The Cloning of a Family of Genes That Encode the Melanocortin Receptors

Kathleen G. Mountjoy, Linda S. Robbins, Marty T. Mortrud, Roger D. Cone*

Melanocyte-stimulating hormone (MSH) and adrenocorticotropic hormone (ACTH) regulate pigmentation and adrenal cortical function, respectively. These peptides also have a variety of biological activities in other areas, including the brain, the pituitary, and the immune system. A complete understanding of the biological activities of these hormones requires the isolation and characterization of their corresponding receptors. The murine and human MSH receptors (MSH-Rs) and a human ACTH receptor (ACTH-R) were cloned. These receptors define a subfamily of receptors coupled to guanine nucleotide–binding proteins that may include the cannabinoid receptor.

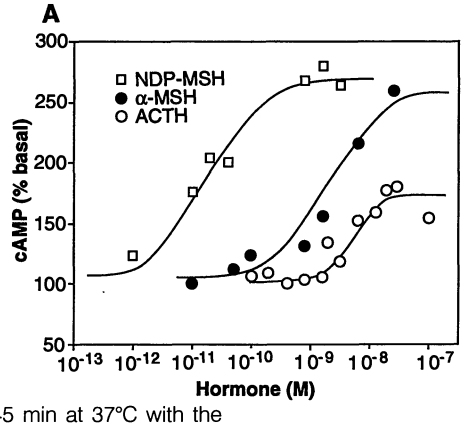
Proopiomelanocortin (POMC) is expressed primarily in the pituitary and in limited regions in the brain and periphery. It is processed into a large and complex family of peptides with an array of biological activities (*1*). The three major activities of these peptides include regulation of adrenal glucocorticoid and aldosterone production (through ACTH), control of melanocyte growth and pigment production (through α-MSH, β-MSH, β-lipotropic hormone, and ACTH), and analgesia (through β-endorphin). ACTH and the opiate peptide β-endorphin are co-expressed in the anterior pituitary in response to corticotropin-releasing factor. The orchestrated production of these two peptides induces increased metabolism and analgesic effects in response to stress. A number of biological activities are attributed to the melanocortins, ACTH and MSH, in tissues other than the melanocyte and adrenal cortex. Binding sites for ACTH and MSH are found throughout the brain (*2*) where they affect learning, memory, and attention (*3*); α-MSH regulates temperature control in the septal region of the brain (*4*) and stimulates prolactin release from the pituitary (*5*). Both peptides have numerous immunomodulatory properties. For example, α-MSH inhibits the inflammatory and immunostimulatory actions of interleukin-1 on murine fibroblasts and thymocytes, respectively (*6*). Receptors for the melanocortins in the nervous system and the immune system also exhibit distinct pharmacological properties when compared with those on melanocytes and adrenocortical cells, which suggests the existence of multiple melanocortin receptors (*2*, *3*, *6*, *7*).

K. G. Mountjoy, L. S. Robbins, M. T. Mortrud, Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences University, Portland, OR 97201.
R. D. Cone, Vollum Institute for Advanced Biomedical Research and Department of Cell Biology and Anatomy and Department of Biochemistry and Molecular Biology, Oregon Health Sciences University, Portland, OR 97201.

*To whom correspondence should be addressed.

MSH and ACTH bind to receptors that couple to heterotrimeric guanine nucleotide–binding proteins (G proteins) that activate adenylyl cyclase (*8–11*) and were therefore expected to have sequence similarity with other members of this large gene family (*12*). A DNA fragment that encoded part of the MSH-R was isolated by polymerase chain reaction (PCR) (*13*) amplification of cDNA from a human melanoma that contained a large number of MSH binding sites (*14*). Initially, we determined by DNA sequencing that two PCR subclones encoded G protein–coupled receptors related to one another, and we also determined by Northern (RNA) hybridization analysis that these two clones were expressed specifically in melanocytes and adrenal cortex. We then used the subclone expressed in the original melanoma sample to screen a mouse cDNA library made from the Cloudman S91 melanoma cell line (*15*). All eight clones thus identified were encoded by a single gene. A human genomic library was also screened at high stringency with both human PCR fragments in order to isolate the human MSH-R and ACTH-R genes. Two different genes were isolated that corresponded to the two PCR fragments and were found to encode related G protein–coupled receptors.

