4, respectively. 24 h after transfection the cells were washed twice with 1× PBS buffer (pH 8.0) and subsequently placed on ice for 20 min before 0.5 mg/ml NHS-PO₄⁻ Biotin (Pierce) in 1× PBS (ice-cold) was applied and left on the cells for 30 min. The cells were washed once with ice-cold 1× PBS; 20 μl Tris-HCl (pH 8.0) to quench the reaction. After a second wash with ice-cold 1× PBS (pH 8.0), homogenization buffer (1× PBS, 1% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml pepstatin A; pH 7.2) was added, and the cells were scrapped off the plates and subsequently homogenized using a Potter-Elvehjem. After incubating for 30 min on ice, the samples were centrifuged (16,000 × g at +4 °C for 30 min) to pellet cellular debris. Next, protein contents of the samples were determined using a Bio-Rad protein assay. For affinity purification of the FLAG-tagged receptor, 10 μl of FLAG-agarose (Sigma) and 10 μl of neutravidin-agarose (Pierce), respectively, were added to 250 μg each of the whole cell protein extracts and incubated overnight (+4 °C, gentle agitation). Agarose beads were then washed five times with 500 μl of 1× PBS (pH 7.2) containing protease inhibitors. FLAG-agarose beads were treated as before (see “Coimmunoprecipitation”). Neutravidin-agarose beads were resuspended directly in SDS-PAGE loading buffer and heated to 95 °C for 5 min. Western blotting and immunodetection of FLAG-tagged receptors was performed as described for the coimmunoprecipitation procedure.

Expression Analyses—RT-PCR analyses of RTP, REEP, and hTAS2R mRNAs were essentially done as before (20). Primer sequences (5’ to 3’) were: RTP1_for, AAGCGTGAACACAGAGTAGT; RTP1_rev, GAGGAGGCTCCGAGCAC; RTP2_for, AAGCGATCCTGGAGCAGC; RTP2_rev, AGAAGGCGAGCTGAGAAG; RTP3_for, ATGGCTGGGACA-CAGAACT; RTP3_rev, CAGATGTGGTTTGGCATGCG; RTP4_for, TGGACGCTGAGTGTGGATG; RTP4_rev, AGTGGATCTGGGTCTCGACTG; hTAS2R16_for, GCTTTGCCAAATCTTCAGAATTAC; hTAS2R14_rev, CTCTAAATCTTTTGTGACCTGG; hTAS2R16_for, CTTGGAATTTTTTGAATATTT; hTAS2R16_rev, GAAGCGCGTTCTCATGCT; hTAS2R38_for, ACAGTGAATTGTTGCTGT; and hTAS2R38_rev, GTCTCTCTCAGAATT. Amplification of RPE1–6 cDNA was done using the same set of oligo-nucleotides as used for cDNA cloning.

10-μm sections of human circumvallate papillae from biopsy material were used for in situ hybridization analyses with homologous, digoxigenin-labeled RTP3 and RTP4 riboprobes. The sense and antisense probes corresponded to nucleotides 1–350 of the human RTP3 cDNA and to nucleotides 1–368 of the human RTP4 cDNA, respectively. In situ hybridization was performed mainly as described before (20), except for the duration of the RNase A digestion step, which has been shortened to 5 min.

Coimmunoprecipitation—For immunoprecipitation of hTAS2R16, the carboxyl-terminal HSV tag was replaced by an additional FLAG tag to improve affinity purification of the receptor further. HEK 293T-Ga16gust44 cells were cotransfected with hTAS2R16-FLAG-FLAG and HA-RTP3 or HA-RTP4. Approximately 24 h after transfection cells were harvested, and the proteins were extracted. Briefly, the cells were homogenized on ice in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml pepstatin A using a glass homogenizer. After 30 min of incubation on ice, the cell debris was removed by centrifugation (30 min, 16,000 × g, +4 °C). Protein concentrations of supernatants were determined by Bio-Rad protein assay, and 1 mg of each protein extract was subjected to immunoprecipitation using either anti-FLAG agarose beads or anti-HA-agarose beads (Sigma). After extensive washing with TBS, bound proteins were eluted for 30 min at 56 °C with standard SDS-PAGE loading buffer without reducing agents. Following a brief centrifugation the supernatants were transferred to fresh tubes, and dithiothreitol was added to a final concentration of 200 mM. Next, the samples were heated to 95 °C for 5 min and subjected to SDS-PAGE and Western blotting according to standard procedures. Detection of FLAG and HA epitopes was done using 1:1000 rabbit anti-FLAG/1:5000 sheep anti-rabbit conjugated to alkaline phosphatase (Sigma) and 1:1000 mouse anti-HA (Covance, Berkeley, CA) with 1:5000 goat anti-mouse conjugated to alkaline phosphatase (Sigma), respectively, in combination with standard colorimetry.

