Functional expression of the taste specific G-protein, α-gustducin

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The taste-specific G-protein α-subunit, α-gustducin, was expressed using a baculovirus based system. α-Gustducin was demonstrated to be myristoylated and was also palmitoylated in insect larval cells. Recombinant α-gustducin was purified to homogeneity. Neither receptors nor effectors that interact with gustducin in taste are known. However, α-gustducin has a close structural similarity to the visual G-protein, α-transducin. Therefore α-gustducin was reconsitituted with components of the visual system to determine the degree of its functional similarity with α-transducin. Despite the fact that the sequences of α-gustducin and α-transducin share only 80% identity with each other, the interactions and functions of these two proteins were quantitatively identical. These included the interaction with receptor, bovine rhodopsin, with effector, bovine retinal cyclic GMP-phosophodiesterase, and with bovine brain and retinal G-protein βγ-heterodimers; receptor-catalysed GDP–GTP exchange and the intrinsic GTPase activity of α-gustducin and α-transducin were also identical. $G_i\alpha$ which is 70% identical with α-transducin interacts with different receptor and effector proteins and has very different guanine-nucleotide binding properties. Therefore, the functional equivalence of α-gustducin and α-transducin suggest that taste buds are likely to contain receptor and effector proteins that share many properties with their retinal equivalents.

INTRODUCTION

Heterotrimeric G-proteins mediate the signalling from a variety of cell surface receptors to specific effector systems [1,2]. A classification of G-proteins divides G-protein α-subunits into four main classes according to their sequence similarity [2]. The largest of these groups comprises G-protein α-subunits that are pertussis toxin sensitive and related to $G_i\alpha$. As well as the different $G_i\alpha$ sub-types, this class includes $G_o\alpha$, $G_z\alpha$ and the photoreceptor-specific α-transducins. Despite their close sequence relationship, $G_i\alpha$-class G-proteins have diverse and specific functions [1,2].

Recently a new G-protein α-subunit, α-gustducin, was identified in taste receptor cells using molecular biology techniques [3]. The sequence of α-gustducin places it in this $G_i\alpha$-related class of G-proteins with 80% identity to both rod and cone α-transducins (which are also 80% identical to one another). The expression of α-gustducin mRNA appears to be limited to about 40% of the taste receptor cells. Immunocytochemistry, using sequence-specific antibodies to α-gustducin, also localizes the protein to rat and human taste receptor cells [4,5]. This specific localization of α-gustducin strongly suggests that it may mediate taste signal transduction.

Biochemical studies of signal transduction in taste have been hampered by the small quantity of material that is available: a rat has less than 10^5 taste receptor cells in its entire tongue and many of these are scattered as isolated taste buds within individual papillae. There are reports that sweet compounds (e.g. saccharin) evoke G-protein-dependent cyclic AMP responses from regions of the tongue containing taste buds [6]. However, saccharin also elicits similar responses from non-taste tissue [7]. Other studies have pointed to a G-protein-dependent rise in Ins(1,4,5)P_3 when taste cell membranes are treated with bitter compounds [8,9]. Therefore it has been suggested that α-gustducin may function in sweet and/or bitter taste sensation [3].

No functional studies of gustducin have been reported. Experiments using synthetic peptides suggest large differences between the taste-specific α-gustducin and the visual G-protein, α-transducin [10]. A 22-residue peptide, based on the sequence of transducin, activated retinal phosphodiesterase (PDE) [11]; the corresponding peptide from gustducin did not [10]. However, the studies with synthetic peptides do not fully reflect G-protein effector interactions. For example, the crystal structure of α-transducin shows that there is no conformational change in the region from which the peptide was taken, between the GDP-form (which does not activate PDE) [12] and the GTP-form (which does) [13]. Therefore we have expressed α-gustducin in *Spodoptera frugiperda* (*Sf9*) cells using baculovirus. In this report we have examined to what extent the distinct, but similar, G-protein α-subunits, retinal α-transducin and α-gustducin, resemble each other in their functions as GTP-binding and -hydrolysing proteins and, most importantly, in their interactions with receptor, effector and βγ-subunits.

EXPERIMENTAL

Construction of recombinant baculoviral α-gustducin

A cDNA encoding α-gustducin [3] was amplified by PCR using oligonucleotide primers: AACAGCTGATCATGGGAAGTG-GAATTAGTTCA and CTATAGTGATCACAAGTGGTAG-CAAACA (20 ng of plasmid template and 200 pmol of each primer in a Perkin-Elmer 9600 thermocycler: 95 °C, 270 s; then 30 cycles of 95 °C, 30 s, 50 °C, 30 s, 72 °C, 60 s; followed by 72 °C, 600 s). The amplified product was cloned in pBluescript (Stratagene) by blunt-end ligation and the sequence of the

Abbreviations used: *Sf9*, *Spodoptera frugiperda*; DTT, dithiothreitol; GTP[γS], guanosine-5′-[γ-thio]triphosphate; ROS, rod outer segments; cGMP, cyclic GMP; PDE, phosphodiesterase; AEBSF, 4-(2-aminoethyl)benzenesulphonyl fluoride.

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amplified cDNA was confirmed by sequencing both strands. The coding region of α-gustducin was sub-cloned into pBacpak for transfer to baculovirus. Construction and propagation of recombinant baculovirus was essentially as described elsewhere [14]. For expression studies, suspension cultures of *Sf*9 cells were infected at a multiplicity of infection of ~ 1 with recombinant baculovirus at a cell density of 1×10^6 cells/ml using serum-free medium (*Sf*9-II, Gibco-BRL).

