Biochimica et Biophysica Acta 1828 (2013) 535–542

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

The melanocortin 4 receptor: Oligomer formation, interaction sites and functional significance

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ARTICLE INFO

Article history:
Received 2 May 2012
Received in revised form 14 September 2012
Accepted 12 October 2012
Available online 23 October 2012

Keywords:
Melanocortin 4 receptor
Oligomerization
Trans-activation
GPCR

ABSTRACT

This study involves the structural and functional properties of the recombinant melanocortin 4 receptor (MC_4R) expressed in the HEK-293 cell line. Using co-immuno-purification approaches, the receptor appears to be an oligomer, which can be crosslinked through disulphide bonds involving a native cysteine residue (84) to give a dimeric species. This position is located near the cytosolic region of transmembrane segment 2 and it is suggested that this is an interacting interface between MC_4R monomers. Using co-expression of the native protein and a C84A mutant, it appears that the receptor also forms higher order oligomers via alternative interfaces. Interestingly, disulphide crosslink formation does not occur if the receptor is uncoupled from its G-protein, even though the oligomeric state is preserved. This suggests that the conformational changes, which occur on activation, affect the TM2 interface. The pharmacology of the agonist, NDP-MSH, indicates that the MC_4R retains high affinity for the ligand in the absence of the G-protein but occupancy for the ligand is increased. The data can be interpreted to suggest that the G-protein exerts a negative allosteric effect on the receptor. Co-expression of one receptor lacking the ability to signal with another, which cannot bind the agonist, restored ligand-dependent activation of the G-protein to situations in which neither receptor on its own could activate the G-protein. Such transactivation suggests meaningful cross talk between the receptor subunits in the oligomeric complex. These studies demonstrate further unique features of the MC_4R.

1. Introduction

The MC_4R belongs to Family A of the G-protein coupled receptors (GPCRs) and is conventionally activated by peptide agonists inducing coupling to $G\alpha_s$, although not exclusively [1]. The receptor is located primarily in the brain and shown to have an important regulatory role in energy homeostasis and food intake [2–4]. It is, therefore, a potential therapeutic target for a variety of conditions including anorexia and obesity [5]. Understanding its functionality may be the key to successfully exploiting the receptor as a druggable target. MC_4R possesses unique structural features not seen in other Family A members. These include some highly conserved key residues in TM regions which have been substituted including; $Pro^{4.59,\ 5.50}$ residues at TM4 and TM5 respectively and the $Cys^{3.25}$ residue located in the extracellular portion of TM3 which generally forms a disulphide bond to a Cys residue in ECL2. The Family A conserved $Cys^{3.25}$ residue is substituted in the MC_4R by $Asp^{3.25}$ (Asp122) which plays a key role in interacting with the agonist peptides [6–8]. The atypically small ECL2 lacks the second conserved Cys residue in the disulphide bond, the Asp189 in its place also being critical for agonist binding [6–8].

The MC_4R endogenous agonists are the melanocortin peptide which contains a conserved tetrapeptide sequence His-Phe-Arg-Trp- [9,10] and the pharmacological tool, NDP-MSH, developed from the endogenous peptide α-MSH, whereby the essential Phe^7 in the tetrapeptide sequence is replaced with D-Phe^7 to give an agonist of increased potency [9,10].

Not only is MC_4R structurally disparate from other Family A GPCRs but the receptor is also unusual in that it also has a native antagonist, the 132 residue agouti related protein (AgRP) which is expressed in the neurones of the arcuate nucleus of the hypothalamus [11]. It has been shown to lower constitutive levels of cAMP [12–14].

It is well reported, though still not universally accepted, that many GPCRs appear to form homo- and hetero-dimers. The research emphasis now has shifted to the structural characteristics and functional significance of oligomeric states. Several roles for dimerization have been suggested but there are no consistent commonalities amongst families [15,16]. Conventionally, it is thought that one receptor sub-unit promotes GDP-GTP exchange on one G-protein. However, it has been proposed that one receptor molecule may not be sterically able to provide simultaneous interaction with all the G-protein

Abbreviations: ACTH, Adrenocorticotropic hormone; AgRP, Agouti-related protein; CRE-Luc cells, cAMP response element luciferase cells; CuP, copper 1–10 phenanthroline; ECL, Extracellular loop; Gα, G-protein α-subunit; G-protein, guanine nucleotide-binding regulatory protein; GPCR, G-protein-coupled receptor; Gpp(NH)p, guanosine-5′-(βγ-imino)triphosphate; GTPγS, Guanosine 5′-[γ-thio]triphosphate; HA epitope, human influenza haemagglutinin; MC_4R, Melanocortin 4 receptor; NDP-MSH, [Nle^4, D-Phe^7] α-MSH; TM, transmembrane segment; POMC, proopiomelanocortin

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http://dx.doi.org/10.1016/j.bbamem.2012.10.011

subunits (both Gα and Gβγ) [17]. This raises the possibility of trans-activation between subunits in a complex and the potential for discrete signaling pathways [18,19].

Several TM domain interfaces have been predicted for Family A receptors, including TM4 and TM5 in rhodopsin [20,21] and in the D_2 dopamine receptor [22–25]. Further to this, by employing a bioinformatics and molecular modeling approach to current available data, Simpson et al., have implicated TM4 to have a key role in dimerization across family A GPCR's [26]. Cysteine mutagenesis and cross-linking studies with the D_2 dopamine receptor have discriminated between the "active" R* state (agonist bound) conformation [24] represented by a TM4 only interface and the "inactive" R state (inverse agonist bound) conformation, demonstrating TM4 and possible TM5 involvement [20,21]. In rhodopsin, through disulphide bond formation at helix 8, Cys316 was implicated as a contact site in the homo-dimer [27]. Recent crystal structure of the dimeric μ-opioid receptor bound to the antagonist morpinan, has shown TM6-TM5 and TM1-TM2-H8 as potential interface regions [28]. Finally, a recent exploration of ligand bias has re-examined the possibility of oligomerization and multiple conformation involvement in G-protein directed signaling [18].

There is evidence from enzyme linked immunosorbent assays and fluorescence energy transfer studies [29] to suggest that MC_4R assembles as a homo-dimer, but the sites of interaction and the functional significance are slightly understood [29,30]. Additionally, binding kinetics for the agonist, NDP-MSH, has suggested a tandemly-arranged binding site across the homo-dimer [31,32]. Dominant negative mutations of the MC_4R including D90N and S136P have been used to model interaction between dimers that stabilize the TM helical arrangement. These data suggest that TM2, TM3 and TM7 are important in the dimeric complex [33] and raise the possibility of multi-activated states and differential G-protein coupling which result in biased agonistic action [34].