RESULTS

Functional Characterization of Recombinant hTAS2R16—To investigate the dependence of hTAS2R16 function in heterologous cells on the presence of export tags, we performed functional calcium imaging experiments comparing sst3-hTAS2R16-FLAG-hsv with hTAS2R16-FLAG-hsv. The dose-response curves obtained by stimulating receptor-transfected cells with different concentrations of the agonist salicin demonstrate the different responsiveness of both receptor variants. Compared with sst3-hTAS2R16-FLAG-hsv (Fig. 1, circles), the construct hTAS2R16-FLAG-hsv is much less sensitive (Fig. 1, triangles). However, high agonist concentrations lead to activation of hTAS2R16-FLAG-hsv, showing that the receptor is functional. The signal amplitude obtained for hTAS2R16-FLAG-hsv stimulated with 10 mM salicin is ~15-fold smaller when compared with the sst tag-containing construct (Fig. 1, inset).

Subcellular Localization of hTAS2R16—To investigate the reason for the decreased responsiveness of hTAS2R16-FLAG-hsv, we performed immunocytochemical colocalization analyses using antibodies for subcellular marker proteins in combination with anti-FLAG staining of receptor transfected HEK 293T cells (Fig. 2). Coimmunostaining of hTAS2R16-FLAG-hsv (red) with the ER-marker anti-calnexin (23) (green) (Fig. 2, A–C) shows limited colocalization. The lack of colocalization of
Identification of Factors Involved in Cell Surface Targeting of Chemoreceptors in hTAS2R-expressing Tissues—Recently, it was reported that members of the RTP and REEP gene families improve cell surface expression of ORs (17). By RT-PCR analysis of a human CV, which is a site of bitter receptor gene expression (4–6,8), we detected expression of RTP2 and RTP4 and all six REEP genes (Fig. 3A). Another tissue expressing hTAS2R genes is testis, where we analyzed for hTAS2R14, -16, and -38 (Fig. 3C). In contrast to CV, RTP1–4 and REEP1–6 followed by calcium imaging is shown in Fig. 4.