Analysis of α-gustducin expression

*Sf*9 cells were harvested, washed once in PBS, resuspended in cell lysis buffer: 20 mM Tris-Cl (pH 8.0), 3 mM EGTA, 1 mM EDTA, 0.1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF), 100 μM L-1-chloro-3-tosylamido-7-amino-2-heptanone, 100 μM L-1-tosylamido-2-phenylethyl chloromethyl ketone, 2 μg/ml leupeptin and 30 μg/ml benzamide, and lysed by passage through a 25-gauge needle (three times). Centrifugation at 3000 *g* yielded a cell pellet fraction. The supernatant was centrifuged further at 100000 *g* for 1 h to obtain the soluble and membrane fractions.

Immunoblot analysis was carried out with an antiserum $G_i\alpha_{1-2}$(UBI); the antiserum was raised against the C-terminal decapeptide of $G_i\alpha_{1-2}$, which is almost identical with the C-terminal decapeptide of α-gustducin and α-transducin. In addition, an antiserum to α-transducin ($G_t\alpha$-9) was used. Detection was with horseradish peroxidase-coupled donkey anti-(rabbit secondary antibody) (Jackson Labs.) and enhanced chemiluminescence (Amersham). For biosynthetic radiolabelling, 1 ml of cells (1×10^6 cells/well) were transferred to 6-well plates containing 25 μg of cerulenin and either 100 μCi of [^{3}H]myristic acid (NEN) or 500 μCi of [^{3}H]palmitic acid (NEN) 54 h after infection. Cells were incubated in the presence of radiolabel for 8 h, washed three times with PBS and lysed in SDS/PAGE sample buffer. Cell lysates (from 1.2×10^5cells) were subjected to SDS/PAGE; gels were soaked in Autofluor (National Diagnostics), dried and autoradiographed at −80 °C.

Guanosine-5′-[γ-thio]triphosphate (GTP[γS])-binding assay

The [γ-^{35}S]GTP[γS]-binding assay was based on that described previously [15]. Rhodopsin-catalysed GTP[γS]-binding to α-transducin or α-gustducin was carried out in a 10 μl assay volume in 20 mM MOPS (pH 7.5), 3 mM $MgSO_4$, 1 mM EDTA, 3 mg/ml BSA, 1 mM dithiothreitol (DTT), 3.6 mM CHAPS and 0.1 μM [γ-^{35}S]GTP[γS]. Reaction mixtures contained 17.5 nM α-gustducin or α-transducin and varying concentrations of bleached rhodopsin in urea-washed rod outer segments (ROS). Assays were performed at 30 °C under normal laboratory illuminations for 1 h. Reactions were terminated by adding 2 ml of ice-cold wash buffer [20 mM Tris-Cl (pH 8.0), 100 mM NaCl and 25 mM $MgCl_2$]. Samples were filtered rapidly through 0.45 μm type HA nitrocellulose filters (Millipore) and were washed seven times with ice-cold wash buffer. Filters were dried for liquid scintillation counting.

All stages of α-gustducin purification were monitored by using a minor modification of the rhodopsin-catalysed GTP[γS]-binding assay. Fractions were assayed in a 50 μl volume in the presence of 1 μM rhodopsin and 0.05 μM GTP[γS] to increase sensitivity.

Purification of α-gustducin

*Sf*9 cells infected with recombinant α-gustducin baculovirus were grown for 64 h. Infected cells were harvested by centrifugation at 3000 *g* for 10 min, washed with PBS and resuspended on ice in cell lysis buffer using a glass–Teflon homogenizer. Lysed cells were centrifuged at 3000 *g* for 10 min; cell debris was washed with lysis buffer (75 ml of buffer/1×10^9 cells) and centrifuged a second time. Supernatants were combined, fortified to 20 μM $AlCl_3$, 10 mM NaF, 5 mM $MgCl_2$, 1 mM DTT, 3 mM CHAPS and centrifuged at 143000 *g* for 1 h. Glycerol (to 25%) was added to the supernatant.

Ion-exchange chromatography of α-gustducin was carried out using 50 ml of DEAE-Sephacel (Pharmacia) pre-equilibrated with 20 mM Tris-Cl, pH 8.0/1 mM EDTA/4 mM $MgCl_2$/15 mM NaCl/3 mM CHAPS/25% glycerol. The column was developed by washing with 150 ml of buffer followed by a 180 ml linear gradient from 15 to 300 mM NaCl in buffer. α-Gustducin eluted as a broad peak between 140 and 200 mM NaCl.

α-Gustducin-containing fractions were pooled, diluted with 2 vols. of 20 mM MOPS, pH 7.5/1 mM EDTA/4 mM $MgCl_2$/1 mM DTT and loaded onto a 25 ml Phenyl-Sepharose (Pharmacia) column equilibrated in the same buffer. The column was washed with 75 ml of buffer containing 0.2% Na-cholate, then with 75 ml of buffer containing 0.2% Na-cholate/400 mM NaCl. α-Gustducin was eluted with a linear gradient of 400 mM NaCl/0.2% Na-cholate to 150 mM NaCl/1% Na-cholate in buffer. α-Gustducin eluted as a peak centred at 300 mM NaCl/0.65% Na-cholate.

Peak fractions were concentrated to 2 ml (Amicon PM30 membrane) for chromatography over HR-100 Sephacryl (Pharmacia) in 20 mM MOPS, pH 8.0/1 mM EDTA/4 mM $MgCl_2$/200 mM NaCl/1.0% Na-cholate. α-Gustducin eluted as a single peak at ~ 60–50 kDa.