A 2.1-kb melanoma cDNA clone was inserted into the pcDNA I-Neo vector (Invitrogen); we demonstrated that the clone encodes an MSH-R with functional coupling to the stimulatory G protein (G_s) (Fig. 1). We analyzed intracellular adenosine 3′,5′-monophosphate (cAMP) concentrations in transfected 293 cells incubated with the melanotropic peptides α-MSH and β-MSH; the MSH analog Nle^4, D-Phe^7-α-MSH (NDP-MSH) (*16*); and γ-MSH and ACTH. None of these peptides induced any change in the amount of intracellular cAMP in cells that contained the vector alone. Cells that expressed the



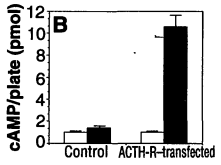


Fig. 1. (**A**) Functional expression of the murine MSH-R. The *x*-axis indicates the concentration of each hormone, and the *y*-axis indicates the percent of basal concentrations of intracellular cAMP after each treatment. Points indicate the mean of duplicate incubations, and the standard error did not exceed 15% for any data points. Human 293 cells were transfected with vector alone or vector plus the 2.1-kb MSH-R cDNA with a calcium phosphate procedure (*29*), and stable populations of transfected cells were selected in medium containing G418 (Gibco). Cells (~1 × 10^6) were plated in six-well dishes, washed once with Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin and 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX), and then incubated for 45 min at 37°C with the concentrations of the melanotropic peptides shown (Bachem, Torrance, California). After hormone treatment, the cells were washed twice with PBS, and intracellular cAMP was extracted. The cells were lysed with 60% ethanol (1 ml) and centrifuged to remove cellular debris, and the supernatant was lyophilized. Intracellular cAMP concentrations were determined with an assay (Amersham) that measures displacement of [5,8-^{3}H]cAMP from a high-affinity cAMP-binding protein (*30*). No increase in the amount of cAMP was detected after the same treatment of cells transfected with the vector that lacked the MSH-R cDNA. (**B**) Functional expression of the human ACTH-R. Bar graph shows the elevation of intracellular cAMP in Cloudman S91 melanoma cells transfected with vector alone (Control) or with the expression vector containing a human genomic ACTH-R fragment (ACTH-R–transfected) in response to a mock treatment (open bars) or 1 nM ACTH (solid bars). Each bar shows the mean and standard error of duplicate incubations and is representative of three independent experiments. A 2.9-kb Acc I–Hind III human genomic DNA fragment with approximately 200 nucleotides of sequence 5′ of the apparent translational start site was inserted into the pcDNA I-Neo expression vector. Cloudman S91 melanoma cells were transfected with this vector or the control vector with no insert. Cells were selected in G418 (Gibco), stimulated with ACTH, and analyzed for intracellular cAMP content as in (A).

