Biochemical Pharmacology, Vol. 58, pp. 633–639, 1999.
© 1999 Elsevier Science Inc. All rights reserved.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00134-3

Differential Signaling of Human Mel1a and Mel1b Melatonin Receptors through the Cyclic Guanosine 3′-5′-Monophosphate Pathway

Laurence Petit, Isabelle Lacroix, Pierre de Coppet, A. Donny Strosberg and Ralf Jockers

LABORATOIRE D'IMMUNO-PHARMACOLOGIE MOLÉCULAIRE, CNRS UPR0415, ICGM, 75014 PARIS, FRANCE

ABSTRACT. Cyclic guanosine 3′-5′-monophosphate (cGMP) has recently been shown to constitute a second messenger for *Xenopus laevis* melatonin Mel1c receptors. To verify whether cGMP levels are also modulated by mammalian melatonin receptors, we cloned the genes encoding the human Mel1a and Mel1b receptor subtypes and expressed them in human embryonic kidney cells. Pharmacological profiles and inhibition of forskolin-stimulated adenosine 3′-5′-cyclic monophosphate levels by melatonin confirmed functional expression of high-affinity melatonin receptors. Mel1b receptor-transfected cells modulated cGMP levels in a dose-dependent manner via the soluble guanylyl cyclase pathway. In contrast, Mel1a receptors had no effect on cGMP levels. These results demonstrate that mammalian melatonin receptors modulate cGMP levels and reveal for the first time differences in signaling between melatonin receptor subtypes, which may explain the necessity to express different receptor subtypes. BIOCHEM PHARMACOL **58**;4:633–639, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. G protein-coupled receptors; cGMP; cAMP; circadian rhythm; receptor subtypes; melatonin

Melatonin, the hormone secreted by the pineal gland and the retina, is involved in a number of physiological processes such as circadian rhythm, retinal physiology, reproduction, sleep, and modulation of the immune and vascular systems [1, 2]. Melatonin exerts its functions through specific G protein-coupled receptors [3]. Several of these receptors have now been cloned from different species [4–7] and classified into three subtypes called Mel1a, Mel1b (mammals and chicken), now also called mt1 and MT2, and Mel1c (Xenopus and chicken). Little is known about subtype-specific differences of melatonin receptors. Cloned receptors have affinities in the nanomolar range for the natural ligand, melatonin. Among the three subtypes, Mel1a receptors are the most widely expressed [8–14], whereas Mel1b and Mel1c receptor expression seems to be restricted to a smaller number of tissues [5, 8, 13, 15, 16]. Mel1a and Mel1b receptors are co-expressed at major sites of melatonin function such as the retina and the SCN†, which contain an internal clock generating the circadian rhythm [4, 16]. Mel1a and Mel1c co-localize in various areas of the chick brain [13].

The existence of subtype-specific differences in the signaling properties of melatonin receptors has been suggested, i.e. by the phenotype of Mel1a receptor knockout mice [17]. Whereas the melatonin-induced phase shift in SCN's circadian rhythm was still observed in these mice, the effect of melatonin on the electric activity of the SCN was absent. These results suggest that melatonin's effect on electric activity is specifically mediated by the Mel1a receptor and that the phase-shifting effect may be due to the activation of the Mel1b receptor alone or of both the Mel1a and Mel1b receptors. The Mel1b receptor has indeed recently been shown to be involved in the phase-shifting properties of melatonin [16].

Functional studies have revealed that all three receptor subtypes inhibit adenylyl cyclase via pertussis toxin-sensitive G proteins. Other signaling pathways such as potentiation of phospholipase C activation and arachidonic acid release by Mel1a receptors [18] and activation of Kir3 potassium channels by both Mel1c and Mel1a receptors have been reported [19]. However, no signaling differences between subtypes have been found so far. We previously showed that both allelic variants of the Mel1c receptor from *Xenopus laevis*, Mel1c(α) and Mel1c(β), modulate intracellular cGMP levels when transfected into cell lines [5]. We thus investigated whether human Mel1a and Mel1b subtypes could modulate this signaling pathway.

