Chimeric Melatonin $m_{t1}$ and Melatonin-related Receptors

IDENTIFICATION OF DOMAINS AND RESIDUES PARTICIPATING IN LIGAND BINDING AND RECEPTOR ACTIVATION OF THE MELATONIN $m_{t1}$ RECEPTOR*

Melatonin receptors bind and become activated by melatonin. The melatonin-related receptor, despite sharing considerable amino acid sequence identity with melatonin receptors, does not bind melatonin and is currently an orphan G protein-coupled receptor. To investigate the structure and function of both receptors, we engineered a series of 14 chimeric receptor constructs, allowing us to determine the relative contribution of each transmembrane domain to ligand binding and receptor function. Results identified that when sequences encoding transmembrane domains 1, 2, 3, 5, or 7 of the melatonin $m_{t1}$ receptor were replaced by the corresponding domains of the melatonin-related receptor, the resultant chimeric receptors all displayed specific 2-[125I]iodomelatonin binding. Replacement of sequences incorporating transmembrane domains 4 or 6, however, resulted in chimeric receptors that displayed no detectable 2-[125I]iodomelatonin binding. The subsequent testing of a “reverse” chimeric receptor in which sequences encoding transmembrane domains 4 and 6 of the melatonin-related receptor were replaced by the corresponding melatonin $m_{t1}$ receptor sequences identified specific 2-[125I]iodomelatonin binding and melatonin-mediated modulation of cyclic AMP levels. To further investigate these findings, site-directed mutagenesis was performed on residues within transmembrane domain 6 of the melatonin $m_{t1}$ receptor. This identified Gly358 (Gly6.49) as a critical residue required for high affinity ligand binding and receptor function.

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**To whom correspondence should be addressed: Molecular Neuroendocrinology Group, Div. of Appetite and Energy Balance, Rowett Research Inst., Greenburn Rd., Bucksburn, Aberdeen AB21 9SB, UK. Tel.: 44-1224-712751; Fax: 44-1224-716687; E-mail: sco@rri.sari.ac.uk.

†The abbreviations used are: TM, transmembrane; GPCR, G protein-coupled receptor; PCR, polymerase chain reaction; STM, segment including transmembrane; IL, intracellular loop; EL, extracellular loop.

Shaun Conway‡§, Janice E. Drew‡, Elaine S. Mowat‡, Perry Barrett‡, Philippe Delagrange†, and Peter J. Morgan‡

From the Molecular Neuroendocrinology Group, Division of Appetite and Energy Balance, Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB21 9SB, United Kingdom and the Institut de Recherches Internationales Servier, 6 place des Pléiades, F-92415 Courbevoie cedex, France

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tigate the relative contribution of each TM domain to ligand binding and receptor activation. The results from these studies were used to design site-directed mutagenesis experiments on the human melatonin mt1 receptor. The data generated by site-directed mutagenesis identified a residue in TM6 as being critical for both ligand binding and receptor function.

**EXPERIMENTAL PROCEDURES**

*Materials—*Tissue culture media, supplements, and sera were purchased from Life Technologies Inc. 2-(10)C-lodomelatonin (2200 Ci/mmol) was obtained from NEN Life Science Products. Oligonucleotides for use as primers in PCR were synthesized and purified by Biosearch. Other oligonucleotides were from Sigma-Genosys. Melatonin and general reagents were obtained from Sigma. Molecular biology reagents and enzymes were purchased from Promega.

Engineering the FLAG Epitope onto the Human Melatonin mt1 Receptor—The DNA sequence encoding the human melatonin mt1 receptor (19) was engineered by PCR to introduce a FLAG epitope sequence (5′-GACTACAGAGCAGCATGACAAG-3′) directly after the ATG start codon. This was confirmed by DNA sequencing, and the engineered receptor sequence was ligated as a HindIII/KpnI restriction fragment into pcDNA3 (Invitrogen). This plasmid was named pcDNA-mt1.