A βγ-affinity resin was prepared by covalently linking bovine brain βγ to ω-aminobutyl agarose (Sigma) [16]. α-Gustducin was concentrated by ultrafiltration (Amicon PM30), with exchange of buffer to 20 mM MOPS, pH 7.5/200 mM NaCl/1 mM EDTA/1 mM DTT/5 mM GDP/0.2% Lubrol PX (Sigma). α-Gustducin (2 ml) was added to 2 ml of βγ resin. The resin was rotated overnight, then poured into a column and washed with 20 ml of the same buffer. The column was brought to room temperature and α-gustducin was eluted with column buffer fortified with 30 μM $AlCl_3$/10 mM NaF/50 mM $MgCl_2$. α-Gustducin fractions were diluted in 7 vols. of 20 mM Tris-Cl, pH 8.0/1 mM EDTA/4 mM $MgCl_2$/1 mM DTT/12 mM CHAPS applied to a 0.2 ml DEAE-Sephacel column, the column was washed with three column vols. of buffer and α-gustducin was eluted with a single step of buffer containing 300 mM NaCl. The concentration of α-gustducin was determined by the microbicinchoninic acid assay (Pierce) using BSA as a standard. α-Gustducin was snap-frozen in liquid nitrogen and stored at −80 °C.

Purification of rhodopsin, α-transducin and cyclic GMP-PDE

Bovine ROS were prepared from light-adapted retina as described previously [17]. ROS were washed four times in isotonic buffer (10 mM Hepes, pH 7.5/100 mM NaCl/5 mM $MgSO_4$/0.1 mM EDTA/1 mM DTT/0.1 mM AEBSF) and three times with hypotonic buffer (10 mM Hepes, pH 7.5/0.1 mM EDTA/1 mM DTT/0.1 mM AEBSF). Cyclic GMP (cGMP)-PDE was purified from the pooled hypotonic washes by size exclusion on a 200 HR-Sephacryl (Pharmacia) column pre-equilibrated with 20 mM MOPS, pH 7.5/500 mM NaCl/1 mM EDTA/1 mM DTT. Fractions were pooled, concentrated (Amicon PM30 membranes), fortified with 50% glycerol and stored at −20 °C. The concentration of cGMP-PDE was determined from Coomassie-stained SDS/PAGE relative to βγ-transducin. Transducin was

eluted from the hypotonic washed ROS membranes using hypotonic buffer fortified with 50 μM GTP. α-Transducin and transducin βγ-subunits were resolved on ω-amino-octylamine agarose [18]. Washed ROS were stripped using urea, treated with hydroxylamine and regenerated using 9-*cis*-retinal [18]. The rhodopsin content of the stripped membranes was determined using Amido Black. The concentration of α-transducin was determined by the micro-bicinchonic acid assay (Pierce) using BSA as a standard.

GTPase assay

The GTPase activity of α-gustducin and α-transducin were measured by the release of $[^{32}P]P_i$ from [γ-^{32}P]GTP [19]. Reaction mixtures contained 3 μM rhodopsin and 17.5 nM α-gustducin or α-transducin in GTP[γS]-binding buffer in a final volume of 5 μl. Assays were initiated by adding 20 μM [γ-^{32}P]GTP and were stopped by the addition of 0.2 M perchloric acid, followed by ammonium molybdate precipitation [20]. GTP[γS]-activated α-gustducin and α-transducin were quantified by incubation (1 h, 30 °C) of the assay mixture used to determine GTPase activity with GTP[γS] instead of with GTP. Phosphate precipitates were collected by filtration on GF/C glass-fibre filters (Whatman), that were dried for liquid scintillation counting.

Activation of cGMP-PDE

Stimulation of cGMP hydrolysis by α-gustducin or α-transducin on cGMP-PDE was used to assess their interaction. α-Gustducin and α-transducin were activated as described in the GTP[γS]-binding assay using 3 μM rhodopsin (1 h incubation at 30 °C). After GTP[γS] binding was determined, rhodopsin-containing membranes were removed by centrifugation (100000 *g*, 30 min). PDE assays were carried out in a 40 μl volume of 20 mM MOPS (pH 7.5), 3 mM $MgSO_4$, 1 mM EDTA, 2.25 mg/ml BSA, 2 mM DTT, 2.7 mM CHAPS, 0.2 mg/ml snake venom (*Ophiophagus hannah*) and 2 mM cGMP. Reactions were for 15 min at 30 °C and were terminated by adding 56 μl of 28% perchloric acid. Samples were vortex mixed, centrifuged to remove precipitated protein and the phosphate was determined using ammonium molybdate [21].

Pertussis toxin-catalysed ADP-ribosylation

Pertussis toxin-catalysed ADP-ribosylation of *Sf*9 cell extracts, purified α-gustducin and α-transducin was examined under a number of conditions [15]. The extent of modification of soluble cell extracts (20 μl) and purified G-protein (5 nM) was determined with and without addition of either bovine brain or retinal βγ-dimers (0.3 μM).

Pertussis toxin was pre-activated for 20 min at 30 °C in 10 mM Tris-Cl, pH 7.5/10 mM DTT/100 mM ATP. Activated toxin was added to G-protein samples in a reaction buffer containing 20 mM Tris-Cl (pH 7.5), 5 mM DTT, 10 μM GDP, 1 mM $MgCl_2$, 10 mM thymidine, 10 mM arginine, 0.2% Na-cholate and 20 μg/ml pertussis toxin, containing 2.5 μCi [^{32}P]NAD$^+$ (final concentrations in 50 μl). The reactions were incubated at 30 °C for 30 min and were terminated by the addition of 25 μl of 30% trichloroacetic acid. Samples were resuspended in SDS/PAGE sample buffer and subjected to SDS/PAGE. Gels were fixed in 10% acetic acid for 30 min, dried and autoradiographed.