* Corresponding author: Ralf Jockers, Ph.D., Laboratoire d'Immuno-Pharmacologie Moléculaire, CNRS UPR0415, Institut Cochin de Génétique Moléculaire, 22, rue Méchain, 75014 Paris, France. Tel. 33-140516434; FAX 33-140517210; E-mail:jockers@icgm.cochin.inserm.fr

† *Abbreviations:* SCN, suprachiasmatic nucleus; cGMP, cyclic guanosine 3′-5′-monophosphate; cAMP, cyclic 3′-5′ adenosine monophosphate; IBMX, 3-isobutyl-1-methylxanthine; HEK293, human embryonic kidney cells; RT-PCR, reverse transcriptase-polymerase chain reaction; Mel, melatonin; NAS, *N*-acetyl serotonin; GC, guanylyl cyclase; SNP, sodium nitroprusside; and ODQ, 1*H*-[1,2,4] oxadiazolo [4,3-*a*] quinoxalin-1-one.

Received 1 November 1998; accepted 19 February 1999.

MATERIALS AND METHODS

Cloning and Expression of Human Mel1a and Mel1b Receptors

The human Mel1a receptor cDNA was cloned by RT-PCR from human embryonic RNA and by PCR from human genomic DNA. Human embryonic brain RNA was reverse transcripted as described [5]. Mel1a-specific primers were selected from the published sequence [8]. DNAs were amplified with high-fidelity Pfu or Pwo DNA polymerase as described previously [5]. The N-terminal part corresponding to the first exon was amplified using PCR primers 23S(5′-AACAAAGCTTATGCAGGGC-3′) and 203AS (5′-GATGTTGCCGGCGTTCCTGAGC) (annealing temperature: 58.5°). An NgoMI restriction site was created to facilitate ligation with exon 2. Exon 2 was obtained in three steps. The first 380 bp of exon 2 were amplified using primers 207S (5′-AATTTAGCCGGCAACATCTTT-GTGGTG-3′) and 563AS (5′-TGACGGACTGGGC-GAAGGTG-3′) (annealing temperature: 59°) using a 5kb fragment as template. This 5 kb fragment was amplified from genomic DNA using primers 153S (5′-GACATCCT-GGGCAACCTCCTG-3′) and 750AS (5′-AGGACAA-AAACCACAA ACAT-3′) (annealing temperature: 55.6°) by using the Expand™ long template PCR system (Boerhringer Mannheim). The central part of the exon 2 was obtained with primers 400S (5′-TCAACCGCTACT-GCTACATC-3′) and 1031AS (5′-GAAACCGTCTC-CACTGATGA-3′) (annealing temperature: 55.7°). The 3′ part of the coding region and the 3′ untranslated region were obtained by a modified PCR reaction using the polyadenylation site at the 3′end of the cDNA as primer and the Mel1a-specific primer 700S (5′-CTGACCG-CAAACCCAAACT-3′) (annealing temperature: 56°) located in the coding region. The Mel1b cDNA was isolated from genomic DNA by PCR. This gene contains two exons separated by an intron [4]. The first exon was obtained with primers 1S (5′-CGCCAAGCTTCGAT-GTCAGAGAACGGCTCCTT-3′) and 205AS (5′-TGCGTTCCGGAGCTTGCGATTCCTGAGCAC3′) (with created BspE1 restriction site) (annealing temperature: 67.3°) following the same PCR protocol used for the cloning of the Mel1a receptor. The second exon was obtained with primers 220S (5′AAGCTCCGGAACG-CAGGTAATTTGTTCTTGG-3′) and 1086AS (5′-CTG AGGTACCAGGCTAGAGAGCATCTG-3′) (containing a KpnI cloning site) (annealing temperature: 60.9°). All fragments were ligated with appropriate restriction enzymes and cloned in the expression vector pcDNA3/RSV [5]. The identity of cloned DNAs was verified by dideoxy sequencing. HEK293 cells were transfected using transfection reagent DOTAP. Clones of transfected cells were grown in monolayers in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 4.5 g/liter glucose, and 500 mg/liter G418 (geneticin) at 37° in humidified atmosphere of 5% Co_2. Cell lines expressing melatonin receptors were screened on the basis of 2-(^{125}I)iodomelatonin binding.

(^{125}I)Iodomelatonin Binding Studies

Membrane preparation and 2(^{125}I)iodomelatonin binding were carried out as described recently [5].

Determination of Intracellular cAMP and cGMP Levels

Intracellular cAMP and cGMP levels were determined as described previously [5].

Drugs

2-(^{125}I)Iodomelatonin was purchased from NEN. Melatonin, 6-OH-melatonin, NAS, SNP, and forskolin were from Sigma Chemical Co. 6-Cl-melatonin was from Tocris Cookson. S22153, S20928, and S20098 were supplied by IRIS. Liposomal transfection reagent was from Boehringer Mannheim. Geneticin (G418), penicillin, and streptomycin were from Life Technologies. Foetal bovine serum and Dulbecco's modified Eagle's medium were from GIBCO.

