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Expression cloning of a high-affinity melatonin receptor from *Xenopus* dermal melanophores

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ABSTRACT **Using an expression cloning strategy, a high-affinity melatonin receptor cDNA has been isolated from *Xenopus laevis* dermal melanophores. Transient expression of the cDNA in COS-7 cells resulted in high-affinity 2-[^{125}I]-iodomelatonin binding (K_d = 6.3 ± 0.3 × 10^{-11} M). In addition, six ligands exhibited a rank order of inhibition of specific 2-[^{125}I] iodomelatonin binding that was identical to that reported for endogenous high-affinity receptors. Functional studies of CHO cells stably expressing the receptor cDNA showed that melatonin acting through the cloned receptor inhibited forskolin-stimulated cAMP accumulation in a dose-dependent manner. Northern blot analysis showed that melatonin receptor transcripts are moderately expressed in *Xenopus* dermal melanophores. The cDNA encodes a protein of 420 amino acids, which contains seven hydrophobic segments. Structural analysis revealed that the receptor protein is a newly discovered member of the guanine nucleotide binding protein-coupled receptor family.**

Melatonin, the principal hormone of the vertebrate pineal gland, elicits potent neurobiological effects. The hormone influences circadian rhythms in some lizards, birds, and mammals and mediates the profound effects of photoperiod on reproductive function in seasonally breeding mammals (for reviews, see refs. 1–3). Over the past 7 years, the ligand 2-[^{125}I]iodomelatonin (^{125}I-Mel) has revealed the presence of high-affinity melatonin receptors ($K_d < 2 \times 10^{-10}$ M) (4–6). Receptor affinity is sensitive to guanine nucleotides and activation of the receptors consistently leads to inhibition of adenylyl cyclase through a pertussis toxin-sensitive mechanism (7–11). High-affinity melatonin receptors thus appear to belong to the superfamily of guanine nucleotide binding protein (G protein)-coupled receptors. To better understand how melatonin acts at a cellular and molecular level, it is important to determine melatonin receptor structure.

One of the earliest described actions of melatonin is its ability to cause melanin aggregation in dermal melanophores of amphibians (for review, see ref. 12). This action is mediated through a high-affinity melatonin receptor that is coupled to inhibitory G protein (G_i) (13, 14). Using a cDNA library constructed from an immortalized cell line of *Xenopus* dermal melanophores and a mammalian cell expression cloning strategy, we have succeeded in cloning a high-affinity melatonin receptor. The cDNA encodes a protein that is a newly discovered member of the G protein-coupled receptor family.‖

MATERIALS AND METHODS

Expression Cloning. *Xenopus laevis* dermal melanophores were cultured as described (15). The melanophores were derived from a clonal line that was isolated from a primary culture. Total cellular RNA was isolated from melanophores by extraction with guanidinium thiocyanate and separation in cesium chloride. Melanosomes were removed as described before centrifugation on the cesium chloride gradient (16). Poly(A)$^+$ RNA was isolated by established methods (17).

A random-primed cDNA library was constructed with a kit from Pharmacia. Double-stranded cDNA was ligated with nonpalindromic *Bst*XI/*Eco*RI adaptors (Invitrogen). The cDNA was size-fractionated on an agarose gel, and cDNA ≥ 2 kb was recovered by electroelution. The size-selected cDNA was ligated into the expression vector pcDNAI (Invitrogen) and introduced into *Escherichia coli* MC1061/P3 by electroporation.

A total of 4 × 10^5 recombinants were obtained from 5 μg of poly(A)$^+$ RNA and divided into 54 pools of ≈7400 clones each. Plasmid DNA was prepared from each pool by the alkaline lysis method and transfected into COS-7 cells by the DEAE-dextran method (18). Three days after transfection, cells were incubated with 90 pM ^{125}I-Mel in 50 mM Tris·HCl (pH 7.4) containing 100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 5% Nu-Serum I (Collaborative Biomedical Products, Bedford, MA) for 2 hr at room temperature. Cells were washed, air dried, and exposed to x-ray film for 14 days. A pool of clones that showed positive signals was subdivided, and the transfection procedure was repeated. This subdividing process was continued until a single clone was isolated.