RESULTS

Characterization of expression of recombinant α-gustducin

The sequence similarity of α-gustducin and α-transducin suggested that rhodopsin might be able to stimulate GTP[γS] binding to α-gustducin. Therefore, cellular fractions of *Sf*9 cells, infected with several different recombinant viruses, were tested by assaying rhodopsin-catalysed GTP[γS] binding. Binding was observed in the soluble fraction of *Sf*9 cells that had been infected with virus containing the coding sequence of α-gustducin under the control of the polyhedrin promoter. This binding indicated that approximately 1.7 nmol of functional α-gustducin/10^9 cells were expressed. No significant binding was observed to the cell pellet fraction of cells infected with recombinant α-gustducin, or in any cellular fractions from infections with β1γ2 or Golfα viruses (Figure 1a). Cell pellets and membranes from α-gustducin-infected cells solubilized with 12 mM CHAPS or with 1% cholate showed no rhodopsin-catalysed GTP[γS] binding (results not shown). Combined infections with viruses containing the coding sequences of α-

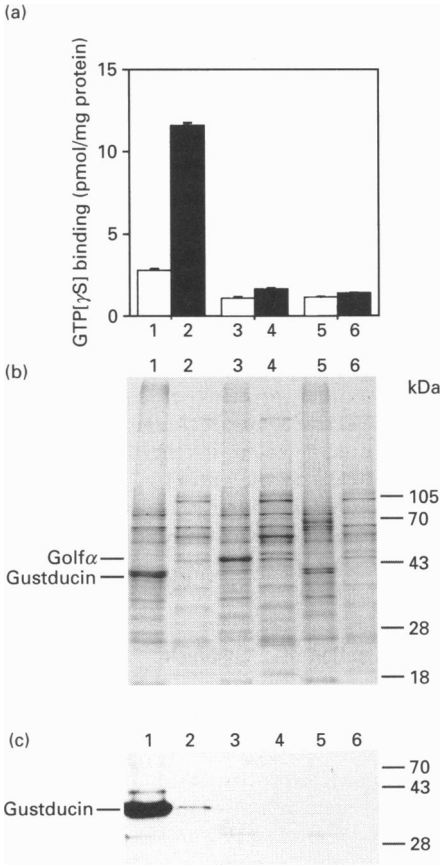

Figure 1 Expression of α-gustducin in *Sf*9 cells

Cellular extracts of *Sf*9 cells infected with recombinant α-gustducin (lanes 1 and 2), Golfα (lanes 3 and 4) and β1γ2 (lanes 5 and 6) viruses were analysed. (**a**) GTP[γS]-binding assays were performed on soluble cell extracts using 0.05 μM [γ-^{35}S]GTP[γS] for 1 h at 30 °C in the presence of 1 μM rhodopsin (shaded) or without rhodopsin (open). Determinations are the means of duplicate measurements. Cell pellet from 6×10^4 cells (lanes, 1, 3 and 5) and soluble fractions from 3.5×10^4 cells (lanes 2, 4 and 6) were separated on SDS/PAGE; (**b**) stained with Coomassie Blue; (**c**) immunoblotted with anti-$G_i\alpha_{1-2}$ antibody (UBI). The positions of α-gustducin, Golfα and molecular mass standards (kDa) are indicated.

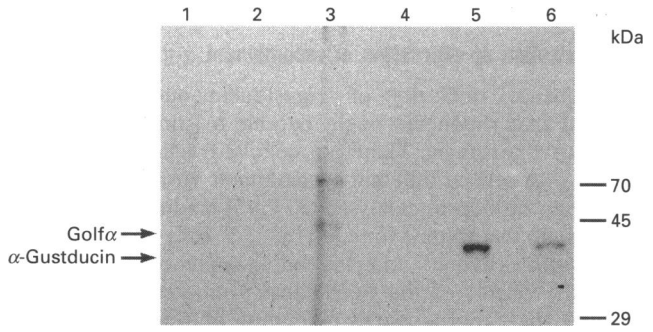

Figure 2 Biosynthetic acylation of *Sf*9 cells infected with recombinant α-gustducin, Golfα and lacZ baculoviruses

Cultures of *Sf*9 cells were infected with recombinant lacZ (lanes 1 and 2), Golfα (lanes 3 and 4) and α-gustducin (lanes 5 and 6) viruses and 54 h later either 500 μCi/ml of [^{3}H]palmitic acid (lanes 1, 3 and 5) or 100 μCi/ml of [^{3}H]myristic acid (lanes 2, 4 and 6) in the presence of 25 mg/ml cerulenin was added to the culture medium for 8 h. Cells were lysed in sample buffer, protein was resolved by SDS/PAGE and gels subsequently visualized by fluorography (7 day exposure). The positions of α-gustducin, Golfα and molecular mass standards (kDa) are indicated.

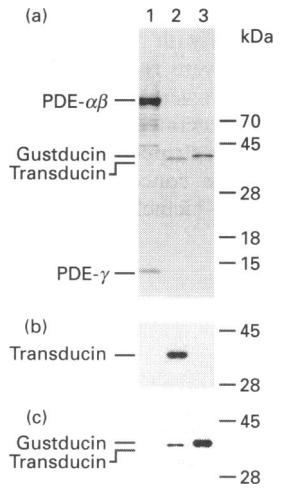

Figure 3 SDS/PAGE of purified α-gustducin, α-transducin and cGMP-PDE

Purified ROS cGMP-PDE (lane 1), α-transducin (lane 2) and recombinant α-gustducin (lane 3) were subjected to SDS/PAGE and: (**a**) stained with Coomassie Blue or (**b** and **c**) immunoblotted. Immunoblots were probed either with (**b**) a polyclonal anti-α-transducin antiserum [19] or (**c**) a polyclonal anti-$G_i\alpha_{1-2}$ decapeptide antibody (UBI). Positions of α-gustducin, α-transducin, cGMP-PDE and molecular mass standards (kDa) are indicated.

gustducin, the G-protein β1-subunit and the G-protein γ2-subunit under the control of the polyhedrin promotor, failed to produce larger quantities of functional soluble α-gustducin (results not shown).