RESULTS

Cloning, Expression, and Pharmacological Characterization of Human Mel1a and Mel1b Receptors

The coding region and the 3′ untranslated region of the Mel1a receptor were cloned by RT-PCR from human embryonic brain RNA. Mel1a-specific PCR primers were selected from the recently published sequence [8]. The human Mel1b receptor gene was isolated from human genomic DNA by PCR using Mel1b-specific primers selected from the recently published sequence [4]. Both receptor DNAs were cloned into the expression vector pcDNA3/RSV and their identity was confirmed by sequencing. No amino acid differences were observed for either receptor when compared to the published sequences. The 3′ untranslated region of the Mel1a receptor had an overall length of 926 bases terminating with a polyA stretch and contained at position 365 a perfect nonameric consensus sequence (TTATTTATA) implicated in mRNA destabilization (Genbank accession number AF085498). Both receptors have now been cloned from several sources, including genomic DNA [8] this report), hypothalmic [8], cerebellum [12], and foetal brain RNA (this report). No variation in the sequence was found, in contrast to the allelic variants observed for the ovine Mel1a and Xenopus Mel1c receptors [5, 7]. Receptor cDNAs were stably transfected into HEK293 cells and their pharmacological profile and signaling properties were studied. Saturation binding studies were performed on membranes prepared from melatonin receptor-expressing HEK293 cells with increasing concentrations of 2-(^{125}I)iodomelatonin (2–1500 pM). From Scatchard representations K_d values of 152 ± 27 pM and 367 ± 9 pM (N = 3) were calculated for Mel1a and

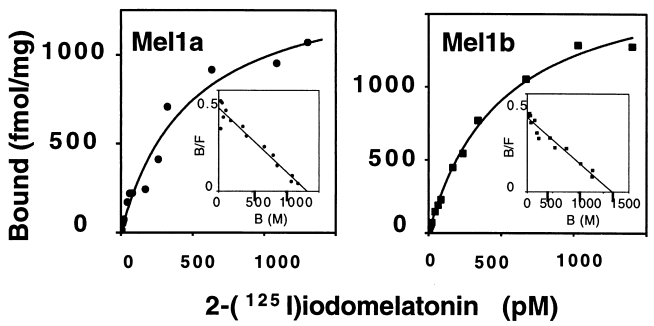

FIG. 1. Saturation isotherms of 2-(^{125}I)iodomelatonin binding in HEK293 cells expressing Mel1a or Mel1b receptors. Membranes prepared from HEK293 cells stably expressing Mel1a or Mel1b receptors were incubated with increasing concentrations of 2-(^{125}I)iodomelatonin. Specific binding is shown; non-specific binding was determined in the presence of 10 μM melatonin. Inset, Scatchard plot of saturation data. Data shown are representative of three experiments.

Mel1b receptors, respectively. In agreement with values in the literature, affinities were higher for Mel1a than for the Mel1b subtype, although the absolute values appear to be higher in HEK293 cells compared to other cell types [20]. Comparable amounts of maximal specific binding sites (B_{max}) of 1270 + 120 and 1500 + 200 fmol/mg of protein (N = 3) for Mel1a and Mel1b, respectively were obtained (Fig. 1). Competition binding studies of 2-(^{125}I)iodomelatonin (Fig. 2) showed the following order of inhibitory potencies: S20098 > melatonin > 6-Cl-Mel > S22153 > 6-OH-Mel > S20928 > NAS for the Mel1a receptor and 6-Cl-Mel > S20098 > Mel > S22153 > 6-OH-Mel > S20928 > NAS for the Mel1b receptor (Table 1). These pharmacological profiles are characteristic for high-affinity melatonin receptors and thus confirm functional receptor expression.