Expression Studies. COS-7 cells were grown as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml), in 5% CO_2/95% air at 37°C. CHO cells were grown similarly in Ham's F-12 medium supplemented with 10% fetal calf serum and antibiotics.

For ligand binding studies, the cDNA in pcDNAI was introduced into COS-7 cells by the DEAE-dextran method (18). Three days after transfection, medium was removed, dishes were washed with phosphate-buffered saline, and cells were harvested. The cells were then pelleted (1600 × *g*; 10 min; 4°C) and stored at −80°C. Whole cell binding studies were performed by thawing the cells and resuspending them in binding buffer [50 mM Tris·HCl (pH 7.4) with 5 mM $MgCl_2$] at a concentration of 456 μg of protein per ml. The cell suspension was incubated with ^{125}I-Mel with or without drugs in a total reaction vol of 0.2 ml of binding buffer; the suspension was incubated in a shaker bath for 1.5 hr at 25°C. All determinations were done in duplicate or triplicate. Protein measurements were performed using the Pierce BCA protein assay. Binding data were analyzed by computer by using the LIGAND program of Munson and Rodbard (19).

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Abbreviations: ^{125}I-Mel, 2-[^{125}I]iodomelatonin; G protein, guanine nucleotide binding protein.

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. U09561).

For cAMP studies, the receptor cDNA was subcloned into pcDNAI/NEO (Invitrogen) and introduced into CHO cells by calcium phosphate-mediated transfection (17). Transformed CHO cells resistant to neomycin were isolated and single colonies expressing high levels of melatonin receptor binding (>10 fmol/60-mm dish of cells using 100 pM ^{125}I-Mel) were isolated.

cAMP Studies. Transformed CHO cells were plated on 35-mm dishes. Forty-eight hours later, the cells were washed twice with F-12 medium. Cells were then incubated with or without drugs (diluted in F-12 medium) for 10 min at 37°C. At the end of treatment, the medium was aspirated and 1 ml of 50 mM acetic acid was added. The cells were collected, transferred to an Eppendorf tube, boiled for 5 min, and centrifuged (12,500 × *g* for 15 min). The supernatant was collected and assayed for cAMP. All determinations were done in triplicate. cAMP levels were determined in duplicate by radioimmunoassay (New England Nuclear).

Northern Blot Analysis. Poly(A)$^+$ RNA was subjected to electrophoresis through a 1% agarose-formaldehyde gel, blotted onto GeneScreen (New England Nuclear), and hybridized with a fragment of the coding region of the receptor cDNA labeled with [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq) by the method of random priming (Pharmacia) (20). Hybridizing conditions were 50% formamide/1 M sodium chloride/1% SDS/10% dextran sulfate/100 μg of denatured salmon sperm per ml, at 42°C overnight. The final washing of the blot was in 0.2× standard saline citrate/0.1% SDS at 65°C for 40 min. Blots were exposed at −80°C to x-ray film with an intensifying screen.

DNA Sequencing. Nucleotide sequences were analyzed by the dideoxynucleotide chain-termination method of Sanger *et al.* (21) using Sequenase (United States Biochemical). Sequencing template was double-stranded plasmid. Primers were synthetic oligonucleotides that were either vector specific or derived from sequence information.

Drugs. ^{125}I-Mel was purchased from New England Nuclear. 2-Iodomelatonin was purchased from Research Biochemicals (Natick, MA). 6-Chloromelatonin was generously supplied by J. A. Clemens (Eli Lilly). All other drugs were purchased from Sigma.