Here, we characterize the oligomeric form of the MC_4R, including the identification of one of its contact sites, the presence of higher order oligomeric species and the novel involvement of the G-protein in conformational rearrangements. We have also explored co-operative behavior between the receptor and the G-protein and between individual protein subunits.

2. Materials and methods

2.1. Materials

The expression vector, pcDNA3, containing cDNA for the human wild type (WT)-MC_4R was provided by Dr. Sharon C. Cheetham (BASF Pharma, Nottingham). The pcDNA3.1 Hygromycin© vector was purchased from Invitrogen (Paisley, UK). [^{125}I] NDP-MSH and [^{125}I] SHU9119 were obtained from Perkin Elmer Life Sciences (Boston, MA, USA). Non-radioactive peptides were purchased from Bachem (St. Helens, UK), cell culture reagents from Invitrogen (Paisley, UK) and protease inhibitor cocktail tablets from Roche (Lewes.UK). Oligonucleotide primers were synthesized by Sigma-Genosys Ltd. (Pamisford, UK).

2.2. Amino-terminal tagging

The HA (YPYDVPDYA) and c-Myc (EQKLISERDL) N-terminally tagged WT-MC_4R was generated using the polymerase chain reaction (PCR). The sequence contained a *Hind* III restriction site. A *Bam*H I restriction site was incorporated into the 3′-carboxyl terminus primer at the stop codon. The constructs were sub-cloned into pcDNA3 vector and pcDNA3.1 Hygromycin©.

2.3. Mutagenesis

Point mutations were introduced into the WT-MC_4R sequence using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) with the MC_4R-pcDNA3 vector and c-Myc-MC_4R-pcDNA3.1 Hygromycin© as template.

2.4. HEK-293 cell transfection

HEK-293 cells were passaged 24 h prior to transfection, and seeded at 70% confluency. On the day of transfection, 6.5 μg/ml of DNA was added to serum-free DMEM with 2% of Plus reagent (Invitrogen) and 1.25% of Lipofectamine™ (Invitrogen) according to the manufacturer's instructions. The reaction was terminated by the replacement of the media with complete DMEM. Two days after transfection, cells were harvested using 0.02% EDTA in PBS, and pelleted by centrifugation at 4000 rpm for 5 min (Hettich, Rotofix 32, swingout rotor). The cell pellets were washed twice with PBS (Dulbecco's) and re-suspended in an appropriate solution according to the investigation to be carried out. Alternatively, cells were harvested for propagation of stable transformants in the appropriate selection media, depending on single or dual transfection. If dual transfection occurred, both pcDNA3 and pcDNA3.1 Hygromycin© were utilized simultaneously during DNA/Lipofectamine™ complex formation.

2.5. Cell growth

HEK-293 cells were routinely maintained in DMEM medium supplemented with 10% fetal calf serum, 100 μg/ml penicillin, 100 μg/ml streptomycin and Glutamax™ (Invitrogen). Stably transfected cells were grown in the same medium supplemented with 800 μg/ml Geneticin G418 sulfate or/and 200 μg/ml Hygromycin. Cells were grown at 37 °C in 5% CO_2.

2.6. Membrane preparation

Cell membranes were prepared from cells grown as a monolayer to confluency. The growth medium was removed and the cells were washed three times with 10 ml cold PBS. The cells were then treated with 0.02% EDTA solution for 15 min, harvested, and centrifuged at 5000 rpm for 5 min. The cell pellet was re-suspended in assay buffer (25 mM HEPES-KOH, 1.5 mM $CaCl_2$ 1 mM $MgSO_4$ 100 mM NaCl, and pH 7.0 plus protease inhibitors), and homogenized using a teflon/glass homogenizer. The homogenate was then centrifuged for 10 min at 31000 rpm, at 4 °C. The supernatant was discarded and the pellet was re-homogenized in assay buffer and centrifuged as before. Finally, the supernatant discarded the pellets homogenized in 25 mM HEPES-KOH (pH 7.0), containing 10% sucrose and the membranes frozen at −70 °C.

2.7. Cross-linking

Membranes (50 μg of protein) were diluted to a concentration of 25 μg/ml with binding buffer (25 mM HEPES-KOH, 1.5 mM $CaCl_2$, 1 mM $MgSO_4$, 100 mM NaCl, and pH 7.0 plus protease inhibitors). Cross-linking was induced using 5 mM copper 1–10 phenanthroline (CuP) at 22 °C for 1 h. The reaction was terminated by addition of 5 times SDS sample buffer (60 mM Tris–HCl, pH 6.8, 2% SDS, and 10% glycerol) containing 25 mM N-ethyl maleimide (NEM) to react with any free sulfhydryl groups.

2.8. Co-immuno-purification

1000 μg of CuP treated membranes expressing HA- and c-Myc tagged WT-MC_4R were incubated with 3:1 ratio of immunopurification buffer (25 mM HEPES-KOH, 1.5 mM $CaCl_2$, 1 mM $MgSO_4$, 100 mM NaCl, 1% Triton-X, and pH 7.0 plus protease inhibitors). 100 μl of Anti-c-Myc protein G-conjugated beads was added overnight at 22 °C, after which, the beads were washed with 0.5 ml of immunopurification buffer, and centrifuged (12,000 *g*) for 2 min. The supernatant was discarded and the wash step was repeated 10 times. Finally

the Protein G bound complex was incubated with 25 μl of SDS-PAGE sample buffer (62.5 mM Tris/HCl pH 6.8, 2% SDS, 5% sucrose and 0.009% bromo blue) without β-mercaptoethanol, for 30 min at 37 °C. The samples were centrifuged and the supernatants were collected. This was repeated twice and the supernatants were pooled. The samples were resolved by 12.5% SDS-PAGE, and Western blotting was carried out with antibodies to the HA epitope.