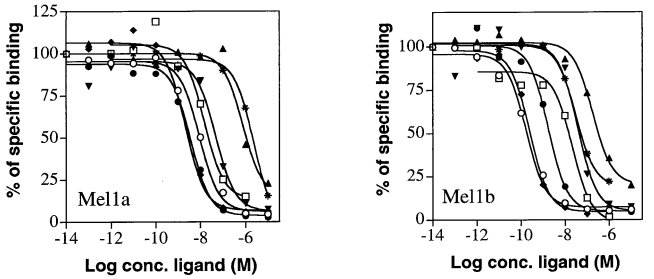

FIG. 2. Competition binding of 2-(^{125}I)iodomelatonin (300 pM) on membranes of HEK293 cells expressing Mel1a or Mel1b melatonin receptors with 6-Cl-melatonin (○), melatonin (●), 6-OH-melatonin (▼), S22153 (□), S20098 (◆), S20928 (❋), and *N*-acetyl-5-OH-tryptamine (▲). Data represent the mean values of duplicates of a representative experiment performed three times and are fitted with Graph Pad Prism version 2.b (1998).

Modulation of cGMP Levels by Melatonin Receptors

We tested whether Mel1a and Mel1b receptors may modulate the cGMP signaling pathway. HEK293 cells expressing either Mel1a, Mel1b, or Mel1c receptors were incubated in the presence of IBMX, a non-specific inhibitor of phosphodiesterases, and increasing concentrations of melatonin. Incubation with IBMX alone increased basal cGMP levels by approximately 10-fold. Stimulation of Mel1c(β) receptors inhibited, as expected, cGMP levels in a dose-dependent manner, with an IC_{50} of approximately 2×10^{-9} M and a maximal inhibition of 60% (Fig. 3). Stimulation of Mel1a receptors had no significant effect on cGMP levels, while activated Mel1b receptors inhibited cGMP levels with an IC_{50} value of approximately 1×10^{-10} M and a maximal inhibition level of 40%. Melatonin had no effect on either basal cGMP levels (unstimulated by IBMX) in any cell line expressing melatonin receptors or on IBMX-stimulated cGMP levels in non-transfected HEK293 cells (data not shown). These results demonstrate that the human Mel1b receptor modulates intracellular cGMP concentration in HEK293 cells when stimulated with physiological melatonin concentrations. Cyclic GMP may be generated from two types of enzymes, soluble and membrane-bound GC. Soluble GC are activated by NO. Incubation of HEK293 cells with the NO donor SNP increased basal cGMP levels approximately 5-fold, indicating a functional soluble GC in these cells (Fig. 4). In contrast, incubation with the atrial naturiuretic peptide, an activator

TABLE 1. Competition binding with 2-(^{125}I)iodomelatonin on HEK 293 cell membranes expressing human Mel1a or Mel1b receptors

	K_i (nM)		
Ligand	Mel1a	Mel1b	Mel1c [See Ref. 5]
6-Cl-melatonin	4.75 ± 0.378	1.1 ± 0.9	ND
Melatonin	3.60 ± 2.32	2.10 ± 0.56	1.30
6-OH-melatonin	19.20 ± 8.70	14.90 ± 4.32	35.70
S22153	8.33 ± 1.48	6.18 ± 2.89	195
S20928	79.00 ± 24.00	37.50 ± 23.5	ND
S20098	0.33 ± 0.064	0.28 ± 0.15	ND
NAS	324.00 ± 61.51	228.00 ± 118	1425

Various ligands were incubated with 300 pM of 2-(^{125}I)iodomelatonin and various concentrations of drugs. K_i values were calculated from IC_{50} values using the Cheng–Prussof formula: $K_i = IC_{50}/1 + L + K_d$. ND, not determined.

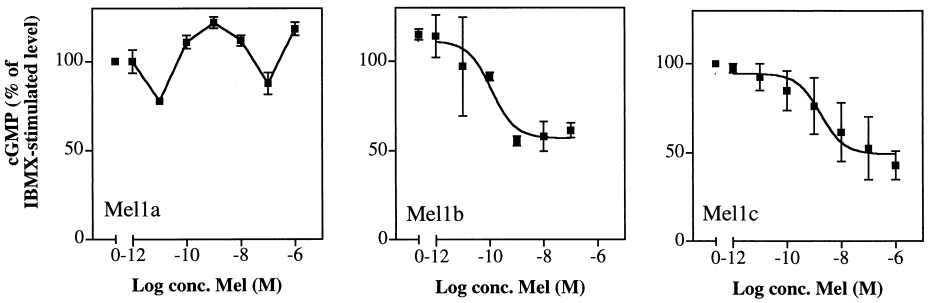

FIG. 3. Subtype-specific modulation of cGMP levels by Mel1a, Mel1b, and Mel1c(β) receptors expressed in HEK cells. HEK293 cells stably transfected with Mel1a, Mel1b, or Mel1c(β) receptor cDNAs were incubated for 15 min at 37° with IBMX (1 mM) in the absence or presence of indicated concentrations of melatonin. Data are presented as % of the IBMX-stimulated level (approximately 10-fold over basal levels). Data presented are the means ± SEM of duplicates of a representative experiment performed four times.