RESULTS

Isolation of a Melatonin Receptor Clone. A cDNA library was constructed in the eukaryotic expression vector pcDNAI. The poly(A)$^+$ RNA used for library synthesis was derived from an immortalized cell line of *Xenopus* dermal melanophores, which was found to express a high level of ^{125}I-Mel binding (≥100 fmol per mg of total cell protein using 50 pM ^{125}I-Mel; S. A. Rivkees and S.M.R., unpublished data). After pools of the library were transfected into COS-7 cells, the cells were incubated with ^{125}I-Mel and screened by film autoradiography. After several rounds of pool subdivision, a single recombinant clone was identified that conferred specific ^{125}I-Mel binding to COS-7 cells.

Ligand Binding Studies. To establish the binding characteristics of the encoded receptor, the cDNA in pcDNAI was transiently expressed in COS-7 cells. Three days after transfection, saturation studies (Fig. 1 *Upper*) were performed using increasing concentrations of ^{125}I-Mel (5–1280 pM). Scatchard analysis (Fig. 1 *Lower*) revealed that transfected COS-7 cells bound ^{125}I-Mel with high affinity (K_d = 6.3 ± 0.3 × 10^{-11} M; n = 3 experiments). The B_{max} value using the whole cell binding assay was 67 ± 7 fmol per mg of protein. No specific binding of ^{125}I-Mel were found in mock-transfected COS-7 cells (data not shown).

The pharmacological characteristics of specific ^{125}I-Mel binding in acutely transfected COS-7 cells were next examined (Fig. 2). The rank order of inhibition of specific ^{125}I-Mel

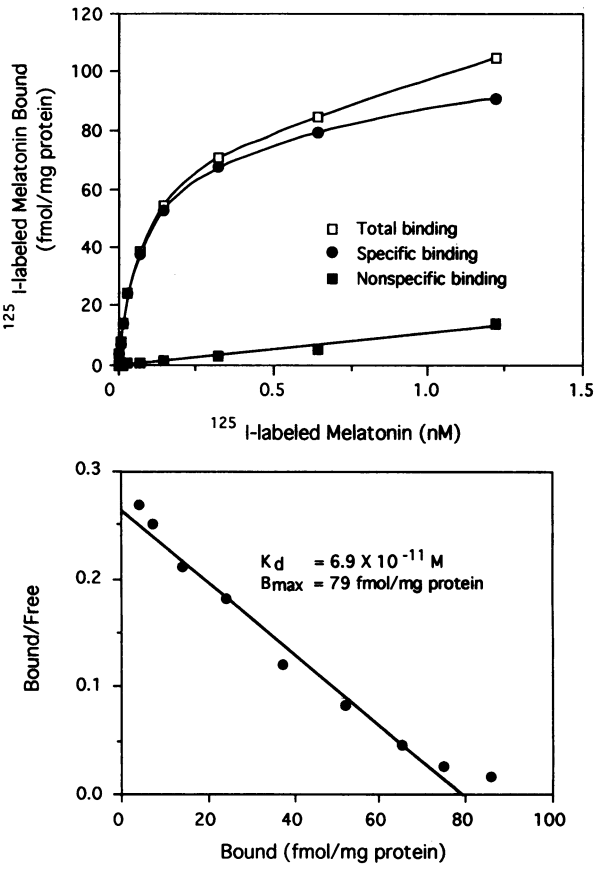

FIG. 1. Expression of the *Xenopus* melatonin receptor cDNA in COS-7 cells assayed by ^{125}I-Mel binding. (*Upper*) Saturation curve. Nonspecific binding was determined by using 10 μM melatonin. (*Lower*) Scatchard plot of saturation data. Data shown are representative of three experiments.

binding by six ligands was characteristic of a high-affinity melatonin receptor (4, 7) with 2-iodomelatonin > melatonin > 6-chloromelatonin > 6-hydroxymelatonin > *N*-acetyl-5-hydroxytryptamine > 5-hydroxytryptamine. Thus, the cDNA we have isolated encodes a protein with the affinity