2.9. Immunoblotting

Proteins were resolved on a 12.5% SDS-PAGE gel and electroblotted on to polyvinylidinedifluoride (PVDF) membranes (Fluoro Trans^r). The membrane was blocked for 1 h at room temperature with Super Block in PBS (Roche Molecular Biochemicals). The MC_4R was identified using anti-HA-HRP (Sigma) to the N-terminal tag at a dilution of 1:1000, and antibody AB5134 (Chemicon international) was raised to the rat MC_4R at a 1:1000 dilution in antibody buffer (PBS plus 10% BSA). Immuno-reactivity was determined with anti-rabbit horseradish peroxidase-conjugated antibody (Amersham Bioscience) in antibody dilution buffer (1:5000) by Chemiluminescence (Roche Molecular Biochemicals).

2.10. Radio-ligand binding assays

Homologous competition binding assays were performed by incubating membranes (5–50 μg) in binding buffer, in triplicate with (40,000–60,000 cpm) 0.2 nM of [^{125}I] NDP-MSH or [^{125}I] SHU9119 (2000–6000 cpm counts for total binding respectively). Several known concentrations of equivalent ligand were allowed to compete for the radio-ligand in a volume of 100 μl. The mixture was incubated for 45 min at room temperature. The reaction was stopped by filtration through 0.5% polyethyleneimine and 1 mg/ml BSA pre-treated filters and washed with 200 μl ice-cold PBS. Non-specific binding was measured using 1 μM SHU9119.

2.11. Cyclic AMP response (CRE)-luciferase assay

24 h post-transfection, HEK-293 CRE-luc cells were plated into 96 well blackwall, clear bottom, and poly-D-lysine coated microplates (Corning Costar) at a density of 50,000 cells/well and cultured for a further 12–18 h. Media were replaced with 100 μl phenol-red-free culture medium including IBMX and the appropriate concentration of agonist NDP-MSH. [35]. The microtitre plates were incubated for 4 h at 37 °C after which Luclite® reagent (Perkin Elmer) was added to each well, the plates were sealed and subjected to dark adaptation for 5 min at room temperature. Luciferase activity was determined by luminescence using NOVAstar (BMG).

2.12. Data analysis

All measurements, except where stated otherwise, were carried out in triplicate in three independent experiments. The values quoted and depicted graphically are the means of the independent determinations with the corresponding SEM. In competition binding studies, counts were normalized to the maximum and non-specific binding within each data set and the pIC_{50} values were calculated with a single site-binding model, fitted with the aid of GraphPad PRISM 3.0 software (San Diego, CA). The pK_d values were obtained from the IC_{50} using the following equation $K_d = IC_{50} - [\text{radio-ligand}]$ [36] and $pK_d = -\log[K_d]$. In homologous binding assays, B_{max} values were determined using $B_{max} = B_0{}^*IC_{50}{}^*[L^{-1}]$ [37]. The significance of differences between values was determined by comparing mean values using two tailed unpaired or paired Student's *t* test.

3. Results

3.1. Identification of a wild type MC_4R homodimer

Membranes prepared from HEK-293 cells stably transfected with both HA and c-Myc tagged forms of the cloned human WT-MC_4R, were isolated, solubilized in mild detergent and subjected to immuno-purification using immobilized anti-c-Myc antibody. The bead-retained fraction was solubilized with SDS and subjected to PAGE, followed by Western blotting using an anti-HA antibody. This revealed a clear band migrating with a mass equivalent to 36 kDa (Fig. 1), identical to that seen using antibody to the wild-type receptor or to the c-Myc tag. The same result was obtained when membranes were incubated with 1 mM GTPγS (binds irreversibly to the G-protein, uncoupling it from the receptor) plus 0.5 mM NDP-MSH prior to solubilization and immuno-purification (Fig. 1). This suggests that MC_4R is a stable dimer, in the plasma membrane with and without the G-protein or the ligand.

Solubilization and mixing of membranes from cell-lines expressing either HA-WT-MC_4R or c-Myc-WT-MC_4R, prior to immuno-purification and Western blotting as above, demonstrated that no aggregation of the HA- with the c-Myc-tagged protein occurred during solubilization and incubation (Fig. 1), reinforcing the formation of stable dimers in the intact cell.

Further controls included immuno-purification of the dimeric form of the receptor under different detergent conditions (n-dodecyl-beta-D-maltoside and *n*-octylglucoside) to exclude any possibility of receptor co-location in triton-resistant lipid domains.

Homologous binding studies using either the agonist [^{125}I] NDP-MSH or antagonist [^{125}I] SHU9119 with cell lines stably expressing either the tagged or untagged WT-MC_4R indicated that addition of the tags had no effect on the functional properties of the receptor (Table 1).

3.2. Cross-linking the dimeric MC_4R complex

Membranes prepared from HEK-293 cells stably expressing the WT-MC_4R were incubated for 1 h at room temperature without and with 5 mM CuP (Fig. 2), washed, solubilized in mild detergent and subjected to SDS-PAGE under reducing and non-reducing conditions. Subsequent Western blotting with a polyclonal antibody to the MC_4R

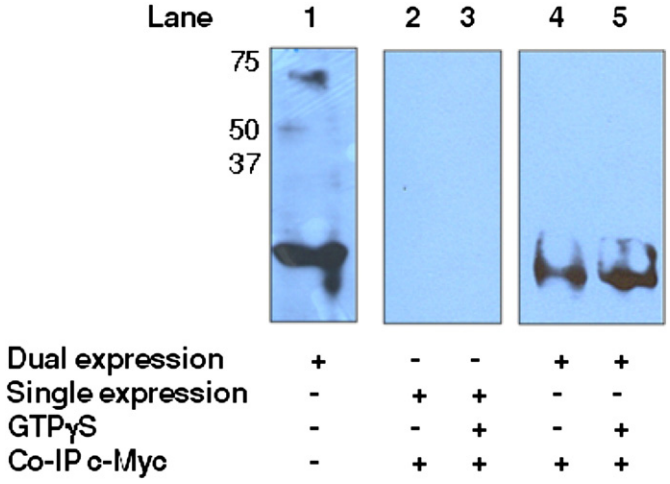

Fig. 1. Immuno-precipitation of the human WT-MC_4R oligomer in the presence and absence of the Gα-protein. The human WT-MC_4R was tagged with either the HA or the c-Myc epitope at the N-terminus. Both forms were stably expressed together in HEK-293 cells (lanes 1, and 4 and 5). Immuno-purification was performed on the dual expressed membranes using anti-c-Myc conjugated Protein A beads. The purified proteins were resolved on SDS-PAGE and subjected to Western blotting with anti-HA-HRP to identify the co-purifying HA-WT-MC_4R (36 kDa) that was indicative of homo-dimer formation between c-Myc-WT-MC_4R and HA-WT-MC_4R (lanes 1, 4 and 5). Addition of 1 mM GTPγS (plus agonist) to uncouple the Gα-protein did not affect dimer formation (lane 5).