Whereas for three bitter taste receptors differences in the responses after agonist stimulation were observed upon cotransfection with RTP/REEP factors, the sweet taste receptor heteromer was not influenced, indicating an interaction with hTAS2Rs but not hTAS1Rs. Surprisingly, of the five bitter taste receptors tested, hTAS2R14 and hTAS2R43 showed robust responses when transfected alone (ΔF/F = 0.27 and 0.2, respectively), whereas hTAS2R10 and hTAS2R38 were inactive (not shown). The hTAS2R16 response is very small (ΔF/F = 0.07) corresponding well to its predominant intracellular expression (Fig. 2). Cotransfection of hTAS2R16FLAG-hsv with RTP3 and RTP4 resulted in a considerable increase in responses, whereas RTP1, which has been demonstrated to increase OR function (17), shows only a small effect. None of the six REEP constructs enhanced hTAS2R16FLAG-hsv responsiveness significantly, whereas REEP6 reduced the responses even further. Analyses of the dose–response relationships of the receptor alone or in combination with one of the RTP3, RTP4, REEP1, or REEP3 constructs revealed that signal amplitudes and EC_{50} values are affected differently by RTP and REEP proteins. Whereas the EC_{50} value of cells cotransfected with hTAS2R16FLAG-hsv and RTP3 is 1.3 ± 0.1 mM matching EC_{50} values published for the sst3-tagged receptor variant (1.4 mM) and determined in human psychophysical studies (1.1 mM) (5), the other tested combinations were less sensitive (hTAS2R16FLAG-hsv 3.1 ± 0.4 mM, plus RTP4 8.2 ± 3.5 mM, plus REEP1 10.6 ± 11 mM, plus REEP3 3.0 ± 0.6 mM). Both RTP3 and RTP4, increased the observed signal amplitudes over a wide concentration range, whereas neither REEP1 nor REEP3 showed a comparable effect. Similar, although less pronounced activating effects were observed for hTAS2R43 only. In the case of hTAS2R43 only RTP3, but not RTP4, increased responsiveness to agonist stimulation significantly. The determination of EC_{50} values for the receptor transfected alone, and together with RTP3, RTP4, REEP1, and REEP3 resulted in 0.63 ± 0.18, 1.92 ± 0.59, 0.48 ± 0.19, 1.39 ± 0.19, and 0.99 ± 0.1 μM, respectively. Although these values are quite similar to each other, they differ considerably from data obtained for the sst3-tagged receptor (8). In contrast to hTAS2R10 and hTAS2R38, hTAS2R14 also responded to cotransfection with RTP and REEP constructs. The dose-re-

** Auxiliary Factors for hTAS2R Trafficking**

**FIGURE 1.** Dose–response relationship of hTAS2R16 depends on amino-terminal residues. HEK 293T-Ga16qust44 cells were transfected with hTAS2R16 constructs and loaded with Fluo4-AM, and calcium traces were monitored before and after stimulation with different salicin concentrations. The responses of sst-hTAS2R16FLAG-hsv (circles) and hTAS2R16FLAG-hsv (triangles) are plotted as changes in fluorescence (ΔF/F). Single representative traces of sst-hTAS2R16FLAG-hsv (solid line) and hTAS2R16FLAG-hsv (dotted line) are shown in the inset.
Auxiliary Factors for hTAS2R Trafficking
Antibodies in combination with antibodies for subcellular marker proteins. FLAG immunoreactivity (FIGURE 2.

Values (hTAS2R14, 13 ± 2.3 μM; +RTP3, 18.7 ± 1.5 μM; +RTP4, 13.9 ± 2.6 μM; +REEP1, 23.9 ± 4.2 μM; +REEP3, 11.4 ± 1.7 μM) do not differ much from the sst3-tagged hTAS2R14 (4). Surprisingly, RTP2, REEP2, -4, and -6 reduce hTAS2R14 activation. As shown by RT-PCR analyses (Fig. 5) some REEP mRNAs are present in HEK 293T cells, perhaps not only explaining the apparent independence of hTAS2R14 function but also the negative effects observed for single RTP/REEP proteins by competition between endogenous and transfected factors. The sweet taste receptor heteromer hTAS2R12/3 response is not influenced by any of the RTP or REEP proteins as indicated by single-dose experiments and dose-response curves for stevioside (EC_{50} hTAS2R12/3, 0.06 ± 0.01 mM; +RTP3, 0.06 ± 0.01 mM; +RTP4, 0.07 ± 0.02 mM; +REEP1, 0.06 ± 0.01 mM; +REEP3, 0.07 ± 0.01 mM).