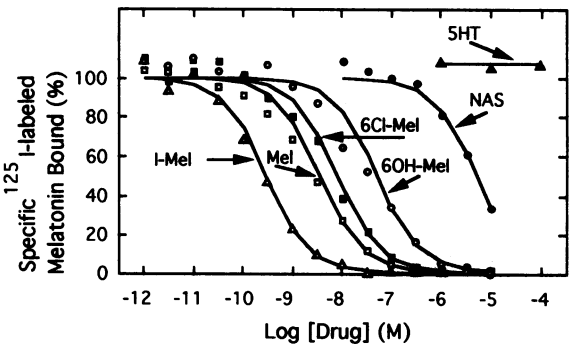

FIG. 2. Competition by various ligands for ^{125}I-Mel binding in COS-7 cells transfected with the melatonin receptor cDNA. Cells were incubated with 100 pM ^{125}I-Mel and various concentrations of 2-iodomelatonin (I-Mel), melatonin (Mel), 6-chloromelatonin (6Cl-Mel), 6-hydroxymelatonin (6OH-Mel), *N*-acetyl-5-hydroxytryptamine (NAS), or 5-hydroxytryptamine (5HT). Nonspecific binding was determined in the presence of 10 μM melatonin. K_i values are as follows: I-Mel, 1.1 × 10^{-10} M; Mel, 1.3 × 10^{-9} M; 6Cl-Mel, 3.0 × 10^{-9} M; 6OH-Mel, 2.0 × 10^{-8} M; NAS, 2.0 × 10^{-6} M; 5HT, >1.0 × 10^{-4} M. Inhibition curves were generated by LIGAND using a one-site model. Data shown are representative of three experiments.

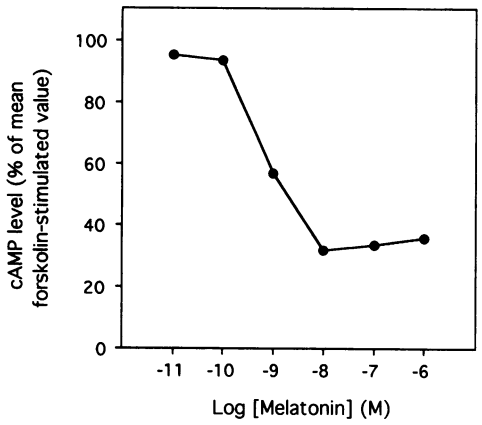

FIG. 3. Melatonin inhibition of forskolin-stimulated cAMP accumulation in CHO cells stably transfected with melatonin receptor cDNA. The 100% value is the mean cAMP value induced with 10 μM forskolin. Data shown are representative of three experiments.

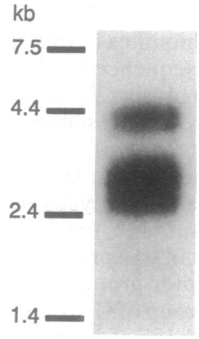

FIG. 4. Northern blot analysis of melatonin receptor transcripts in *Xenopus* dermal melanophores. Lane contained 3 μg of poly(A)$^+$ RNA. Locations of RNA size markers (GIBCO/BRL) are indicated. Blot was exposed to x-ray film overnight.

and pharmacological properties expected of a high-affinity melatonin receptor.