of membrane-bound GC, was without effect (not shown). l-nitro-l-arginine, a competitive inhibitor of NO synthases or ODQ, a selective inhibitor of sGC, prevented the IBMX-induced and SNP-induced increase in cGMP accumulation (Fig. 4). This indicates that basal cGMP levels are due to a basal activity of the soluble GC pathway. Although melatonin inhibited the IBMX-promoted increase in cGMP accumulation, it was without significant effect on SNP-stimulated cGMP levels. This suggests that Mel1b receptors interfere with the soluble GC pathway at a site upstream of sGC.

Functional Coupling of Mel1a and Mel1b Receptors to the cAMP Pathway

To confirm that the Mel1a receptor is functional in HEK293 cells despite the fact that it does not modulate cGMP levels, we verified the melatonin-induced inhibition of forskolin-stimulated cAMP accumulation in HEK293 cells expressing Mel1a receptors (Fig. 5). An IC_{50} value of approximately 1×10^{-10} M was obtained, one comparable to values obtained for Mel1b and Mel1c receptors (IC_{50} value of approximately 5×10^{-11} M). No effect was observed on forskolin-stimulated levels in non-transfected HEK293 cells. All IC_{50} values are in good agreement with values reported for endogenous and transfected high-affinity melatonin receptors [8, 21], confirming functional expression of all three melatonin receptors subtypes in HEK293 cells.

DISCUSSION

Cyclic GMP is an important second messenger in the central nervous system [22], in blood vessels, and in the retina, locations where melatonin receptors are expressed [4, 6, 23, 24]. Melatonin is secreted from the pineal gland in a circadian manner with maximal levels during the night. Cyclic cGMP levels have also been shown to vary in a circadian manner in the cerebral cortex of the chick, where they are correlated to blood rhythms of melatonin [25]. Interestingly, the cycle of cGMP and melatonin concentrations are inversely correlated. This observation is consistent with the inhibition of cGMP levels by activated

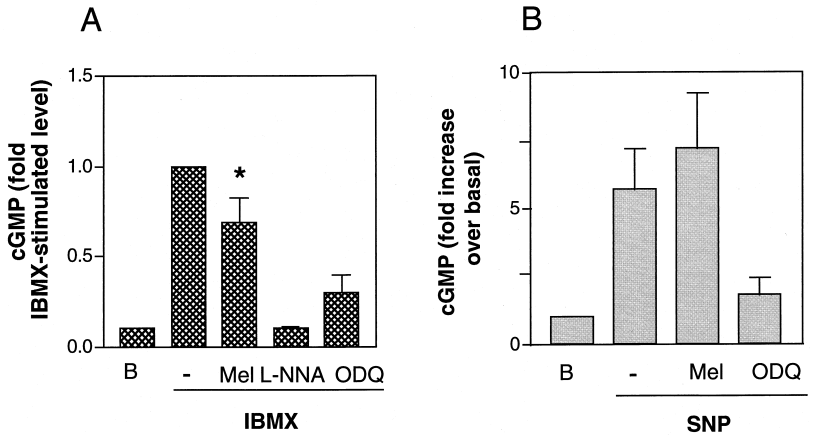

FIG. 4. Modulation of cGMP levels by human Mel1b receptor expressed in HEK293 cells via the soluble guanylyl cyclase pathway. (A) HEK293 cells stably transfected with Mel1b melatonin receptor cDNA were incubated for 15 min with IBMX (1 mM) in the presence or absence of melatonin (1 μM) or L-nitro-L-arginine (L-NNA) (10 μM) or ODQ (1 μM). (B) HEK293 cells stably transfected with Mel1b melatonin receptor cDNA were incubated for 15 min with SNP (300 μM) in the presence or absence of melatonin (1 μM) or ODQ (1 μM). Data represent the means ± SEM of three experiments performed in duplicate.