Functional Interaction between hTAS2R16 and RTP3/4—To better understand the mechanism by which RTP3 and RTP4 increase hTAS2R responses, we performed immunocytochemistry of HEK 293T cells transfected with HA-tagged RTP3 or RTP4 and hTAS2R16FLAG-hsv. Staining of cells cotransfected with RTP3 (green) and hTAS2R16FLAG-hsv (red) reveals a partial overlap between both proteins (Fig. 6A, panels a–c), although the distribution of RTP3 is more restricted. Similar observations can be made for cells cotransfected with RTP4 and hTAS2R16FLAG-hsv, although this time both signals appear to overlap almost completely (Fig. 6A, panels d–f). Coinmunoprecipitation experiments demonstrate that both RTP3 and RTP4 interact directly with hTAS2R16 (Fig. 6B). Interestingly, multiple bands below the expected molecular mass of ~35 kDa are observed in lanes containing extracts of hTAS2R16-transfected cells, indicating that intracellular processing of “export tag-free” hTAS2R16 occurs. The intensity of hTAS2R16-specific bands obtained by coinmunoprecipitation with HA-RTP3 (lane 11, top panel) and vice versa (lane 5, bottom panel) is reduced compared with bands obtained by receptor-RTP4 coinmunoprecipitations (lane 12, top panel, and lane 6, bottom panel). Therefore, the different degree of overlap seen for hTAS2R16 protein and RTP3 on one hand and hTAS2R16 and RTP4 on the other hand (Fig. 6A) corresponds well to the results of the coinmunoprecipitations. A change in cell surface expression of hTAS2R16 by RTP3/4 coexpression is demonstrated by cell surface biotinylation experiments (Fig. 6C). Here, the membrane-impermeable compound NHS-PEO₄-biotin was used to biotinylate cell surface proteins of cells transfected with hTAS2R16FLAG-FLAG and cells that have been cotransfected with the receptor and RTP3/4. The amount of labeled proteins was determined by affinity purification using FLAG-agarose (upper panel) and neutravidin-agarose (lower panel), respectively, followed by Western blotting to detect the FLAG-tagged hTAS2R16. Densitometry revealed RTP3 and RTP4 increased the amounts of hTAS2R16 at the cell surface by ~4-fold and ~3-fold, respectively (Fig. 6D).

**FIGURE 2.** Subcellular localization of hTAS2R16 with its native amino terminus. HEK 293T cells expressing hTAS2R16FLAG-hsv were stained with anti-FLAG antibodies in combination with antibodies for subcellular marker proteins. FLAG immunoreactivity (red) and marker proteins (green) were visualized by confocal microscopy. A–C, anti-calnexin staining of the ER (A), hTAS2R16FLAG-hsv (B), and corresponding overlay shown in C. D–F, anti-EEA1 labeling of early endosomes (D), hTAS2R16FLAG-hsv (E), and overlay (F). G–J, cis-Golgi compartment stained with anti-GM130 (G), hTAS2R16FLAG-hsv (H), overlay (I), and low magnification of overlay (J). K–N, same as G–J, but cells treated with brefeldin A to cause dissociation of Golgi-structures. O–R, trans-Golgi network stained with anti-p230 (O), hTAS2R16FLAG-hsv (P), overlay (Q), and low magnification of overlay (R). S–V, same as O–R but after brefeldin A treatment. W–Y, hTAS2R16FLAG-hsv transfected cells stained without addition of primary antibodies (negative control). The asterisks indicate colocalization of the Golgi-markers GM130 and p230, respectively, with hTAS2R16; dots indicate a lack of colocalization (U, N, R, and V). Scale bars, 20 μm except for J, N, R, and V (40 μm) and insets (7 μm).
Auxiliary Factors for hTAS2R Trafficking

Dose-response curves of hTAS2R16FLAG-hsv cotransfected with either RTP3 or RTP4 and stimulated with the different agonists salicin, helicin, and arbutin indicate that the general agonist profile of hTAS2R16FLAG-hsv is not affected by coexpression of the receptor with RTP3 and RTP4, respectively (Fig. 6E).

DISCUSSION

In the present study we identified members of the RTP and REEP gene families as cofactors for functional expression of some bitter taste receptors. To investigate whether hTAS2Rs exhibit similar problems with intracellular retention as seen for OR (14), we expressed hTAS2R16, a receptor specific for bitter β-glucopyranosides (5), without export tag in HEK 293T cells.
Auxiliary Factors for hTAS2R Trafficking

We observed a strongly reduced responsiveness to salicin (Fig. 1), and by coinmunostaining with subcellular markers, we localized the majority of receptor proteins within the trans-Golgi compartment.

Whereas the ER is important for biosynthesis, folding, and post-translational modifications of proteins, the trans-Golgi compartment is devoted to trafficking of proteins that have passed the ER quality control already (32). Therefore, recombinant hTAS2R16, which reaches the trans-Golgi network and even the plasma membrane to a limited extent as indicated by small functional responses toward salicin (Fig. 1), appears to suffer from mislocalization rather than from misfolding.