cAMP Studies. We next examined whether the recombinant melatonin receptor is coupled to inhibition of adenylyl cyclase as occurs with the endogenous receptor in *Xenopus* dermal melanophores (13, 15). For these studies, we used a clonal line of CHO cells stably transfected with the receptor cDNA. Melatonin (1 μM) did not alter basal cAMP levels in stably transfected CHO cells (data not shown). Melatonin did cause a dose-dependent inhibition of the increase in cAMP induced by 10 μM forskolin (Fig. 3); the maximal inhibition of the mean forskolin-stimulated cAMP value was 68% at 10^{-8} M melatonin. The eye-fitted IC_{50} value of this response ($\approx 8 \times 10^{-10}$ M) was very similar to the computer-generated K_i value (1.3×10^{-9} M) determined for melatonin inhibition of specific ^{125}I-Mel binding shown in Fig. 2. The melatonin-induced inhibition of forskolin-stimulated cAMP accumulation in CHO cells was dependent on the cloned receptor, because 1 μM melatonin did not inhibit the forskolin-stimulated increase in cAMP levels in CHO cells stably transfected with vector lacking the *Xenopus* cDNA. Thus, the recombinant melatonin receptor is negatively coupled to the cAMP regulatory system.

Expression of Melatonin Receptor Transcripts. Northern blot analysis of *Xenopus* dermal melanophores revealed at least three hybridizing transcripts between 2.4 and 4.4 kb under conditions of high stringency (Fig. 4). The presence of multiple hybridizing bands may represent posttranscriptional modifications of the same gene or the presence of transcripts from different but structurally similar genes.

Melatonin Receptor Structure. Both strands of the coding region of the ≈2.2-kb cDNA were sequenced. The cDNA encodes a protein of 420 amino acids with an estimated

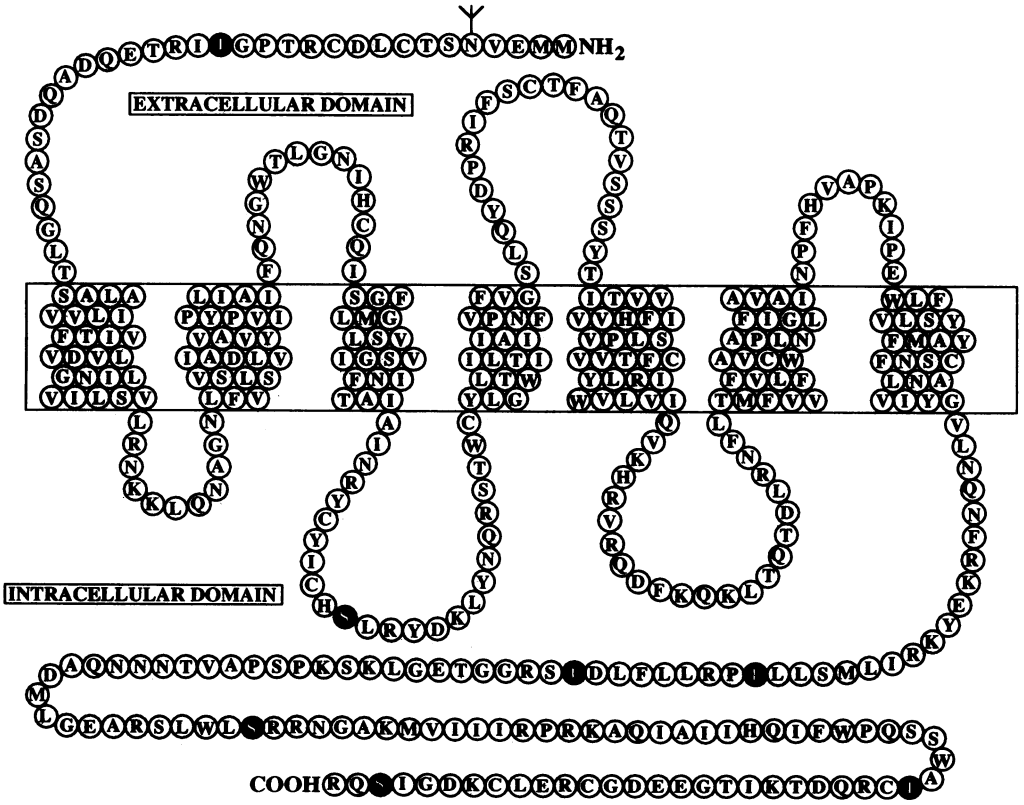

FIG. 5. Proposed membrane structure of the *Xenopus* melatonin receptor. Deduced amino acid sequence is shown. Y, Potential N-linked glycosylation site. Solid circles, consensus sites for protein kinase C phosphorylation.