Small-scale experiments showed that a major 36 kDa protein was present in the cell pellet of cells infected with recombinant α-gustducin virus (Figure 1b). Its expression was dependent on the infection of cells with α-gustducin recombinant baculovirus and reached a maximum 60–72 h post-infection. This protein was not found in the cellular fractions of cells infected with Golfα or β1γ2 viruses and was detected by a polyclonal anti-$G_i\alpha_{1-2}$ antibody (Figure 1c). Together these data indicate that the 36 kDa protein was probably non-functional α-gustducin. In the soluble fractions, other proteins of similar size were more abundant than the active α-gustducin. However, a low level of a 36 kDa protein was detected in the soluble fraction of α-gustducin-infected cells by the anti-$G_i\alpha_{1-2}$ antibody (Figure 1c). The amount of soluble gustducin was estimated to be less than 1% of the total from a comparison of its GTP[γS] binding with the amount of insoluble $G_i\alpha_{1-2}$-gustducin detected by SDS/PAGE.

Biosynthetic modification of α-gustducin

The biosynthetic acylation of α-gustducin in *Sf*9 cells was investigated because the N-terminus of $G_i\alpha_{1-2}$-gustducin has a consensus site for myristoylation and because palmitic acid modification of many G-protein α-subunits has been found. α-Gustducin virus-specific myristoylation and palmitoylation of a 36 kDa protein was detected (Figure 2). In contrast, a 42 kDa protein was labelled only by palmitic acid in Golfα-infected cells. This indicates that α-gustducin can be modified by both myristate and palmitate whereas Golfα is only palmitoylated in *Sf*9 cells. The modification of α-gustducin by palmitate was not sensitive to treatment of the gel with 1 M hydroxylamine at pH 7.5 (results not shown).

Purification of α-gustducin

A four-step procedure was employed to purify α-gustducin from large batch cultures. Scanning of the SDS/PAGE gel shown in Figure 3 indicated that α-gustducin was more than 80% pure. A typical purification from 2×10^9 infected *Sf*9 cells yielded 2 μg of α-gustducin (Table 1). Equal quantities of α-gustducin and α-transducin (determined by protein assay) gave identical GTP[γS] binding, showing that the recombinant α-gustducin had the same specific activity as purified bovine α-transducin. SDS/PAGE showed that the purified α-gustducin migrated with an apparent molecular mass slightly greater than that of α-transducin (Figure 3a). Purified α-gustducin and α-transducin were equally strongly detected by the anti-$G_i\alpha_{1-2}$ C-terminal decapeptide antiserum (Figure 3c). The sequence of the C-terminal decapeptide against which the antiserum was raised differs at only one residue from that of α-transducin and α-gustducin. However, a polyclonal antiserum raised against purified α-transducin, detected α-gustducin only very weakly (Figure 3b). Presumably, the principal epitopes that this antibody reacts with in α-transducin are not found in α-gustducin.

Rhodopsin-catalysed GTP[γS] binding of α-gustducin

The binding of GTP[γS] to purified α-gustducin was determined as a function of rhodopsin concentration and this was compared with its binding to α-transducin (Figure 4). Half-maximal binding of GTP[γS] to both these G-protein [γS]-subunits was achieved by a rhodopsin concentration of 200–300 nM. This indicates that rhodopsin is equally efficient at catalysing the exchange of GDP with GTP[γS] for α-gustducin and α-transducin. However, in the absence of receptor, the level of GTP[γS] binding to α-gustducin was substantially greater than that observed with α-transducin. Approx. 15% of the maximum binding was attained in the absence of rhodopsin for α-gustducin, whereas less than 1% of maximal GTP[γS] binding to α-transducin was measured in the absence of rhodopsin.

GTPase activity of α-gustducin

No basal GTPase activity was detectable for either α-gustducin or α-transducin in the absence of rhodopsin. This demonstrates

Table 1 Summary of purification of α-gustducin from lysates of *Sf9* cells infected with recombinant baculovirus

	Total protein* (mg)	Specific rhodopsin dependent GTP[γS] binding† (nmol/mg)	Yield‡ (%)	Purification factor‡
143000 *g* supernatant	316	0.011	100	–
DEAE-Sephacel	225	0.013	84	1.2
Phenyl-Sepharose	15	0.107	54	8.2
Sephacryl S-100 HR	1.65	0.133	14	1.2
βγ-Agarose	0.0032	14.3	21	107
			Total 1.3	Total 1300

*Total protein was estimated by the micro-bicinchoninic acid assay (Pierce) using BSA as a standard.
† GTP[γS] binding was determined for an aliquot of the pooled fraction using 1 μM rhodopsin and 0.05 μM GTP[γS]; the reaction was incubated for 1 h at 30 °C.
‡ For each stage of purification; the total is for the whole purification.

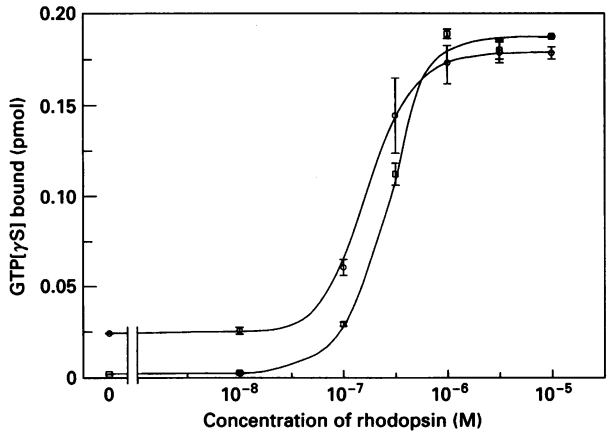

Figure 4 Rhodopsin-dependence of GTP[γS] binding to α-gustducin and α-transducin

α-Gustducin (○) or α-transducin (□) (75 nM) were incubated with rhodopsin in buffer containing 0.1 μM [γ-^{35}S]GTP[γS] for 60 min at 30 °C. The data presented are the mean ± variation between duplicate determinations. Experiments were repeated twice. Non-specific binding was determined without G-protein present and was subtracted for each condition.