Table 1
Antagonist and agonist binding properties of the MC4 receptor. Homologous competition binding studies were performed using [^{125}I] labeled and unlabeled forms of SHU9119 or NDP-MSH. The number of independent experiments is shown in brackets. Data are expressed as pK_d ± S.E.M. or B_{max} ± S.E.M. (fmol/mg of protein). Significant differences were evaluated using the student *t* test and comparisons made between WT-MC$_4$R vs WT-MC$_4$R plus GTPγS, or c-Myc-WT-MC$_4$R vs c-Myc-C84A-MC$_4$R for both [^{125}I] NDP-MSH and [^{125}I] SHU9119 binding. WT = Wild type, HA and c-Myc are the tagged versions and C84A a mutant. N/A indicates no activation, * indicates $p<0.05$ using Student *t* test.

MC$_4$R mutation/assay condition	[^{125}I] SHU9119 binding		[^{125}I] NDP-MSH binding	
	pK_d	B_{max} (fmol/mg of protein)	pK_d (nM)	B_{max} (fmol/mg of protein)
WT	8.32 ± 0.01 (3)	1452 ± 384 (3)	7.98 ± 0.01 (8)	746.0 ± 43.0 (8)
WT(1 mM GTPγS)	8.21 ± 0.27 (7)	1725 ± 90.0 (7)	7.44 ± 0.11 (8)	3532 ± 688* (8)
c-Myc-WT	8.60 ± 0.10 (3)	1699 ± 277 (3)	7.53 ± 0.23 (3)	1234 ± 241 (3)
HA- and c-Myc-WT	N/A	N/A	7.64 ± 0.16 (3)	475.0 ± 34.0 (3)
c-Myc-C84A	8.54 ± 0.28 (3)	1001 ± 145	7.25 ± 0.24 (2)	843.0 ± 33.0 (3)

(Chemicon, AB5236) revealed a band at 70 kDa, in those samples treated with CuP and run under non-reducing conditions. In the absence of CuP only the 36 kDa monomeric receptor is seen (the 40 kDa species is non-specific labeling seen also in the non-transfected cells). The oligomer dissociated under reducing condition confirming the formation of a disulphide bond. Disulphide bond formation occurred in a time-dependent manner and was essentially complete within 30 min with 5 mM CuP at 4 °C.

3.3. Identification of residues involved in cross-linking

There are 15 Cys residues in MC$_4$R. Site-directed mutagenesis was used to introduce single Ala mutations at all these positions (Table 2). A double mutant (C318A, C319A) was also produced. All the mutants except C84A-MC$_4$R retained the ability to form a disulphide bond (Fig. 3). Cys84 resides near the cytoplasmic surface of TM2. No cross-linking was observed on treatment of whole cells with CuP confirming that this residue is only responsive to oxidizing conditions in membrane preparations.

The affinity of the agonist for c-Myc-C84A-MC$_4$R in a stable HEK-293 cell line (pK_d = 7.25 ± 0.24) was the same (*$p<0.05$, unpaired *t* test) as that of c-Myc-WT-MC$_4$R in stable HEK-293 cell line (pK_d = 7.53 ± 0.23). Full characterization of the mutants with Cys residues predicted to be located in the binding pocket is found in Cox et al. (2006).

3.4. Conformation of the cross-linked homodimer and identification of higher order oligomers

Further co-immuno-purification studies were carried out using membranes from cells expressing both c-Myc-WT-MC$_4$R and HA-WT-MC$_4$R. Following treatment with 5 mM CuP, the dimeric species at 70 kDa can be confirmed as a homo-meric complex, purifying with anti-c-Myc and blotting with anti-HA (Fig. 4).

When similar immuno-purification studies were performed on membranes expressing HA-WT-MC$_4$R with c-Myc-C84A-MC$_4$R and subjected to CuP treatment, followed by SDS-PAGE in the absence of reducing agent, the covalent dimeric band was seen following purification via the c-Myc epitope and blotting with the anti-HA antibody (Fig. 4). Since the C84A mutant cannot form covalent dimers (Fig. 3), this implies that cross-linked complex is the HA-WT-MC$_4$R and must have become associated *in-situ* with the c-Myc-C84A-MC$_4$R mutant to enable co-immuno-purification with the anti-cMyc antibody, thereby providing evidence for higher order oligomerization.

When immuno-purification via the c-Myc epitope was performed with both the c-Myc-WT-MC$_4$R/HA-WT-MC$_4$R and c-Myc-C84A-MC$_4$R/HA-WT-MC$_4$R dual expressed membranes in the absence of CuP, the 36 kDa band blotted with the anti-HA antibody in both cases, indicative of a stable complex in mild detergent.

3.5. The significance of uncoupling the G-protein

The nucleotide GTPγS is known to bind to the Gα sub-unit, uncoupling the G-protein from the receptor [38]. Membranes were incubated at room temperature for 30 min with GTPγS at various concentrations from 100 nM to 1 mM in the presence of 0.5 mM NDP-MSH. Western blot analysis (Fig. 5) indicated that 1 mM GTPγS after 30 min completely prevented covalent dimerization on subsequent treatment with CuP.