Construction of a Synthetic DNA Encoding the Human Melatonin mt1 Receptor—A series of 16 overlapping oligonucleotides (60–80 bases each with overlaps of 18–20 bases) were designed to construct the DNA sequence encoding to human melatonin mt1 receptor from the Bpu1102 I restriction endonuclease site (base 203) to one base following the stop codon (base 1054). Two sets of 8 oligonucleotides were assembled in two separate recursive PCRs, essentially by the method of Prytulla et al. (20). Recursive assembly PCR was performed using 5 pmol of each of 8 oligonucleotides in a 100-μl volume with 2.5 units of native Pfu DNA polymerase (Promega). 10 μl of the recursive assembly PCR reactions were then used as the templates in two PCR reactions to amplify the assembled DNA fragments (452 and 429 base pairs). Products were purified, ligated into pc-Script (Stratagene), and confirmed by DNA sequencing. The products were recovered as Bpu1102 B/BamHI and B/BamHI/XhoI restriction fragments, respectively, and ligated into pcDNA-mt1. Correct ligation was confirmed by DNA sequencing, and the plasmid was named pcDNA3-mt1-(syn). The nucleotide modifications present in the synthetic human melatonin mt1 receptor DNA sequence, relative to the published native sequence (2), are listed in Fig. 1.

Cloning and Engineering of the Human Melatonin-related Receptor—The two exons encoding the human melatonin-related receptor (5) were amplified by PCR from a genomic DNA library (CLONTECH). The two fragments were joined and engineered by PCR to introduce a FLAG epitope sequence directly after the ATG start codon. This product was ligated into pcDNA3 and confirmed by DNA sequencing, and the plasmid was named pcDNA3-MAINT. PCR was subsequently used to truncate the melatonin-related receptor coding sequence, engineering a new TGA stop codon at bases 979–982 (numbering excluding the FLAG epitope sequence), prior to the sequence encoding the large carboxy-terminal domain. This PCR also introduced two restriction endonuclease sequences, a silent EcoRV restriction endonuclease site (bases 973–978) was placed immediately before the new stop codon, and a XbaI site was introduced 8 bases following the stop codon. This PCR product was cloned back into pcDNA3 and confirmed by DNA sequencing, and the plasmid was named pcDNA3-MAINT-(syn). The nucleotide modifications present in the synthetic human melatonin mt1 receptor DNA sequence, relative to the published native sequence (2), are listed in Fig. 1.

Generation of Site-directed Mutants—Two site-directed mutants of the human melatonin mt1 receptor, Ala649Cys and Gly655Thr, were constructed using a PCR-based protocol with pcDNA-mt1-(syn) as the template. The template products were restricted, subcloned back into pcDNA3, and confirmed by DNA sequencing.

Transfection of COS-7 Cells—COS-7 cells were grown as monolayers in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum and 1% antibiotic/antimycotic solution, in 5% CO2 at 37 °C. Confluent plates were seeded at 15,000 cells/cm2 and used 24 h later for transfection. Cells were transfected using the DEAE-dextran method of Cullen (21). The expression of all receptor constructs was confirmed by the immunological detection of the engineered FLAG epitope as described previously (6). For ligand binding experiments, transfected cells were cultured for 72 h, washed twice with phosphate-buffered saline (pH 7.4), and harvested. Cells were pelleted at 1,500 g for 10 min, washed twice with 10 mM Tris–HCl (pH 7.4) containing 150 mM NaCl, and harvested. Cell pellets were diluted with 1.5 ml of ice-cold saline (pH 7.4) containing 150 mM NaCl, 10 mM Tris–HCl (pH 7.4), 1.0 mM EDTA, and 1% Triton X-100. Cell pellets were homogenized by sonication for 30 s at 4 °C, and the supernatant was collected. The homogenates were centrifuged at 13,000 g for 20 min at 4 °C. The supernatants were collected.

**Table:**

| Sequence | Created | Nucleotide Modifications | Nucleotide Positions |
|----------|---------|--------------------------|---------------------|
| Hind III | aagcct  | Addition prior to base 1  |                     |
| FLAG     | gactacaaggac-gacagtgacaag | Insertion between bases 3 and 4 |                     |
| BstE II  | c → t  | Base 288                  |                     |
| Age I    | c → t  | Base 291                  |                     |
| Hpa I    | c → g  | Base 459                  |                     |
| Base 460  | c → g  | Base 462                  |                     |
| KpnI     | g → c  | Base 498                  |                     |
| Base 501  | g → t  | Base 550                  |                     |
| Xho I    | a → t  | Base 580                  |                     |
| Acc III  | a → c  | Base 693                  |                     |
| Apa I    | t → c  | Base 756                  |                     |
| Cla I    | c → t  | Base 798                  |                     |
| Base 800  | g → c  | Base 799                  |                     |
| Nhe I    | c → t  | Base 801                  |                     |
| t → c    | Base 840  |                         |                     |
| EcoR I   | g → a  | Base 907                  |                     |
| Xba I    | a → g  | Base 905                  |                     |
| Base 1053 | a → g  | Base 905                  |                     |
| a → c    | Base 748  | (polymorphic variant)     |                     |
| Other Changes | a → g  | Base 90 (polymorphic variant) |                     |
| base 411 (synthesis variant) |                     |
| base 748 (polymorphic variant) |                     |