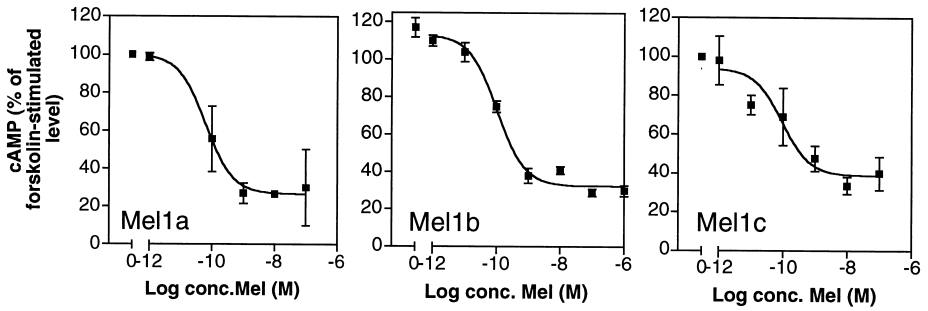

FIG. 5. Melatonin inhibition of forskolin-stimulated cAMP accumulation in HEK293 cells transfected with Mel1a, Mel1b, or Mel1c melatonin receptor cDNAs. Cells stably transfected with Mel1a, Mel1b, or Mel1c melatonin receptor cDNAs were stimulated with forskolin (10 μM) in the presence of an increasing concentration range of melatonin. The stimulated level with 10 μM forskolin represent the 100% value. Data represent the means ± SEM of three experiments performed in duplicate.

melatonin receptors during the night. Furthermore, melatonin has been shown to modulate cGMP levels in cells expressing endogenous receptors such as in neonatal rat pituitary cells and in human prostate [26, 27], suggesting that modulation of cGMP levels may be receptor-mediated. We recently reported that cloned Mel1c receptors from *Xenopus laevis* decrease cGMP levels through the soluble guanylyl cyclase pathway when transfected in different cell lines [5], indicating direct modulation of cGMP levels by high-affinity melatonin receptors. We show here that mammalian melatonin receptors, namely the human Mel1b subtype, also modulate cGMP levels when expressed in HEK293 cells. The IC_{50} value of approximately 1×10^{-10} M is in good agreement with values for inhibition of cAMP accumulation and is in the range of circulating melatonin concentrations and thus of potential physiological relevance.

The Mel1b receptor subtype is expressed at two major sites of melatonin action, the SCN and the retina [4, 16]. In these tissues the second messenger cGMP plays a major role. In the retina of vertebrates, cGMP is involved in light-triggered rhodopsin signaling [28] and in the SCN regulates the circadian rhythm [29–31]. It is important to note that modulation of the circadian rhythm of the SCN by cGMP is restricted to night times and thus correlates well with the time-frame of melatonin receptor activation. Based on our results, the role of melatonin in these and other tissues would be to inhibit cGMP levels which have been stimulated by other means.

The existence of receptors subtypes is a typical feature of G protein-coupled receptors [32] and may confer some of the following advantages on the cell: (i) selectivity for the natural ligand(s); (ii) differential regulation of receptor expression in time (during development) and in a tissue-specific manner; and (iii) selective intracellular signaling due to coupling to different effectors and selective regulation of signaling by desensitization or sensitization. We show here that modulation of cGMP levels is specific for the Mel1b subtype since Mel1a receptors are ineffective in modulating intracellular cGMP levels. These results indicate for the first time the existence of functional differences between the two mammalian melatonin receptor subtypes.