Immuno-purification studies with membranes treated with 1 mM of GTPγS, demonstrated that although the receptor could no longer be covalently cross-linked, the dimer still existed (Fig. 1). The Gα_s-protein purifies with the dimeric form of the receptor but not in the presence of

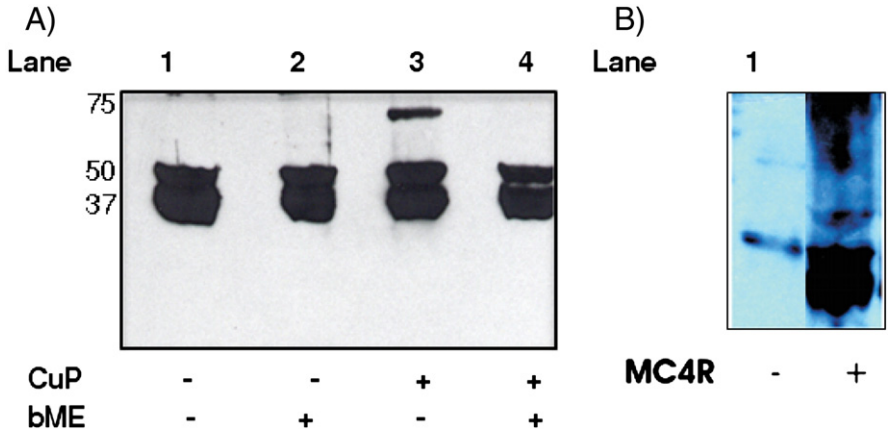

Fig. 2. Western blotting of cross-linked human WT-MC$_4$R. (Panel A) Membrane samples prepared from HEK-293 cells expressing the human WT-MC$_4$R were incubated with 5 mM copper 1–10 phenanthroline (CuP) for 1 h at room temperature. The samples were resolved on 12.5% SDS-PAGE, and detected using a polyclonal antibody (Chemicon AB5134) to the MC$_4$R. Untreated membranes revealed the monomeric MC$_4$R, with a MW of 36 kDa (lane 1) and a non-specific band at 45 kDa. Incubation with CuP caused the MC$_4$R to dimerise (lane 3), via a covalent bond, which was reduced by the addition of βME (lane 4). Untreated membranes were also subjected to βME to reduce any internal disulphide bonds (lane 2). (Panel B) It demonstrates that the band at 40 kDa is present when HEK-293 cells are not transfected with MC4R, and therefore is non-specific. The experiment was repeated on at 4 independent occasions. Molecular mass is indicated as kDa.

Table 2
Table of all 15 Cys residues located on the MC_4R.

Cys residue on receptor	Position
40	N-terminus
84	TM2
130	TM3
138	TM3
172	TM4
177	TM4
196	TM5
256	TM6
271	ECLIII
277	ECLIII
279	ECLIII
293	TM7
318	C-terminus
319	C-terminus
326	C-terminus

1 mM GTPγS (data not shown). The level of $G\alpha_s$-protein association to the MC_4R is reduced dose dependently with GTPγS (Fig. 6). The two condition-covalent crosslinking and G-protein association are inversely correlated and presumably reflect a subtle conformational difference, affecting this region of the receptor.

In further studies to examine the concentration-dependent effect of GTPγS on $G\alpha_s$ coupling, membranes (expressing both HA-WT-MC_4R and cMyc-WT-MC_4R) were treated with increasing concentration of GTPγS (1 μM–1 mM) for 30 min and the receptor immuno-purified using the c-Myc epitope. Western blotting of $G\alpha_s$ and the HA epitope of the MC_4R, demonstrated a concentration-dependent decrease in Gα association with the MC_4R in the presence of 1 μM to 1 mM GTPγS, but there was no change in the levels of the MC_4R on immuno-purification (Fig. 6).

Analysis of the affinity of the receptor for both the agonist and antagonist in the presence of 1 mM GTPγS indicated that the pK_d for SHU9119 was unaltered (8.32 vs. 8.21, without and with 1 mM GTPγS respectively) (Fig. 7a), while the pK_d for the agonist was shifted slightly to the right, (7.98 ± 0.01 to 7.44 ± 0.11 for NDP-MSH without and with 1 mM GTPγS respectively). This is validated by historic data where only a 3-fold change in the K_d occurred in the presence of 30 μM GTPγS [6,39].

Further experiments were carried out at increasing concentrations of GTPγS with 0.2 nM [^{125}I] NDP-MSH (Fig. 7b) or [^{125}I] SHU9119. At high concentrations of GTPγS, specific binding of both ligands increased (Fig. 7b). From 1 μM to 1 mM GTPγS, the percentage specific-binding roughly doubled for the agonist. Interestingly, this increase correlated with inhibition of cross-linking of the receptor in the presence of GTPγS as assessed by Western blotting and densitometry. The pEC_{50} for GTPγS on NDP-MSH binding was 4.22 ± 0.21 μM, which is not significantly different (*p < 0.05, paired *t* test) to the effect on the percentage cross-linking inhibition (increase of monomer formation), which was 4.14 ± 0.23.

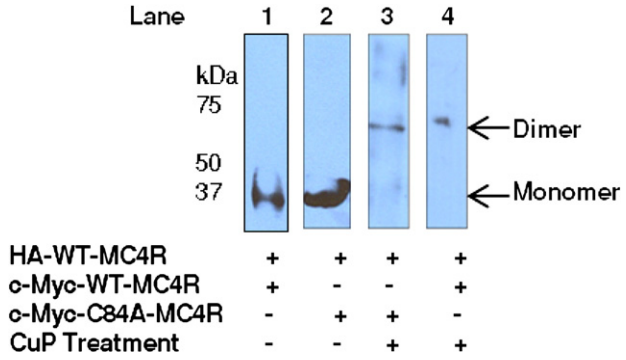

Fig. 4. Identification of higher order oligomers of the MC_4 receptor. Versions of the human WT-MC_4R containing either an HA or a c-Myc epitope on the N-terminus were stably expressed together in the same cells. Membranes isolated from this cell line were treated with and without CuP for 1 h at room temperature. After immuno-purification using the c-Myc epitope, the protein was subjected to SDS-PAGE followed by Western blotting with anti-HA-HRP antibody. In the absence of CuP (lane 1), a band was detected at 36 kDa (as seen in Fig. 1), depicting subunits from the homo oligomer. In the presence of CuP (lane 3), a band was detected at 70 kDa representing a cross-linked species between c-Myc-WT-MC_4R and the HA-WT-MC_4R. This experiment was repeated with membranes stably expressing HA-WT-MC_4R and c-Myc-C84A-MC_4R. A dimeric species was seen with membranes incubated with CuP (lane 4) but only the monomer in the absence of CuP (lane 2) Cys^{84} has been shown to be responsible for CuP induced crosslink, suggesting that high order oligomers were occurring. Experiments were repeated in triplicate. Molecular mass is denoted in kDa.