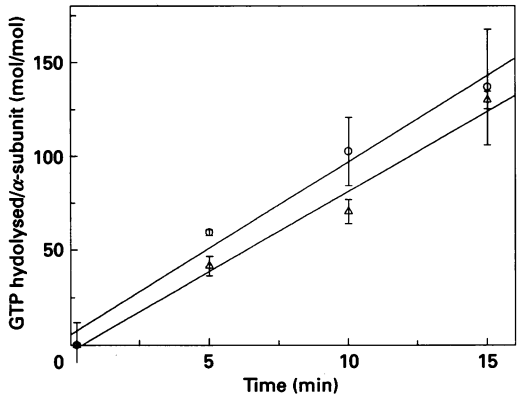

Figure 5 GTPase activity of α-gustducin and α-transducin

Time-course of the hydrolysis of GTP by α-gustducin (○) or α-transducin (△). Reaction mixtures containing 75 nM α-gustducin or α-transducin, and 3 μM rhodopsin were mixed with 20 μM [γ-^{32}P]GTP at 25 °C at time zero. The reactions were quenched in 0.2 M perchloric acid and P_i was assayed by ammonium molybdate precipitation followed by radioactive counting. The data presented are the mean ± variation of duplicate determinations. Experiments were repeated twice.

that a receptor is absolutely required to catalyse the exchange of GDP for GTP on α-gustducin as well as on α-transducin. In the presence of a saturating concentration of rhodopsin, identical rates of GTP hydrolysis were measured for α-gustducin and α-transducin (Figure 5). The concentration of active G-protein was determined in parallel experiments which measured GTP[γS] binding. The turnover number was calculated to be approx. 9 min^{-1} for both α-gustducin and α-transducin.

Activation of cGMP-PDE by α-gustducin

The activation of ROS cGMP-PDE by α-gustducin or α-transducin was examined at a low concentration (1.3 nM) of cGMP-PDE. This is important because purified cGMP-PDE contains two inhibitory subunits, both of which must bind activated α-transducin for full activation [22]. When only one of the inhibitory γ-subunits is bound to α-transducin, the protein is only approx. 10% active [23]. The purified ROS cGMP-PDE contained no contaminating α-transducin, as assessed by immunoreactivity (Figure 3) and also by the absence of any GTP-dependent, rhodopsin-catalysed activity, in the absence of added G-protein α-subunit (results not shown).

Neither α-gustducin nor α-transducin could stimulate cGMP-PDE in the absence of GTP[γS] or rhodopsin (results not shown). However, titration of GTP[γS]-activated α-gustducin or α-transducin stimulated the activity of cGMP-PDE (Figure 6). The concentration dependence of PDE activation was virtually identical for both of these G-protein α-subunits. The complex shape of the concentration dependence is a function of the need to dissociate both PDE γ-subunits to achieve full activation and is very similar to previously published results [23]. This indicates that α-gustducin is as efficient at releasing the inhibitory effects of the cGMP-PDE γ-subunit on the catalytic αβ-subunits as is α-transducin.

Pertussis toxin ADP-ribosylation as a function of interaction with βγ-dimers

Soluble extracts from *Sf9* cells infected with recombinant α-gustducin contained a 36 kDa protein that was a substrate for pertussis toxin-catalysed ADP-ribosylation. In the absence of

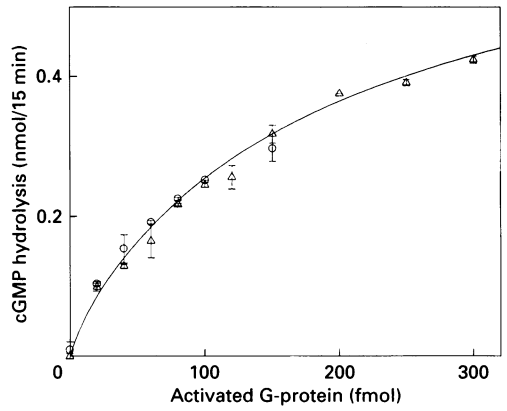

Figure 6 Activation of ROS cGMP-PDE by α-gustducin and α-transducin

cGMP-PDE was mixed with the indicated amounts of GTP[γS]-activated α-gustducin (○) or α-transducin (△) and incubated for 15 min at 30 °C. Phosphate determination was by ammonium molybdate precipitation (mean ± variation of duplicate determinations). Basal activity of cGMP-PDE was subtracted. Basal and G-protein-activated cGMP-PDE activity was linear with time (studied up to 30 min). Experiments were repeated twice.

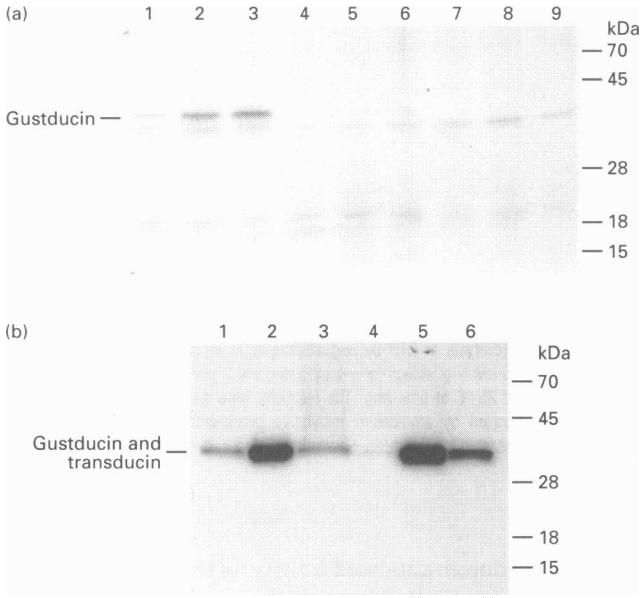

Figure 7 Pertussis toxin-catalysed ADP-ribosylation analysis of βγ-dimer interaction