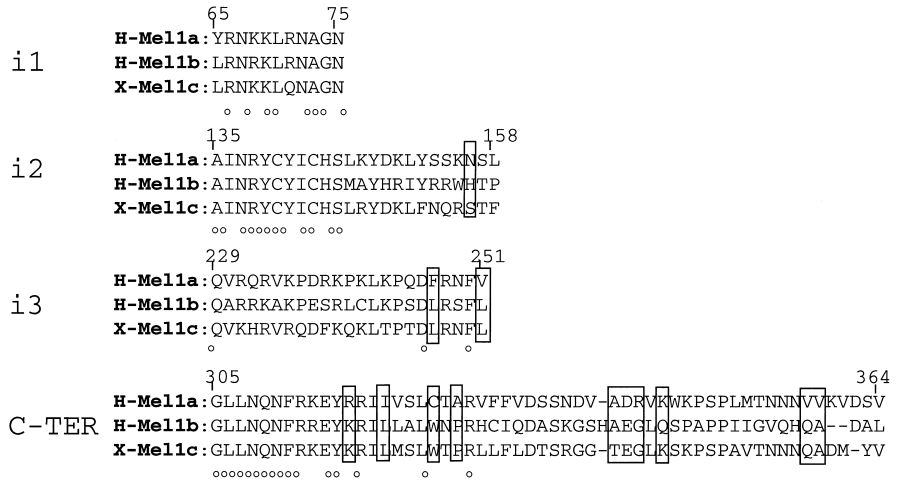

FIG. 6. Alignment of intracellular domains of the human Mel1a and Mel1b receptor and Xenopus Mel1c(β) receptor. i1, i2, i3 are the first, second, and third intracellular loops and C-ter of the carboxy terminal extremity. Amino acid residues identical in all three receptors are dotted. Residues common to Mel1b and Mel1c receptors but different from residue of the Mel1a receptor are boxed. Genbank accession numbers are: U67881 (Mel1c(β); U14108 (Mel1a); U25341 (Mel1b).

Tissues expressing only Mel1a receptors are expected to modulate cAMP levels, whereas in tissues expressing Mel1b and Mel1c receptors both cAMP and cGMP levels may be modulated upon melatonin stimulation. Signaling of melatonin receptors via the cAMP pathway thus appears to be a general feature of melatonin signaling which may be, according to the subtype expressed, complemented by the modulation of the cGMP pathway. Signaling differences may help to identify and classify receptor subtypes in a given tissue.

Molecular determinants involved in receptor signaling are localized in the intracellular receptor domain [32]. Figure 6 shows the alignment of intracellular receptor domains of the human Mel1a and Mel1b and Xenopus Mel1c(β) receptor. Amino acid residues important for cGMP signaling would be expected to be conserved between Mel1b and Mel1c(β) receptors and different from residues of Mel1a receptors. There is a limited set of 10 residues which fulfills these criteria. Further studies will be necessary to determine which residue(s) is/are responsible for signaling differences.

This work was supported by grants from CNRS, the Association pour la Recherche sur le Cancer (N°9890), the University of Paris, and the Intitut de Recherches Internationales Servier (IRIS). We thank Drs. S. Marullo, P. J. Morgan, and P. Barret for their expert advice and help in the initial phase of receptor cloning.