Additionally, the B_{max} values calculated from competition curves without and with 1 mM GTPγS for NDP-MSH were 746 ± 43 fmol/mg and 3532 ± 688 fmol/mg*, respectively and for SHU9119 1452 ± 384 fmol/mg and 1725 ± 89.8 fmol/mg, respectively. The B_{max} for the agonist NDP-MSH, increased significantly ~4 fold (*p < 0.05, unpaired *t* test), which is similar to the increase in specific binding of NDP-MSH in the presence of the equivalent concentration of GTPγS (Fig. 7b). Other nucleotides used in the same concentrations also induced an effect on the level of B_{max}, for example Gpp(NH)p by a similar level to GTPγS, GTP to a lesser extent, and ATP and GDP less still.

3.6. Trans-activation within the MC_4R oligomeric complex

In order to examine the possibility of trans-activation, a series of experiments were carried out utilizing receptors lacking either the ability to activate or to interact with the agonist NDP-MSH. The first receptor characterized was c-Myc-C318A-MC_4R, bearing a mutation at a potential palmitoylation site located on the C-terminal region of the receptor. This mutant is interesting because it cannot be activated (Table 3) but the binding affinity of this receptor for the agonist NDP-MSH was only slightly shifted to the right by approximately ~3.5-fold compared to the c-Myc-WT-MC_4R (pK_d = 7.05 ± 0.35 vs 7.60 ± 0.19) while the pK_d for the antagonist SHU9119 was unaltered. This profile is reminiscent to the uncoupled state of the WT-MC_4R (Table 1). The second groups of receptors lack completely or have a significant reduction in their

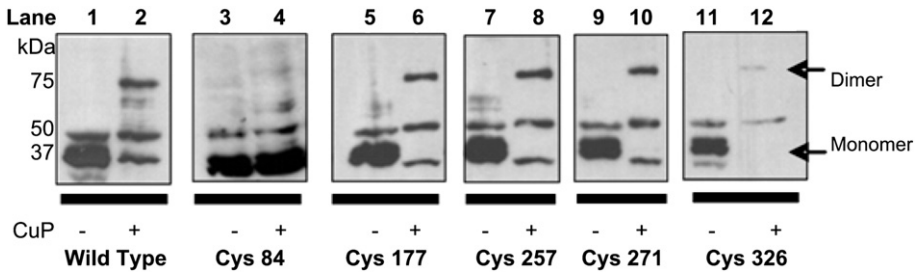

Fig. 3. Identification of the Cys residue responsible for cross-linking the wild type MC_4R in the presence of CuP. Cys residues were mutated in turn to alanine, and stably expressed in HEK-293 cells. Membranes were prepared for each mutant and treated with and without 5 mM copper1-10-phenanthroline (CuP) at 22 °C for 1 h. Western blotting was performed using the antibody to the MC_4R (Chemicon). Molecular mass is denoted in kDa. The mutant c-Myc-C84A-MC_4R did not form a disulphide bond. The experiment was repeated on 3 occasions.

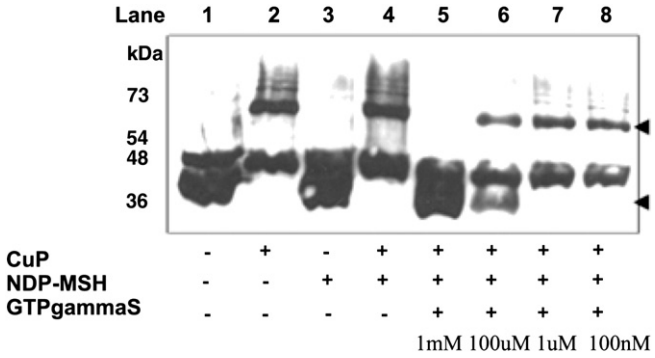

Fig. 5. Western blotting of the cross-linked MC_4R dimer after pre-treatment with GTPγS. Membrane samples prepared from HEK-293 cells expressing the human WT-MC_4R were incubated with 1 mM, 100 μM, 10 μM, and 100 nM of GTPγS (lanes 4–7 respectively) in the presence of 0.5 mM NDP-MSH for 30 min. The samples were then treated with 5 mM copper-1-10 phenanthroline (CuP) prior to SDS-PAGE. Western blotting with the polyclonal antibody to the MC_4R (Chemicon AB5134) revealed monomeric MC_4R (36 kDa) (lanes 1and 3) and dimeric MC_4R (70 kDa) in the presence of CuP alone (lane 2) and CuP plus NDP MSH (lane 4). Pre-treatment of membranes with GTPγS inhibited the formation of the dimeric complex. The experiment was repeated on three separate occasions.

ability to bind the agonist. They include the mutations D126C and M292C (binding not detected in competition studies). NDP-MSH potency was also significantly reduced (D126C; $pEC_{50} = 4.57 \pm 0.05$, M292C; pEC_{50} = No activation). When the mutant c-Myc-C318-MC_4R was transiently expressed in HEK-293 cell lines stably expressing the D126C-MC_4R mutant, the pEC_{50} for NDP-MSH was measured as 8.30 ± 0.07 similar to WT potency. Similarly, when c-Myc-C318-MC_4R was transiently expressed in M292C-MC_4R HEK-293 cell lines, most of the WT-MC_4R NDP-MSH like activation was dramatically restored ($pEC_{50} = 6.72 \pm 0.29$). Ligand binding by membranes from cells expressing the D126C/C318A and M292C/C318A combinations was not significantly different from C318A/WT membranes ($pK_d = 7.52 \pm 0.07$, 7.54 ± 0.04 and 7.53 ± 0.12 respectively), illustrating that the dysfunctional-binding receptors were not perturbing the ligand binding characteristics of the C318 mutant. Whatever the detailed molecular mechanism of this effect, it is clear that cross-talk occurs between the two-receptor monomers which have effectively retained their ability to assume the R* state.