**Fig. 1.** Nucleotide modifications in the synthetic human melatonin mt1 receptor. The nucleotide modifications in the synthetic human melatonin mt1 receptor relative to the native receptor sequence (2) are listed. The restriction endonuclease recognition sequences and the FLAG epitope sequence (FLAG) created by these modifications are also identified.

noted that for some chimeras replacement of TM domains also included some sequence encoding the adjacent IL or EL domains, as illustrated in Fig. 2. In the following text we will therefore refer to chimeric receptors having replaced STM1 (segment including TM1) to STM7, to highlight that these sequences may encode loop(s) as well as the TM domains. TM clones, now only be used where it specifically refers to the transmembrane region.

Generation of Site-directed Mutants—Two site-directed mutants of the human melatonin mt1 receptor, Ala649Cys and Gly655Thr, were constructed using a PCR-based protocol with pcDNA-mt1-(syn) as the template. The template products were restricted, subcloned back into pcDNA3, and confirmed by DNA sequencing.

Transfection of COS-7 Cells—COS-7 cells were grown as monolayers in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum and 1% antibiotic/antimycotic solution, in 5% CO2 at 37 °C. Confluent plates were seeded at 15,000 cells/cm2 and used 24 h later for transfection. Cells were transfected using the DEAE-dextran method of Cullen (21). The expression of all receptor constructs was confirmed by the immunological detection of the engineered FLAG epitope as described previously (6). For ligand binding experiments, transfected cells were cultured for 72 h, washed twice with phosphate-buffered saline (pH 7.4), and harvested. Cells were pelleted at 1.5 ml microcentrifuge tubes (13,000 × g, 1 min, 4 °C) and stored frozen at −80 °C. 2-[[125I]Iodomelatonin Equilibrium Binding—2-[[125I]Iodomelatonin equilibrium binding experiments on transfected COS-7 cells were performed as described previously (22). Protein determinations were performed by the method of Bradford (23). All experiments were performed with duplicate or triplicate determinations, and the data were averaged. Kd and IC50 values were determined by Grafit software (Sigma), and IC50 values were calculated by the method of Cheng and Prusoff (24). All experiments were repeated on three or more occasions, and the mean values ± S.E. were calculated.
Construction of Gs/Gi Chimeric G Protein α-Subunit—The DNA encoding the α-subunit of Gs (25), with the exception of the final 15 bases, was amplified by reverse transcription-PCR using messenger RNA isolated from HEK293 cells as the template. Based on the findings of Komatsuzaki et al. (26), a synthetic DNA linker was ligated onto the amplified product to essentially replace the DNA encoding the final 5 amino acid residues of Gs with that encoding the final 5 amino acids of Gi (27). The product was subcloned into pcDNA3 and confirmed by DNA sequencing.

Determination of Cyclic AMP Levels—COS-7 cells were co-transfected with receptor constructs and the chimeric Gs/Gi construct as described above. Following transfection cells were cultured for 24 h, trypsinnized, and reseeded into 24-well tissue culture plates (1 × 10⁵ cells/well). Following a further 24-h incubation, cells were briefly washed with Dulbecco’s modified Eagle’s medium and incubated with appropriate concentrations of melatonin (in Dulbecco’s modified Eagle’s medium, 10 mM 3-isobutyl-1-methylxanthine) for 2 h at 37 °C. Reactions were stopped by the addition of trichloroacetic acid (final concentration, 5%). Cyclic AMP levels were determined as previously reported (28). All experiments were performed with triplicate determinations, and the data were averaged. EC₅₀ values were determined by Grafit software (Sigma). Experiments were repeated two or three times, and where appropriate the mean values ± S.E. were calculated.