References

1. Cagnacci A, Melatonin in relation to physiology in adult humans. *J Pineal Res* **21:** 200–213, 1996.
2. Viswanathan M, Laitinen JT and Saavedra JM, Expression of melatonin receptors in arteries involved in thermoregulation. *Proc Natl Acad Sci USA* **87:** 6200–6203, 1990.
3. Reppert SM, Melatonin receptors: Molecular biology of a new family of G protein-coupled receptors. *J Biol Rhythms* **12:** 528–531, 1997.
4. Reppert SM, Godson C, Mahle CD, Weaver DR, Slaugenhaupt SA and Gusella JF, Molecular characterization of a second melatonin receptor expressed in human retina and brain: The Mel1b melatonin receptor. *Proc Natl Acad Sci USA* **92:** 8734–8738, 1995.
5. Jockers R, Petit L, Lacroix I, de Coppet P, Barret P, Morgan PJ, Guardiola B, Delagrange P, Marullo S and Strosberg AD, Novel isoforms of Mel1c melatonin receptor modulating intracellular cyclic guanosine 3′-5′-monophosphate levels. *Mol Endocrinol* **11:** 1070–1081, 1997.
6. Mazzucchelli C, Capsoni S, Angeloni D, Fraschini F and Stankov B, Expression of the melatonin receptor in *Xenopus Laevis*: A comparative study between protein and mRNA distribution. *J Pineal Res* **20:** 57–64, 1996.
7. Barrett P, Conway S, Jockers R, Strosberg AD, Guardiola LB, Delagrange P and Morgan PJ, Cloning and functional analysis of a polymorphic variant of the ovine Mel1a melatonin receptor. *Biochim Biophys Acta* **1356:** 299–307, 1997.
8. Reppert SM, Weaver DR and Ebisawa T, Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. *Neuron* **13:** 1177–1185, 1994.
9. Weaver DR and Reppert SM, The Mel1a melatonin receptor gene is expressed in human suprachiasmatic nuclei. *Neuroreport* **8:** 109–112, 1996.
10. Morgan PJ, Williams LM, Barrett P, Lawson W, Davidson G, Hannah L and MacLean A, Differential regulation of melatonin receptors in sheep, chicken and lizard brains by cholera and pertussis toxins and guanine nucleotides. *Neurochem Int* **28:** 259–269, 1996.
11. Drew JE, Williams LM, Hannah LT, Barrett P and Abramovich DR, Melatonin receptors in the human fetal kidney: 2-[^{125}I]Iodomelatonin binding sites correlated with expression of Mel1a and Mel1b receptor genes. *J Endocrinol* **156:** 261–267, 1998.
12. Mazzucchelli C, Pannacci M, Nonno R, Lucini V, Fraschini F and Stankov BM, The melatonin receptor in the human brain: Cloning experiments and distribution studies. *Brain Res Mol Brain Res* **39:** 117–126, 1996.
13. Reppert SM, Weaver DR, Cassone VM, Godson C and Kolakowski LJ, Melatonin receptors are for the birds: Molecular analysis of two receptor subtypes differentially expressed in chick brain. *Neuron* **15:** 1003–1015, 1995.
14. Pang SF, Dubocovich ML and Brown GM, Melatonin receptors in peripheral tissues: A new area of melatonin research. *Biol Signals* **2:** 177–180, 1993.
15. Ebisawa T, Karne S, Lerner MR and Reppert SM, Expression cloning of a high-affinity melatonin receptor from Xenopus dermal melanophores. *Proc Natl Acad Sci USA* **91:** 6133–6137, 1994.
16. Dubocovich M, Yun K, Al-Ghoul W, Benloucif S and Masana M, Melatonin phase-shifts circadian rhythms by MT_2 receptor activation. *FASEB J* **12:** 1211–1220, 1998.
17. Liu C, Weaver DR, Jin X, Shearman LP, Pieschl RL, Gribkoff VK and Reppert SM, Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock. *Neuron* **19:** 91–102, 1997.
18. Godson C and Reppert SM, The Mel1a melatonin receptor is coupled to parallel signal transduction pathways. *Endocrinology* **138:** 397–404, 1997.
19. Nelson CS, Marino JL and Allen CN, Melatonin receptors activate heteromeric G protein-coupled Kir3 channels. *Neuroreport* **7:** 717–720, 1996.
20. Reppert SM, Weaver DR and Godson C, Melatonin receptors step into the light: Cloning and classification of subtypes. *Trends Pharmacol Sci* **17:** 100–102, 1996.
21. Morgan P, Davidson G, Lawson W and Barret P, Both pertussis toxin-sensitive and insensitive G proteins link melatonin receptor to inhibition of adenylate cyclase in the ovine pars tuberalis. *J Neuroendocrinol* **2:** 773–776, 1990.
22. Snyder SH, Nitric oxide: First in a new class of neurotransmitters? *Science* **257:** 94–96, 1992.
23. Krause DN, Barrios VE and Duckles SP, Melatonin receptors mediate potentiation of contractile responses to adrenergic nerve stimulation in rat caudal artery. *Eur J Pharmacol* **276:** 207–213, 1995.
24. Blazynski C and Dubocovich M, Localization of 2-(^{125}I)iodomelatonin binding sites in mammalian retina. *J Neurochem* **56:** 1873–1880, 1991.
25. Guerrero JM, Pablos MI, Ortiz GG, Agapito MT and Reiter RJ, Nocturnal decreases in nitric oxide and cyclic GMP contents in the chick brain and their prevention by light. *Neurochem Int* **29:** 417–427, 1996.
26. Vanecek J and Vollrath L, Melatonin inhibits cyclic AMP and cyclic GMP accumulation in the rat pituitary. *Brain Res* **505:** 157–159, 1989.
27. Gilad E, Matzkin H and Zisapel N, Inactivation of melatonin receptors by protein kinase C in human prostate epithelial cells. *Endocrinology* **138:** 4255–4261, 1997.
28. Yarfitz S and Hurley J, Transduction mechanisms of vertebrate and invertebrate photoreceptors. *J Biol Chem* **269:** 14329–14332, 1994.
29. Liu C and Gillette MU, Cholinergic regulation of the

suprachiasmatic nucleus circadian rhythm via a muscarinic mechanism at night. *J Neurosci* **16:** 744–757, 1996.
30. Liu C, Ding JM, Faiman LE and Gillette MU, Coupling of muscarinic cholinergic receptors and cGMP in noctural regulation of the suprachiasmatic circadian clock. *J Neurosci* **17:** 659–666, 1997.
31. Starkey SJ, Melatonin and 5-hydroxytryptamine phase-advance the rat circadian clock by activation of nitric oxide synthesis. *Neurosci Lett* **211:** 199–202, 1996.
32. Strosberg AD, Structure/function relationship of proteins belonging to the family of receptors coupled to GTP-binding proteins. *Eur J Biochem* **196:** 1–10, 1991.