Pertussis toxin-catalysed ADP-ribosylation of (**a**) soluble cellular extracts of 3×10^4 *Sf9* cells infected with recombinant α-gustducin (lanes 1–3), Golfα virus (lanes 4–6) and β1γ2 (lanes 7–9) were incubated in the presence of pertussis toxin and [^{32}P]NAD$^+$ for 30 min at 30 °C: lanes 1, 4 and 7, no βγ-heterodimer; lanes 2, 5 and 8, 0.2 μM brain βγ; and lanes 3, 6 and 9, 0.2 μM ROS βγ. Proteins were fractionated by SDS/PAGE and visualized by autoradiography. The positions of α-gustducin and molecular mass standards (kDa) are indicated. (**b**) Pertussis toxin-catalysed ADP-ribosylation of recombinant α-gustducin (lanes 1–3) and bovine retinal α-transducin (lanes 4–6) (5 nM each): lanes 1 and 4, no βγ-heterodimer; lanes 2 and 5, 0.2 μM brain βγ; and lanes 3 and 6, 0.2 μM ROS βγ. Proteins were fractionated by SDS/PAGE and visualized by autoradiography. The positions of α-gustducin, α-transducin and molecular mass standards (kDa) are indicated.

βγ-dimers, labelling was inefficient. However, ADP-ribosylation of this protein was facilitated by the presence of either bovine brain or retinal βγ-dimers (Figure 7a). No ADP-ribosylation of G-protein α-subunits was observed in soluble lysates from cells infected with either Golfα or β1γ2 recombinant viruses.

Purified α-gustducin and α-transducin were both good substrates for pertussis toxin-catalysed ADP-ribosylation (Figure 7b). The modification of both proteins was greatly stimulated by the presence of brain βγ-heterodimers. Retinal βγ-heterodimers (mainly β1γ1 from ROS) appeared somewhat less active at facilitating ribosylation. In addition it was noted that α-gustducin was more strongly modified in the absence of βγ-heterodimers than was its visual counterpart α-transducin.

DISCUSSION

Comparison of the sequences of the taste-specific α-gustducin and α-transducin (from vision) suggested that these two G-proteins might share functional properties [3]. However, recent studies of synthetic peptides derived from regions of sequence believed to mediate effector activation [10,11] suggest considerable functional differences. Moreover, $G_i\alpha$, though 70% identical with transducin, couples different receptors and effectors and has different guanine-nucleotide binding properties [1,2,24]. Visual transduction has been studied in detail but no information is available about the signalling pathway mediated by α-gustducin in taste. Therefore functional comparison of α-gustducin and α-transducin could show how closely the receptor(s) and effector(s) from taste cells that interact with α-gustducin resemble those from vision. To carry out these studies, we expressed α-gustducin in *Sf9*-cells and purified recombinant protein.

Purification of functional G-protein α-subunits expressed in heterologous systems has not always been straightforward. Recently, the expression of a number of G-protein α-subunits has been achieved using baculovirus [25–31]. For several of these, co-expression of Gβγ has been required, and even then the yields have been low [28,31]. However, to date, the only expression and purification of functional α-transducin reported [30] used baculovirus. The N-terminus of α-transducin was modified to remove the myristoylation site. The mutated α-transducin could be purified in large amounts (100 μg/10^9 cells) but had lower affinity for rhodopsin and retinal βγ-heterodimer-containing membranes and stimulated retinal cGMP-PDE to a lower level than did native α-transducin [30]. Recombinant baculovirus containing the coding sequence of unmodified α-gustducin under the control of the polyhedrin promoter produced a large quantity of α-gustducin. Only a very small proportion of the expressed protein was in a functional form. Co-expression of Gβγ is necessary to allow purification of G-proteins of the G_q-subfamily [28,31], but did not help in increasing the expression of functional α-gustducin. The amount of purified, active α-gustducin obtained was similar to that reported for other G-proteins [31], but was only 1.3% of the functional α-gustducin in the soluble cellular extract (Table 1).

Covalent modification of G-protein α-subunits by myristic acid has been reported to be critical for membrane association of a number of $G_i\alpha$-related G-proteins [32,33]. We were interested in whether the consensus myristoylation site present at the N-terminus of α-gustducin was modified in the protein produced in *Sf9* cells. To study this, cells were cultured with radiolabelled myristate in the presence of the inhibitor of fatty acid synthesis, cerulenin [34]. α-Gustducin-specific labelling of a 36 kDa protein indicated that at least a fraction of the α-gustducin was modified. More surprising was the finding that α-gustducin was also palmitoylated. Several other G-protein α-subunits have recently been shown to be palmitoylated *in vivo* [35–37]. This modification has also been reported to be linked to the activation state of the G-protein [38,39]. However, in all cases where G-protein palmi-

toylation has been reported, the modified residue appears to be a conserved cysteine that is close to the N-terminus. In rod α-transducin this cysteine is replaced by an alanine, whereas in α-gustducin and cone α-transducin it is a serine and in α-gustducin there are no cysteines in the first 65 residues. Therefore it is possible that Ser-3 in α-gustducin is a potential site for palmitoylation. Consistent with this was the stability to hydroxylamine of the fatty acid labelling. It has also been reported that rod α-transducin is not myristoylated *in vivo* [40,41]. Therefore, it appears that a major difference between α-transducin and α-gustducin may be their fatty acylation and therefore, their relative hydrophobicities.