RESULTS

2-[¹²⁵I]Iodomelatonin Binding Analysis of Chimeric Receptors Expressed in COS-7 Cells—Receptor constructs were transfected into COS-7 cells, and in all cases expression was confirmed by immunological detection of the engineered FLAG epitope (data not shown). The synthetic human melatonin mt₁ receptor displayed a 2-[¹²⁵I]Iodomelatonin Kᵦ affinity value that was identical the native human melatonin mt₁ receptor (Ref. 19, Fig. 3). This demonstrated that modification of the nucleotide sequence of the synthetic mt₁ receptor sequence had no detectable effect on receptor expression. Both the human melatonin-related receptor and the truncated human melatonin-related receptor produced no detectable 2-[¹²⁵I]Iodomelatonin binding (Fig. 3 and data not shown). This confirmed the previous reports for both the human and ovine melatonin-related receptors (5, 6). These data also demonstrated that the lack of observed 2-[¹²⁵I]Iodomelatonin binding to melatonin-related receptors was not due to the presence of the extended carboxyl-terminal domain.
shown to reduce the translation or removal of such glycosylation sequences has been absent from the melatonin-related receptor sequence. The mu-receptors identified in the top two schematics. Each chimera was expressed in COS-7 cells, and a 2-[125I]iodomelatonin saturation binding was performed. The specific 2-[125I]iodomelatonin binding observed for chimera [mt1 (STM1 MRR)] did not reach saturation at 1500 pM; therefore, a \( K_d \) affinity value and an extrapolated estimate of \( B_{\text{max}} \) were determined by competitive displacement of specific 2-[125I]iodomelatonin binding by 2-iodomelatonin. Listed to the right of each chimera receptor is a descriptive name and the mean \( K_d \) or \( K_i \) and \( B_{\text{max}} \) values ± S.E. The number of experiments performed is shown in parentheses.

Chimera [mt1 (STM1 MRR)] displayed a 2-[125I]iodomelatonin \( K_d \) binding affinity close to that determined for the melatonin mt1-synthetic receptor but was expressed at a much reduced level (\( B_{\text{max}} \) values, 14.2 and 506.9 fmol/mg protein, respectively). This implied that none of the residues that were changed in STM1 of this chimera relative to the melatonin mt1 receptor (within 3-fold), demonstrating that the residues that were changed in STM2, STM3, and STM5 of these chimeras relative to the melatonin mt1 receptor sequence did not appear to be critical to either ligand binding or the detectable receptor level in the melatonin mt1 receptor. Chimera [mt1 (STM7+CT MRR)] displayed a 4.5-fold reduction in ligand binding affinity relative to the melatonin mt1-synthetic receptor and was expressed at a reduced level (\( B_{\text{max}} = 44.2 \) fmol/mg protein). This suggested that some of the residues that were changed in STM7 were required for high affinity ligand binding in the melatonin mt1 receptor and that some residues affected the level of receptor which bound the ligand. Chimera [mt1 (STM7+CT MRR)] in which the carboxyl-terminal domain of the melatonin-related receptor was truncated gave \( K_d \) and \( B_{\text{max}} \) values essentially identical to chimera [mt1 (STM7+CT MRR)], indicating that the reduction in \( B_{\text{max}} \), relative to the melatonin mt1 receptor did not appear to be caused by the presence of the carboxyl-terminal domain but was caused by the altered residues within STM7. Competitive displacement of 2-[125I]iodomelatonin binding to chimera [mt1 (STM7+CT MRR)] by a series of melatonin receptor ligands gave a very good correlation with binding data obtained for the synthetic melatonin mt1 receptor (\( r^2 = 0.99; \) slope factor = 0.99). (Fig. 4). This demonstrated that the changes induced in STM7 caused a consistent (4–5-fold) reduction in ligand binding affinity compared with the melatonin mt1 receptor. Chimeras [mt1 (STM4 MRR)] and [mt1 (STM6 MRR)] displayed no detectable 2-[125I]iodomelatonin binding. This implied that some residues that were changed in STM4 and STM6 of these chimeras relative to the melatonin mt1 receptor sequence were critical for high affinity 2-[125I]iodomelatonin binding to the melatonin mt1 receptor.

To expand on these initial findings additional multiple domain chimeras were constructed and tested. Chimeras [mt1...
melatonin mt<sub>1</sub> receptor.

that increased the level of receptor that bound the ligand.
correction (24) states that the IC<sub>50</sub> obtained for the displace -
pM (data not shown). Indeed, Scatchard analysis (30) indicated
was not saturating at the highest concentration tested of 1500

has been shown to cause GPCRs that normally inhibit cyclic

B

or STM7 was independently replaced, large reductions in
wild-type melatonin mt<sub>1</sub> receptor levels. This indicated that