4. Discussion

The work presented here, shown through Western blotting, immuno-purification, cross-linking techniques and agonist activity studies, demonstrates the existence of an MC_4R homo-dimer in cells stably expressing the receptor. Homo-dimer formation is consistent with

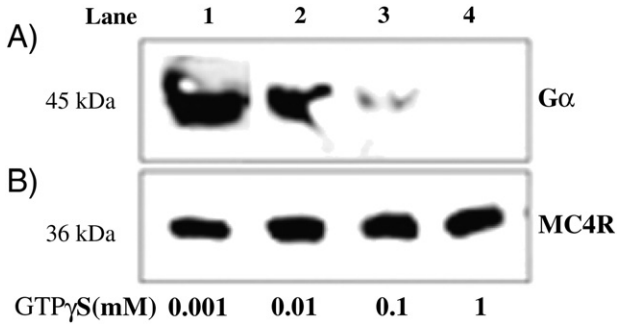

Fig. 6. The effect of GTPγS on G-protein coupling to the human WT- MC_4R. (Panel A) Membrane samples prepared from dual expressed HA and c-Myc-WT-MC_4R HEK-293 cells were incubated with 0.001 mM–1 mM of GTPγS for 30 min. The samples were then solubilized and immuno-purified using the c-Myc epitope. The G-protein was detected using a polyclonal antibody against Gαs (Santa Cruz, Sc-823). (Panel B) The HA-MC_4R was detected using an antibody to the HA epitope (Sigma) from the IP samples to standardize for protein levels.

sandwich enzyme-linked immunosorbent assays and fluorescence energy transfer studies [29,30]. The dimer can be covalently cross-linked though disulphide bond formation under oxidizing conditions. Mutagenesis studies indicate that only the wild type residue Cys84, in near proximity to the cytoplasmic surface of TM2, undergoes cross-linking. The implication of this is that the homo-dimer has an interface in or very near TM2, albeit that Cys84 may not be intimately critical to the interaction since mutation to alanine does not perturb dimer formation. However, the two cysteine residues are sufficiently close that cross-links form in an oxidizing environment without the intervention of a cross-bridge. The –S–S-cross-linked form of the WT-MC_4R immunopurified with the C84A-MC_4R variant suggesting that an oligomeric complex was formed between the WT-MC_4R dimer and the C84A-MC_4R mutant. It is most unlikely that the WT-MC_4R would cross-link preferentially with another –SH on the C84A-MC_4R since this would imply considerable conformational re-arrangements in a dimer species, which is very stable even when the Cys84 residues are replaced with an alanine. Moreover, we detected no evidence that there was any change in the functional characteristics of the mixed species or any cross-linking with the C84A mutant alone as might be expected if conformational changes were occurring. Therefore, the data suggest these dimers can also form higher order oligomers, presumably involving a different interface such as those identified in other GPCR dimeric complexes. In the case of rhodopsin and the D_2 dopamine receptor, the dimer interface is thought to be located on TM4 [21,24–26] or H8 [27], however a recent crystal structure of the opioid receptor has elucidated that TM5–TM6 is important [28].

Both the WT-MC_4R and the TM2 mutant C84A-MC_4R were active and capable of interaction with the G-protein (Fig. 5); illustrated through co-immuno-purification studies. However, cross-linking through this Cysteine residue was completely abolished by incubation with 1 mM GTPγS (agonist stimulated) at room temperature (and in a dose dependent manner), which uncoupled the Gα-protein from the receptor in a similar dose effect (Fig. 7). However, when the receptor is devoid of the G-protein, it still remains oligomeric as demonstrated through Co-IP (Fig. 1). This indicates that the receptor is a constitutive dimer, but the conformation of the interaction site (and potentially of other regions in the whole protein) alters when coupled with the G-protein. It is not yet clear if this dynamic process involves changes in higher levels of oligomerization but the functional consequences are related to the occupancy of the oligomeric complex.

In the coupled form (G-protein associated), MC_4R exists as a dimer (TM2:TM2). When in the uncoupled state, the receptor remains a dimer (with a somewhat lower affinity for the agonist but not the antagonist). Using two methods of quantifying occupancy (competition binding [^{125}I] NDP-MSH binding in the presence and absence of NDP-MSH to elucidate B_{max} or total binding of [^{125}I] NDP-MSH when GTPγS is titrated), it is clear that when the receptor is in an uncoupled state, the B_{max} values are increased compared to the coupled state. This suggests that when in the G-protein coupled form, the receptor appears to exhibit behavior akin to negative co-operativity. Perhaps the agonist binds to only one of the monomers with high affinity. Kopachuk and colleges, through kinetic studies, have suggested that MC_4R binds NDP-MSH with two tandemly arranged, interconnected, mutually dependent binding sites [31,32]. Additionally, only one of the NDP-MSH units dissociates from the dimer; the other has a very slow off rate. Here we have shown that MC_4R, when in the G-protein bound state, exhibits occupancy for NDP-MSH lower than when unbound.

Western blotting demonstrated that the level of G-protein association with the receptor corresponds to the levels of binding and cross-linking. Additionally, the same concentrations of other nucleotides, including GTP, GDP and ATP bring about a similar, though less pronounced effect as GTPγS, implying a clear G-protein-mediated effect. It is possible that the G-protein is rather tightly associated with the receptor in the endogenous situation and that the coupled and uncoupled conformations have similar affinity values. Negative co-operativity between the G-protein and