Recently, it has been demonstrated that the cell surface targeting required for functional OR expression can be improved by coexpression of RTP1, RTP2, and REEP1 (17). To analyze whether proteins of the same gene families also influence taste receptor trafficking, we did coexpression experiments using different hTAS2R constructs without export tags. Surprisingly, hTAS2R14 and hTAS2R43 showed already robust responses upon agonist stimulation when expressed alone, whereas hTAS2R16 strongly depended on coexpression with either RTP3 or RTP4. Although hTAS2R43 responsiveness seems less affected by the presence of an export tag compared with hTAS2R16, RTP3 significantly increased hTAS2R43 activation, confirming the importance of these molecules as auxiliary factors for the functional expression of some bitter taste receptors. The receptors hTAS2R10 and hTAS2R38 neither responded when expressed alone nor in coexpression experiments with RTP/REEP proteins. As shown by RT-PCR analyses (Fig. 5), some REEP mRNAs are present in HEK 293T cells, perhaps not only explaining the apparent independence of hTAS2R14 function but also negative effects observed for single RTP/REEP proteins by competition between endogenous and trans-

![Image](image_url)

**FIGURE 6.** Cellular coexpression of hTAS2R16 and human RTPs. A, HEK 293T-Ga16gust44 cells were transfected with hTAS2R16FLAG-hsv and HA-tagged RTP3 or RTP4. FLAG immunoreactivity (red) and HA immunoreactivity (green) were visualized by confocal microscopy. Cotransfection of hTAS2R16FLAG-hsv and HA-RTP3/4 followed by fixation and permeabilization of cells: panel a, RTP3; panel b, receptor; panel c, overlay; panel d, RTP4; panel e, receptor; panel f, overlay; panels g–i, negative control. Scale bar, 20 μm. B, coimmunoprecipitation of FLAG-tagged hTAS2R16 with HA-tagged RTP3 and RTP4, respectively. HEK 293T cells were either mock-transfected (lanes 1 and 7), or with HA-RTP3 (lanes 2 and 8), HA-RTP4 (lanes 3 and 9), FLAG-tagged hTAS2R16 alone (lanes 4 and 10), or in combination with HA-RTP3 (lanes 5 and 11) and HA-RTP4 (lanes 6 and 12), respectively. Cellular extracts were subjected to affinity purification using either FLAG-agarose beads (lanes 1–6) or HA-agarose beads (lanes 7–12). Following SDS-PAGE and Western blotting the purified proteins were detected with an anti-FLAG antibody (upper panel) or an anti-HA antibody (lower panel). Visualization of bands was done by secondary antibodies linked to alkaline phosphatase and 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) colorimetry. Bands corresponding to copurified hTAS2R16, RTP3, and RTP4 proteins are labeled by arrowheads. C, cell surface biotinylation of HEK 293T cells transfected with hTAS2R16FLAG-hsv alone or in combination with RTP3/4. The cells were treated with membrane impermeable NHS-PEO4-biotin, and whole cell extracts were subsequently subjected to affinity purification using FLAG-agarose (upper panel) and neutravidin-agarose (lower panel), Lane M, molecular weight standard; lane 1, mock transfected (negative control); lanes 2–4, hTAS2R16FLAG-hsv alone; lanes 5–7, hTAS2R16FLAG-hsv + RTP3; lanes 8–10, hTAS2R16FLAG-hsv + RTP4; lanes 11 and 12, hTAS2R16FLAG-hsv nonbiotinylated (negative control). D, densitometric quantification of biotinylated hTAS2R16. Areas of the four prominent bands seen in C were subjected to densitometry. After background subtraction and standardization to the corresponding lane showing total FLAG-tagged hTAS2R16 (C, upper panel), the increase in cell surface localization by RTP3/4 compared with hTAS2R16 alone was calculated and analyzed for statistical significance with Student’s t test. E, Agonist profile of hTAS2R16FLAG-hsv cotransfected with RTP3/4. HEK 293T-Ga16gust44 cells were transfected with hTAS2R16 and RTP3/4 constructs and loaded with Fluo4-AM, and calcium traces were monitored before and after stimulation with different salicin (circles), helicin (squares), and arbutin (triangles) concentrations. The responses are plotted as changes in fluorescence (ΔF/F).
Auxiliary Factors for hTAS2R Trafficking

tected factors. It appears that individual receptors rely on different cofactors for functional expression, a fact observed for other chemoreceptors as well (14, 16, 17).