The final chimeras to be tested were those to further inves-
tigate the roles of STM4 and STM6 in melatonin receptor
ligand binding. Chimeras [MRR (NT+STM4 mt<sub>1</sub>)] and [MRR
(NT+STM6 mt<sub>1</sub>)] displayed no detectable 2-<sup>125</sup>Iiodomelato-
nin binding. Chimera [MRR (NT+STM4+STM6 mt<sub>1</sub>)], however,
displayed specific 2-<sup>125</sup>Iiodomelatonin binding, but this
was not saturating at the highest concentration tested of 1500

The melatonin-mediated effects upon cyclic AMP levels in
COS-7 Cells Transfected with Chimeric Receptors and G<sub>s</sub>/G<sub>i</sub>
chimeric G protein α-subunit—COS-7 cells were co-trans-
fected with the chimeric melatonin mt<sub>1</sub>-melatonin-related receptors
and a chimeric G<sub>s</sub>/G<sub>i</sub> G protein α-subunit construct. In previous
reported studies the use of chimeric G<sub>s</sub>/G<sub>i</sub> α-subunit has been shown to cause GPCRs that normally inhibit cyclic
AMP levels, via activation of G<sub>i</sub>, to produce a ligand-mediated
stimulation of cyclic AMP by promiscuous coupling to the G<sub>i</sub>/G<sub>i</sub>
(26). This system was employed for functional studies in COS-7
cells because this cell line has been previously reported as
being unsuitable for studies on GPCRs that signal via a

these mutants exchanged the native melatonin mt<sub>1</sub> receptor
amino acid residue for that in the same position of the human
melatonin-related receptor. Saturation binding analysis of
Ala6.49Cys produced similar K<sub>D</sub> and B<sub>max</sub> values as those
obtained for the melatonin mt<sub>1</sub>-synthetic receptor (Fig. 6). Therefore
Ala<sup>6</sup>.<sup>49</sup> probably does not play a critical role in
ligand binding to the melatonin mt<sub>1</sub> receptor. Saturation
binding analysis of mutant Gly6.55Thr also identified spe-
cific 2-<sup>125</sup>Iiodomelatonin binding, but this was not saturating
at the highest concentration tested of 1500 pM. Using the
same rationale as described above for chimera [MRR
(NT+STM4+STM6 mt<sub>1</sub>)] the affinity for mutant Gly6.55Thr
was determined by competitive displacement of 2-<sup>125</sup>Iiodome-
latonin binding (1500 pM) by 2-iodomelatonin (1 × 10<sup>-6</sup> to 1 × 10<sup>-9</sup>
M vehicle) for 2 hours at 37 °C. Cyclic AMP
levels were determined by radioimmunoassay. Mean cyclic AMP
values were calculated for the vehicle-treated cells (the basal value), and this
was adjusted to a value of 100% (white bars, means ± S.E.). Cyclic AMP
values for melatonin-treated cells were calculated and normalized relative
to the basal value (gray bars, means ± S.E.). The experiment
shown is representative of two similar experiments.

Dose Response Effect of Melatonin upon Cyclic AMP Levels in
COS-7 Cells Transfected with Site-directed Mutant or Chimeric
Melatonin Receptors and G<sub>s</sub>/G<sub>i</sub> Chimeric G Protein α-Subunit—The dose-response effects of melatonin upon cyclic AMP
levels in COS-7 cells co-transfected with the G<sub>s</sub>/G<sub>i</sub> chimeric G protein α-subunit and either the melatonin mt<sub>1</sub>-synthetic receptor,
selected chimeric receptors, or the 2 site-directed mu-
tant receptors were performed (Fig. 8). The results demon-
strated that melatonin (1 × 10<sup>-12</sup> to 1 × 10<sup>-7</sup> M) caused a
dose-dependent stimulation of cyclic AMP level for all receptor
constructs. The melatonin mt<sub>1</sub>-synthetic receptor had a deter-
med EC<sub>50</sub> affinity value of 186 pM, close to other reported
values for melatonin-mediated activation of mt<sub>1</sub> receptors (2,
4). Chimera [mt<sub>1</sub> (STM7+CT MRR)] produced an EC<sub>50</sub>
affinity value 7.3-fold lower than that of the mt<sub>1</sub>-synthetic receptor.
The aim of this study was to expand the understanding of the molecular processes that underlie how melatonin receptors bind and are activated by melatonin. To achieve this, we employed a strategy involving chimeric receptors constructed from the human melatonin mt₁ receptor and the human melatonin-related receptor. The rationale for these studies was based on the fact that melatonin receptors bind melatonin with high affinity (4), whereas the melatonin-related receptor, which shares considerable sequence identity with melatonin receptors, does not bind melatonin (5, 6). Our hypothesis for why the melatonin-related receptor does not bind melatonin was that this was due to the absence of a limited number of specific amino acid residues present in the melatonin receptor ligand-binding site and not due to a gross difference in the structures of the two receptors. If this hypothesis was accurate it would be possible to identify in which domains of the mt₁ receptor such residues occurred by the construction of chimeric receptors in which small regions of the melatonin mt₁ receptor were replaced with the corresponding region of the melatonin-related receptor. A significant reduction in ligand binding affinity, compared with that of the mt₁-synthetic receptor (within 2.2-fold), whereas mutant Gly6.49Thr displayed a profound loss of melatonin-mediated receptor activation with an EC₅₀ affinity value ~166,000-fold lower than that determined for the melatonin mt₁-synthetic receptor.