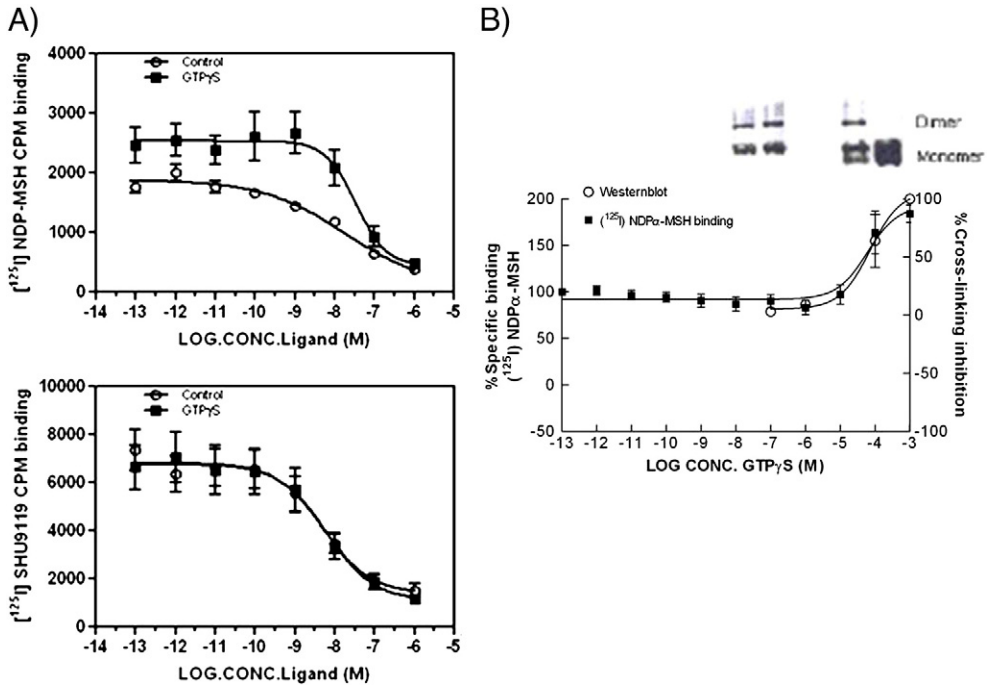

Fig. 7. The effect of GTPγS on agonist and antagonist binding and on the cross-linked conformation of the MC_4R. (Panel A) Homologous competition analysis of WT-MC_4R treated with and without 1 mM GTPγS. [^{125}I] NDP-MSH (0.2 nM; upper) and [^{125}I] SHU9119 (lower) binding was competed with the unlabeled homologous ligand. The data are representative of at least 3 independent experiments performed in triplicate and analyzed in triplicate. (Panel B) Competition studies of [^{125}I] NDP-MSH binding using increasing concentrations of GTPγS (■); left axis. Western blot densitometry of the percentage cross-linking inhibited by increasing concentrations of GTPγS, is shown by (○) taken from Fig. 6; right axis. The EC_{50} values for GTPγS on [^{125}I] NDP-MSH binding, and percentage inhibition of cross-linking were 4.22 ± 0.21 μM and 4.12 ± 0.23 μM respectively. ($p<0.05$, paired t test, $n=4$).

the receptor would allow more subtle modulation of the receptor. When the dimer changes conformation to the uncoupled state (as demonstrated by the cross-linking results), the B_{max} values for both the agonist and the antagonist increase. Whether this is as simple as negative co-operativity within the dimeric complex or related to a more extended oligomeric array will require further pharmacological investigation.

The native, non-activated state of the receptor is in a conformation that facilitates cross-link formation. When activated, the resulting conformation state (associated with uncoupling) is not able to cross-link, presumably due to an altered positioning of Cys84. Thus, it appears that the MC_4R protein is in a dynamic and sensitive state as illustrated by its structural and functional characteristics and by the presence of both a native agonist and inverse agonist.

Our studies show that mutation of C318A, located in the C-terminal region, resulted in a loss of the ability to increase cAMP production, but the binding of the agonist as assessed using competition studies was unaffected. The mutant receptors D126C and M292C showed no detectable binding using competition studies and NDP-MSH potency was significantly reduced or abolished altogether. When the C318A mutant was co-expressed with the MC_4R mutants D126C or M292C, near normal levels of activation were restored. Neither complex caused any significant change in K_d for agonist compared to the C318A mutant alone. It is plausible that the mutation C318A affects G-protein interaction with the receptor rather than changing the active conformation of the receptor so that D126C allows full activation.

Accumulating data have suggested that receptors form at least dimers [40] and that there can be a level of co-operativity within the complex. Again, only one site of action has been assumed in the allosteric modulation by the G-protein of the R* state [41]. However, the possibility of more than one-or-more different sites of interaction should not be ignored. A series of competition experiments and saturation curves have introduced a role for co-operativity, with ligand binding oscillating between two states of cross-talk [42]. Co-operativity was terminated by dissociation of one of the proteins or independent binding of ligand by the receptor. However, this model may over-simplify the system as it does not take into account different interactions between the receptor

Table 3
MC_4 receptor trans-activation. HEK293 cells were stably expressed with the single form of MC_4R (*WT- or C318A- or D126C- or M292C-MC_4R*). These stable cell lines were then transiently transfected with the mutant C318A MC_4R. Membranes were prepared for ligand binding experiments. Homologous competition binding studies were performed using the agonist [^{125}I] NDP-MSH. Data are expressed as $pK_d \pm$ S.E.M. HEK293 cells with CRE-Luc gene were then transiently transfected with either single or dual forms of the MC_4R. CRE-luciferase assays were used to identify receptor activity. Data are expressed as $pEC_{50} \pm$ SEM. The number of independent experiments is shown in brackets. Significant differences were evaluated using the Student t test, and comparisons were made between single and dual expressed membranes (*$p<0.05$) and WT-MC_4R vs WT-MC_4R expressed with mutant (§$p<0.05$). NA = no activation. NB = no binding.

MC_4R expression in HEK-293 cell	[^{125}I] NDP-MSH binding		CRE-Luc cAMP activity assay
	pK_d	B_{max} (fmol/mg protein)	pEC_{50}
WT	7.60 ± 0.19 (3)	1234 ± 241 (3)	8.27 ± 0.27 (3)
C318A	7.05 ± 0.35 (3)	1052 ± 37.1 (3)	NA (3)
D126C	NB		4.57 ± 0.05§ (3)
M292C	NB		NA (3)
D126C/C318A	7.52 ± 0.07 (3)	3050 ± 344 (3)	8.30 ± 0.07* (6)
M292C/C318A	7.54 ± 0.04 (3)	3320 ± 475 (3)	6.72 ± 0.29*§ (6)
WT/C318A	7.53 ± 0.12 (3)	2708 ± 261 (3)	–

and the G-protein, including the ability to interact with more than one type of G-protein [43]. There is also the possibility of domain swapping as postulated previously [44–46] but this seems less likely since these are essentially the same monomers varying only in a single mutation in quite different regions of the molecule. However the recent emerging documentation of biased ligand signaling through multi-state receptor conformations [44,45], may provide insights into the different levels of occupancy for the agonist with regards to the dimeric conformation.

5. Conclusion

The data presented here demonstrate that the MC_4R forms a constitutive dimer (and possibly even a higher order complex) which exists in different conformations depending on association with the G-protein and the agonist i.e. the oligomeric state is both stable and conformationally dynamic. At least one interaction is made between the receptor sub-units; this allows disulphide cross-linking at the cytoplasmic surface of TM2 under oxidizing conditions. This interface reflects the G-protein coupled state, and the dimeric species. This state appears to regulate the ligand occupancy of the receptor. Further studies indicate that there is cross-talk between the monomers such that binding of a single ligand to one monomer can induce the active state in the other. There are, therefore, high levels of modulation not only between two receptor sub-units in a dimeric complex but also between the G-protein and the receptor.

Acknowledgments

We thank Dr D. Donnelly and Dr A Cox for all their advice with this work. This work is supported by the Wellcome Trust [Grant 06680712/02/Z].

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