The final stage in purification of α-gustducin was adsorption to βγ-agarose and elution with aluminium fluoride in the presence of magnesium. This indicated that the purified α-gustducin possessed several of the functional properties of other G-proteins. However, in order to make a direct comparison of α-gustducin with α-transducin, quantitative determination of their interaction with a receptor, bovine rhodopsin, and an effector, bovine retinal cGMP-PDE, were measured. In addition, the intrinsic GTPase activity of these two proteins was assayed as was their susceptibility to, and the βγ-dependence of, pertussis toxin-catalysed ADP-ribosylation. In all respects the proteins were almost identical, except that the *Sf9* cell purified α-gustducin showed higher intrinsic GTP[γS] binding than did α-transducin. This difference most probably results from the difference in the method of purification of the two G-protein α-subunits. The extensive chromatography, in the absence of guanine nucleotides, that α-gustducin was subjected to may have resulted in purification of a fraction of α-gustducin with an empty nucleotide-binding site. This fraction of α-gustducin would bind GTP[γS] rapidly in the absence of rhodopsin. However, once occupied by the hydrolysable GTP molecule, this population of α-gustducin would be indistinguishable from α-gustducin with an occupied site. Consequently, no rhodopsin-independent GTPase activity was measured.

The interaction of α-gustducin with rhodopsin (Figure 4) was measured only in the absence of βγ-heterodimers and was compared with equivalent measurements of α-transducin. It has been shown that βγ-heterodimers reduce the concentration of rhodopsin needed to activate α-transducin [18] and that various βγ-heterodimers differ in their efficacy [15]. However, at saturating rhodopsin concentration, the extent of GTP[γS] binding is approximately equal for heterotrimeric transducin and α-transducin. Similarly, the rate of GTPase activity (Figure 5) was assessed in the absence of βγ-heterodimers. Again, at saturating concentrations of rhodopsin, the GTPase activity of α-transducin has been shown to be limited by the release of phosphate from α-transducin rather than by the rate of α-transducin activation by rhodopsin [19]. The rate that we determined for the GTPase activity of α-transducin was very similar to previous estimates [19]. Therefore these measurements, like the stimulation of cGMP-PDE activity and the interaction of α-gustducin with different βγ-subunits, are direct comparisons of distinct properties of α-gustducin with those of α-transducin.

The functional identity of α-gustducin and α-transducin is surprising given that these two proteins share only 80 % sequence identity and that whereas α-transducin functions in vision, α-gustducin is found only in taste receptor cells [3]. It is therefore tempting to speculate that there is considerable similarity in signal transduction between vision and taste. This is all the more attractive because α-gustducin and the rod and cone α-transducins are all 80 % identical with each other. Rod and cone α-transducins are activated by distinct but closely related receptors and as a result regulate distinct but related cGMP-PDEs. The receptor that activates α-gustducin is unlikely to be a light-activated rhodopsin, but we would predict close similarity to rhodopsin in its cytoplasmic domain which interacts with α-gustducin. Similarly the effector enzyme in taste is likely to contain a domain related to the very basic retinal PDE γ-subunit which interacts with α-transducin.

The identical stimulation of retinal PDE activity by α-gustducin and α-transducin is at odds with the conclusions of a recent study that used synthetic peptides to examine the functional properties of α-gustducin [10]. That study was based on the observation that a 22-amino acid peptide from rod α-transducin had been reported to be sufficient to mimic it in activation of bovine retinal cGMP-PDE [11]. The corresponding peptides from cone α-transducin or from α-gustducin contain several differences in sequence, and in contrast to the rod α-transducin-derived peptide do not stimulate cGMP-PDE [10]. However, this peptide sequence is located in a region that does not change in conformation between the GDP- and GTP-bound structures of α-transducin [12,13]. Thus the identical stimulation of cGMP-PDE that we observe with GTP[γS]-activated α-gustducin and α-transducin (Figure 6) is particularly notable. Almost full activation of 1.3 nM retinal cGMP-PDE was achieved by 5 nM purified α-transducin (Figure 6). However, maximal stimulation of 10 nM cGMP-PDE by the rod α-transducin-derived peptide required more than a 1000-fold molar excess of peptide [10,11]. Therefore it is likely that the mechanisms by which cGMP-PDE is activated by peptides and by α-transducin or α-gustducin are different. It is also premature to conclude that the region of α-transducin sequence that was suggested to interact with, and activate, GMP-PDE [11] actually plays this role.

The functional properties that we have determined for α-gustducin distinguish it from the G-proteins that are not in the α-transducin subclass. For example, $G_i\alpha$, $G_o\alpha$ and $G_s\alpha$ all exchange GTP for GDP and therefore have detectable GTPase activity in the absence of a receptor [24,42,43], whereas α-gustducin requires an activated receptor for this guanine-nucleotide exchange. Similarly G-proteins from other subclasses are not efficiently activated by rhodopsin, and none has been reported to activate retinal cGMP-PDE, let alone with a concentration dependence indistinguishable from that of α-transducin. The converse is also true, α-transducin is not activated by G-protein-linked receptors other than rhodopsin [44]. Whereas the members of the α-transducin subclass share 80 % identity with one another, they also share 70 % identity with $G_i\alpha$. These few additional sequence differences must therefore account for the extensive functional differences that distinguish α-transducin (and its functionally identical homologue, α-gustducin) from $G_i\alpha$.

In summary, we have described an extensive comparison of recombinant α-gustducin with bovine retinal α-transducin that demonstrates that not only are these two proteins structurally similar but that they appear to be functionally interchangeable. This surprising result strongly suggests considerable similarity between taste and visual signalling and in combination with peptide studies [10] raises questions as to which regions of G-proteins interact with effector enzymes.

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