**DISCUSSION**

This was consistent with the observed 4.5-fold reduction in ligand binding affinity previously described for this chimera (Figs. 3 and 4). Chimera [MRR (NT + STM4 + STM6 mt₁)] produced an EC₅₀ affinity value ~830-fold lower than that of the mt₁-synthetic receptor. This demonstrated that dual replacement of STM4 and STM6 of the melatonin-related receptor with the corresponding sequences of the melatonin mt₁ receptor could produce melatonin-mediated receptor activation at high dose. Site-directed mutant Ala6.49Cys produced a similar response to that of the mt₁-synthetic receptor (within 2.2-fold), whereas mutant Gly6.55Thr displayed a profound loss of melatonin-mediated receptor activation with an EC₅₀ affinity value ~166,000-fold lower than that determined for the melatonin mt₁-synthetic receptor.

**FIG. 6.** 2-[¹²⁵I]Iodomelatonin saturation binding to human melatonin mt₁ receptor site-directed mutant Ala6.49Cys. 2-[¹²⁵I]Iodomelatonin saturation binding to COS-7 cells transfected with site-directed mutant Ala6.49Cys. Total binding (white circles), nonspecific binding in the presence of 1 μM melatonin (white squares), and specific 2-[¹²⁵I]Iodomelatonin binding (black circles) are shown. The experiment shown is representative of three similar experiments. The ligand binding parameters (means ± S.E.) for mutant Ala6.49Cys were $K_d = 1.59 \pm 0.26 \times 10^{-10}$ M; $B_{max} = 481.1 \pm 130.7$ fmol/mg protein.

**FIG. 7.** Competitive displacement of specific 2-[¹²⁵I]Iodomelatonin binding to human melatonin mt₁ receptor site-directed mutant Gly6.49Thr. Competitive displacement of specific 2-[¹²⁵I]Iodomelatonin binding by 2-iodomelatonin in COS-7 cells expressing either the synthetic-melatonin mt₁ receptor (black circles) or site-directed mutant Gly6.49Thr (white circles) are shown. The 2-[¹²⁵I]Iodomelatonin tracer concentrations used were 1500 pM for Gly6.49Thr and 19.5 pM for the melatonin mt₁ receptor. The data shown are the averages of duplicate determinations from a single representative experiment. Experiments were repeated three times. The determined $K_i$ affinity values (means ± S.E.) were, synthetic melatonin mt₁ receptor, $8.12 \pm 2.89 \times 10^{-11}$ M; mutant Gly6.49Thr, $1.26 \pm 0.44 \times 10^{-7}$ M.