molecular weight of 47,424 (Fig. 5). The flanking sequence of the first two methionines in this reading frame displayed a Kozak consensus sequence for the initiation of translation (22). Hydropathy analysis (23) of the predicted amino acid sequence revealed the presence of seven hydrophobic segments (data not shown), which likely represent the transmembrane regions of a G protein-coupled receptor. The protein appears to be glycosylated as the N terminus contains a consensus site for N-linked glycosylation, a feature typical of most G protein-coupled receptors (24). The melatonin receptor protein is not similar in identity to any one particular group of G protein-coupled receptors but is similar to a wide range of receptors within the prototypic G protein-coupled receptor family (25); the highest identity scores were ≈25% for both the mu opioid and type 2 somatostatin receptors. Using a G protein-coupled receptor data base (26), the melatonin receptor appears to form a group distinct from the other groups of receptors that comprise the prototypic G protein-coupled receptor family, such as biogenic amine, neuropeptide, photopigment, paracrine/autocrine, and olfactory receptors. No sequence homology was identified between the melatonin receptor and the nonprototypic metabotropic glutamate or parathyroid hormone/calcitonin/secretin families of G protein-coupled receptors (27–29).

The melatonin receptor does have some general structural features in common with amine and peptide receptors. For example, it contains a single cysteine residue in each of the first two extracellular loops that, based on mutagenesis studies of opsin and amine receptors (30, 31), are believed to form a disulfide bridge, which stabilizes receptor structure. Furthermore, proline residues are present in transmembrane domains 4, 5, and 6, which have been suggested to introduce turns in the α-helices that may be important in forming the ligand binding pocket (32, 33). Interestingly, the proline in the NPXXY motif that is found in transmembrane 7 of virtually all other G protein-coupled receptors is replaced by an alanine in the melatonin receptor. The carboxyl tail of the melatonin receptor is 119 amino acids long and contains several consensus sites for protein kinase C phosphorylation that may be involved in receptor regulation (34).

DISCUSSION

The results show that the cDNA we have cloned encodes a high-affinity melatonin receptor. Acute transfection of COS-7 cells with the receptor cDNA results in transient expression of receptors that bind ^{125}I-Mel with high affinity. In addition, specific ^{125}I-Mel binding in acutely transfected cells is inhibited by six ligands in a rank order that is identical to that reported for the endogenous receptor in reptiles, birds, and mammals (4, 7, 9). The ability of the recombinant *Xenopus* melatonin receptor to inhibit the forskolin-stimulated increase in cAMP accumulation in stably transfected CHO cells is consistent with studies of the endogenous receptor showing that a major signal transduction pathway for the high-affinity receptor is inhibition of adenylyl cyclase (13, 15). Finally, receptor mRNA is moderately expressed in the cells whose RNA was used to generate the cDNA library. Thus, the cloned receptor likely mediates the potent effects of melatonin on pigment aggregation in frog melanophores. Structurally, the protein encoded by the melatonin receptor cDNA defines an additional receptor group within the large superfamily of G protein-coupled receptors.

Thirty-six years ago, Lerner and colleagues (35) elucidated the chemical structure of melatonin. Since then, the pineal hormone has been found to have substantial effects on physiology and behavior in a wide range of vertebrates (1–3). Advances in understanding melatonin action at the cellular and molecular level, however, have been slow. With the cloning of a melatonin receptor cDNA, the pace of such advances should quicken. Moreover, knowledge of the *Xenopus* receptor sequence should allow the cloning of melatonin receptor cDNAs from other species, including mammals.

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