Our observation that CV, testis, and HEK 293T cells express several RTP/REEP genes raises the question of whether these genes are cell type-specific factors for functional hTAS2R gene expression. Clearly, TAS2R gene expression is not confined to gustatory tissue (see Fig. 3C and Refs. 33 and 34), which implies additional functions for this gene family. Our screening of mouse and human expressed sequence tag data bases (release 082605) revealed a rather widespread expression of RTP3 (liver, lung, and testis) and especially RTP4 (amygdala, bladder (cancer), bone marrow, colon, kidney, liver, lung, lymph node, macrophages, mammary gland, melanocytes, nasopharynx, pituitary, prostate, retinal pigment epithelium, spinal cord, spleen, testis, thymus, and uterus). A similar widespread expression is found for several REEP genes. This indicates that some of these factors might serve global functions in folding/ trafficking of transmembrane proteins, thereby creating a permissive environment for receptor expression in a variety of tissues and cells. Interestingly, in situ hybridization analyses of human circumvallate papillae revealed expression of RTP3 and RTP4 within a gustatory cell population that appear to be basal cells. These precursor cells give rise to taste receptor cells, which are constantly replaced throughout the life time (35). This would indicate a situation where hTAS2R expression might occur after RTP3 and/or RTP4 are already present.

The mechanisms by which improved functional expression of chemoreceptors is achieved might be as different as the molecules identified for this process. As demonstrated for hTAS2R16 cotransfected with RTP3 and RTP4 (Fig. 6A), both factors colocalize with the receptor and improve hTAS2R16 plasma membrane localization. On the other hand, the increase in sensitivity toward agonist stimulation of cells cotransfected with hTAS2R16 and RTP3 indicates a different potency of RTP3 and RTP4 (Fig. 4). Coinmunoprecipitations of hTAS2R16 with RTP3 and RTP4, respectively, demonstrate that physical interaction between receptor and auxiliary factors occurs (Fig. 6B). This has also been observed by Saito et al. (17) for ORs coexpressed with RTP1 or REEP1 and might therefore be the general mode of action by which RTP/REEP proteins improve chemoreceptor function. Because different chemoreceptors expressed in heterologous cells display individual subcellular localizations ranging from ER retention to plasma membrane localization, the degree of overlap between the predominant expression domains of receptors and auxiliary factors might be as important for functional interaction as compatibility of their peptide sequences.

In analogy to speculations about the functional role of RTP and REEP proteins for OR trafficking (17), several main scenarios for RTP/REEP interaction with hTAS2Rs appear conceivable. 1) The identified auxiliary factors might have chaperone-like function and therefore assist in the folding of the receptors. The properly folded receptor could then be able to reach the cell surface without further contact to additional specific guidance molecules. 2) The receptor does not contain or expose adequate sorting signals. Subsequent binding of a cofactor to the receptor provides the established receptor/factor complex with the necessary signature to be routed to the plasma membrane. In fact, for some human RTP and REEP proteins putative sorting signals like the YXXΦ motif (36) are predicted by pattern recognition programs. Alternatively, binding of the cofactor could expose an existing targeting motif within the receptor or mask a retention signal. 3) The cofactor might be targeted to the same vesicular structure within which the receptor is trapped and provides a sorting signal for vesicular transport. This scenario would not require direct physical interaction between the receptor and the auxiliary factor.

In the future, a detailed study on the mechanism by which the plasma membrane localization of hTAS2Rs is regulated and on the exact roles the different identified auxiliary factors have will be necessary to understand how the function of bitter taste receptors is achieved in vivo.

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