**FIG. 8.** The effects of melatonin concentration upon cyclic AMP levels in COS-7 cells co-transfected with either site-directed mutant or chimeric receptors and G/Gi, chimeric G protein α-subunit. The effect of melatonin concentration upon cyclic AMP levels in COS-7 cells co-transfected with either site-directed mutants or chimeric receptor and G/Gi, chimeric G Protein α-subunit is shown. Experiments were performed essentially as described for Fig. 5 but with melatonin concentrations between $1 \times 10^{-15}$ and $1 \times 10^{-4}$ M. A four-parameter logistic curve was fitted to averaged data for each construct, and the observed stimulation of cyclic AMP levels were normalized to a 100% response. The receptor constructs are indicated by the following symbols: synthetic melatonin mt₁ receptor, black circle (EC₅₀ 1.56 ± 10⁻¹⁰ M); site-directed mutant Ala6.49Cys, white circle (EC₅₀ 4.08 ± 10⁻¹¹ M); chimera [mt₁ (STM7 + CT MRR)], black square (EC₅₀ 1.36 ± 10⁻⁹ M); chimera [MRR (NT + STM4 + STM6 mt₁)], white square (EC₅₀ 1.55 ± 10⁻⁷ M); and site-directed mutant Gly6.55Thr, black triangle (EC₅₀ 3.08 ± 10⁻⁹ M). Data shown were calculated from mean values determined in three experiments.
Chimeric receptors in which STM2, 3, or 5 of the melatonin mt1 receptor were replaced by the equivalent melatonin-related receptor sequences had little effect on either $K_d$ or $B_{\text{max}}$ when compared with the synthetic human melatonin mt1 receptor. This demonstrated that the altered residues within these domains were not critical for high affinity ligand binding in melatonin receptors. It should be noted, however, that these studies were not designed to yield any information on the contribution of the conserved residues toward ligand binding. Indeed His$^{5.46}$ is conserved in both melatonin receptors and in the melatonin-related receptor and has been previously shown to be involved in ligand binding within the melatonin mt1 receptor (7, 8). Our findings do, however, suggest that any further site-directed mutagenesis studies that are designed to investigate possible ligand binding residues in STM2, 3, or 5 of melatonin receptors should concentrate on the residues that are conserved with the melatonin-related receptor. Chimeric replacement of STM1 also displayed little difference in $K_d$ value compared with the melatonin mt1 receptor; however the $B_{\text{max}}$ was reduced by 36-fold. Therefore, one or more of the altered residues within this domain reduced the amount of receptor that was capable of binding the ligand. Immunological detection of the FLAG epitope on this chimera, however, indicated that the expression was indistinguishable from that of the melatonin mt1 receptor; therefore, the loss of observed binding was probably due to a change in the structure of the expressed receptor and not due to impaired expression. Chimeric replacement of STM7 reduced both $K_d$ and $B_{\text{max}}$ values relative to the melatonin mt1 receptor. This suggests that STM7 may involve in the ligand-binding site of melatonin receptors. To further investigate the role of STM7 in the melatonin receptor-binding site, competitive displacement studies were performed using six bioisosteres of melatonin, including drugs that had a modified $N$-acetyl group (S20642, 5-methoxy tryptophol) or a modified 5-methoxy group (Jam09, $N$-acetyl tryptamine). The relative reductions in binding affinities for all of these drugs were identical, indicating that the changes produced in the STM7 chimera affected the general structure of the melatonin receptor-binding pocket and did not appear to affect specific interactions that might occur with, for example, the $N$-acetyl group. The chimeras involving the replacement of either STM4 or STM6 resulted in no detectable $2-[^{125}\text{I}]$iodomelatonin binding. This indicated that one or more nonconserved residues in STM4 and STM6 may be critical in providing ligand binding in the melatonin mt1 receptor.

To expand upon the above data, additional multiple domain chimeras were constructed. All of these chimeras were engineered to have the amino-terminal domain of the melatonin mt1 receptor because of our previous observation that this produced higher expression levels than when the amino-terminal domain of the melatonin-related receptor was present. Two multiple chimeras were designed to investigate whether the reduction in $B_{\text{max}}$ observed for both STM1 and STM7 chimeras were related. These data identified that when both STM1 and STM7 were simultaneously replaced by melatonin-related receptor sequences, the $B_{\text{max}}$ levels were elevated to near melatonin mt1 receptor levels. Therefore, high expression levels were seen when STM1 and STM7 were present as a pair from either the melatonin mt1 receptor or the melatonin-related receptor but not when they were combined. It has previously been shown that TM1 and TM7 are adjacent in rhodopsin-like GPCRs (32, 33). Therefore, the most logical explanation of the above phenomenon is that both melatonin receptors and melatonin-related receptors have some form of close association between TM1 and TM7 and that when this is perturbed the structure of either receptor is compromised. Potentially TM1 and TM7 could form specific interactions between their respective amino acid residues. There is evidence to suggest that different TM domains may interact in GPCRs (34), as illustrated by the reported interaction of TM2 and TM7 in the gonadotrophin hormone releasing hormone receptor (35). Because the melatonin and melatonin-related receptors are, however, not typical GPCRs in possessing Ala$^{7.50}$ in TM7, instead of the otherwise conserved Pro$^{7.50}$, it is possible that this conveys unique aspects to the structure of these receptors, especially involving TM7. It is interesting to note that amino acid positions 1.56 (Ser$^{1.56}$ mt1, Ala$^{1.56}$ melatonin-related receptor) and 7.58 (Gln$^{7.58}$ mt1, Glu$^{7.58}$ melatonin-related receptor) both show nonconservation between the melatonin receptors and the melatonin-related receptor but are potentially closely positioned within the structure of these receptors. It is possible that residues such as these could therefore form unique interactions within these receptors. Additional site-directed mutagenesis studies may resolve the molecular mechanisms underlying the observed association of STM1 and STM7 in melatonin and melatonin-related receptors. The final three chimeric receptors that were constructed were to further investigate the role of STM4 and STM6 to ligand binding in the melatonin mt1 receptor. We found that when STM4 and STM6 were replaced together in chimera [MRR (NT + STM4 + STM6 mt1)], this receptor construct could bind $2-[^{125}\text{I}]$iodomelatonin, albeit with a relatively low affinity, $K_d = 28,800 \pm 10,800$ pM. Replacement of STM4 or STM6 alone resulted in no detectable $2-[^{125}\text{I}]$iodomelatonin binding. The ligand binding results were complemented by measurement of the melatonin-mediated stimulation of cyclic AMP levels in COS-7 cells co-transfected with a Gs/Gi chimeric G protein $\alpha$-subunit. All of the chimeras that bound $2-[^{125}\text{I}]$iodomelatonin stimulated cyclic AMP levels, whereas the receptors that displayed no detectable ligand binding did not. The one exception to this was chimera [mt1 (NT + STM1 MRR)], which bound $2-[^{125}\text{I}]$iodomelatonin at a very low level (1.14 fmol/mg protein) but did not display changes in cyclic AMP level. This would appear to be solely due to the low expression level, because HEK293 cells that express mt1 receptors at 1 fmol/mg protein also failed to display melatonin-mediated effects upon cyclic AMP levels.

The observation that chimera [MRR (NT + STM4 + STM6 mt1)] bound $2-[^{125}\text{I}]$iodomelatonin and demonstrated melatonin-mediated stimulation of cyclic AMP levels identified that...
certain residues that were altered within these exchanged domains (STM4 and STM6) must be critical for providing ligand binding to the melatonin mt1 receptor. The altered residues in STM4 mainly occurred within IL2 and EL2 and not TM4. These loop domains are not thought to be directly involved in ligand binding in rhodopsin-like GPCRs; therefore, the most probable explanation is that residues in these domains are required for the formation or maintenance of the melatonin receptor structure. This may possibly occur by interactions with other IL and EL domains. The altered residues present in STM6 included two residues within TM6 as well as some residues in IL3 and EL3. It is possible that residues in IL2 or EL2 may interact with residues in IL3 or EL3, respectively, thereby explaining why STM4 and STM6 were both required to produce detectable 2-[^125]Iiodomelatonin binding in the chimeric receptors. Such putative interactions could be explored using more refined chimeras or by site-directed mutagenesis. To study altered residues within TM6, two site-directed mutants were constructed in the melatonin mt1 receptor, Ala6.49Cys and Gly6.55Thr. These exchanged the melatonin mt1 receptor residues for those present in the melatonin-related receptor. Mutant Ala6.49Cys displayed both a $K_d$ binding affinity and a functional $EC_{50}$ value close to those determined for the melatonin mt1 receptor. This suggested that Ala$^6.49$ was not critical for ligand binding in the melatonin receptor. Mutant Gly6.55Thr, however, displayed a ~970-fold reduction in $K_d$ binding affinity and a ~166,000-fold reduction in functional $EC_{50}$ value relative to the determined melatonin mt1 receptor values. Therefore, replacement of Gly$^6.55$ with a Thr residue severely compromised both high affinity ligand binding and ligand-mediated signal transduction in the melatonin mt1 receptor. The positions of Gly$^6.55$, Ala$^6.49$, and His$^5.46$ are shown on a schematic projection of the human melatonin mt1 receptor (Fig. 9). As can be seen, Gly$^6.55$ is predicted to point toward the central receptor core, and this suggests that mutant Gly6.55Thr affected ligand binding and receptor function by directly perturbing the structure of the melatonin receptor ligand-binding pocket. The most probable explanation for this would appear to be that the Thr residue was not tolerated in the melatonin receptor-binding pocket because of its hydroxyl containing side-chain structure.

We have presented data that Gly$^6.55$ is an important conserved residue required for ligand binding and receptor activation in melatonin receptors. Additional site-directed mutagenesis could further expand our knowledge of the molecular structure of TM6 and its role in the melatonin receptor ligand-binding site.