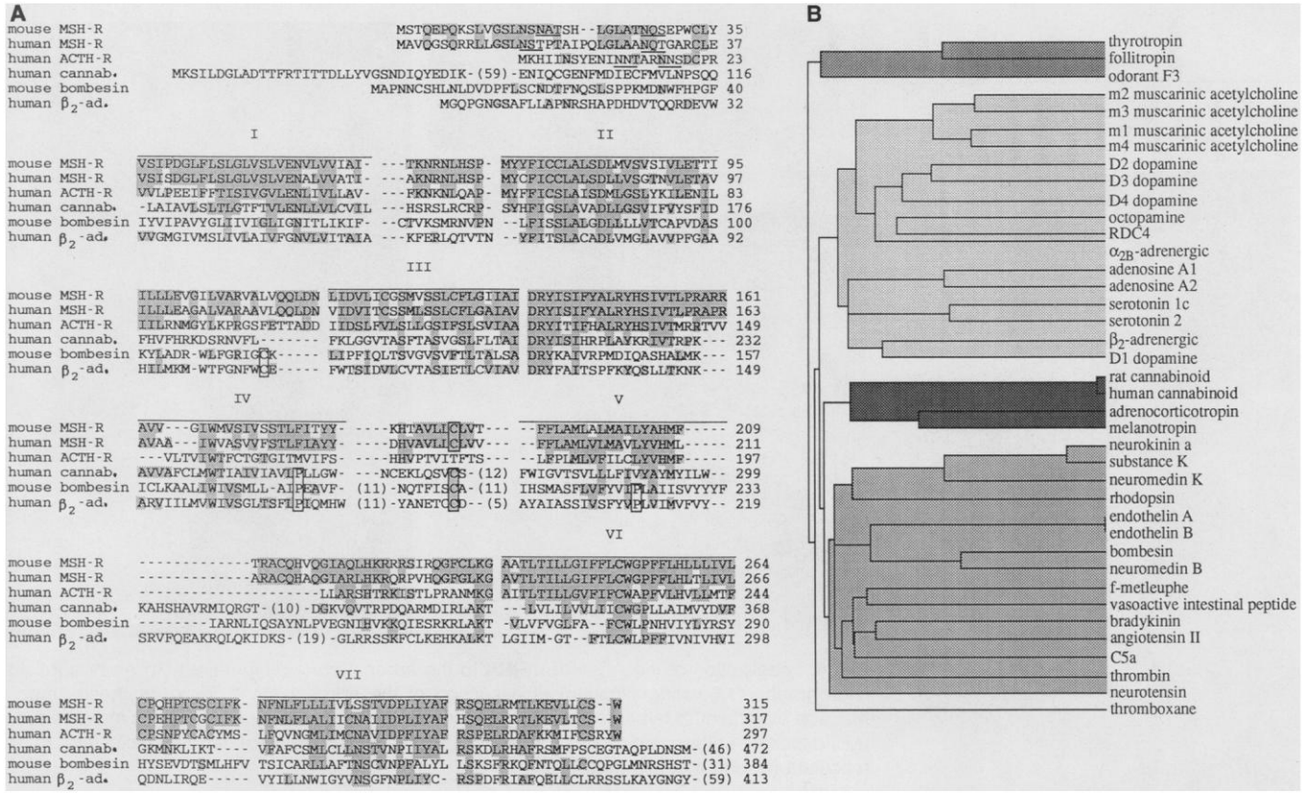


Fig. 2. (**A**) Amino acid sequences of the murine MSH-R, the human MSH-R, and the human ACTH-R. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. Polypeptide sequences were aligned with the Genalign program, version 5.4 (Intelligenetics, Mountain View, California). Amino acid identities among the melanocortin receptors or between the melanocortin receptors and the cannabinoid (cannab.), bombesin, or β_2-adrenergic receptors (β_2-ad.) are shaded. Predicted transmembrane domains are numbered and indicated by a solid black line. The numbers in parentheses indicate the number of amino acids that have been deleted in order to align the sequences shown. The extracellular cysteine residues and the fourth and fifth transmembrane domain proline residues conserved in most G protein–coupled receptors are boxed. Putative N-linked glycosylation sites in the melanocortin receptors are underlined. We used PCR to generate fragments of the MSH and ACTH receptors, which we in turn used to screen genomic and cDNA libraries for full-length clones (*31*). The entire coding sequence of the human ACTH-R was found in a 3.9-kb Hind III fragment, whereas the human MSH-R coding sequence was found in a 7.5-kb Hind III fragment. The human MSH-R and ACTH-R genes did not contain introns in the coding sequence; they were colinear with the coding sequences of the mouse MSH-R cDNA and a bovine ACTH-R cDNA (*18*). The predicted molecular sizes for the mouse and human MSH receptors (~35 kD) are less than values reported from biochemical characterization of the receptor (~45 kD) (*32*), which suggests that glycosylation of these receptors occurs at consensus N-linked glycosylation sites (Asn–any amino acid–Ser or Thr) present in the NH_2-terminus. The nucleotide sequences of the mouse MSH-R, the human MSH-R, and the human ACTH-R have been submitted to the GenBank/European Molecular Biology Laboratory database under acquisition numbers X65633, X65634, and X65635. (**B**) Multiple sequence alignment of melanocortin receptors and other representative members of the G protein–coupled receptor family. Amino acid sequences were obtained from the Protein Identification Resource (PIR) database and aligned with the melanocortin receptors with the Pileup program, version 7.0, from the Genetics Computer Group analysis software (Madison, Wisconsin) (*27*). The branch length is proportional to the similarity between sequences, which suggests that the melanocortin and cannabinoid receptors are members of a distinct subfamily of the G protein–coupled receptors.

murine MSH-R responded to melanotropic peptides with a two- to threefold elevation of intracellular cAMP. The median effective concentration (EC_{50}) values determined for α-MSH (2.0×10^{-9} M), ACTH (8.0×10^{-9} M), and NDP-MSH (2.8×10^{-11} M) correspond to reported values (*7, 16*). The β-MSH peptide had an EC_{50} value comparable to that of α-MSH, whereas γ-MSH had little or no activity (*17, 18*). Thus, this receptor is specific for peptides with melanotropic activity.

One of the two human genomic clones encodes a human MSH-R gene, whereas the other encodes an ACTH-R that also has functional coupling to G_s (Fig. 1). Intracellular cAMP concentrations were examined in Cloudman S91 melanoma cells transfected with the pcDNA I-Neo vector with a 2.9-kb Acc I–Hind III genomic DNA fragment. Stimulation of these cells with 1 nM ACTH produced a greater than tenfold increase in intracellular cAMP (Fig. 1). Stimulation of cells transfected with the vector alone had no effect. The Cloudman S91 melanoma cell line was used because it was a more sensitive assay system for ACTH-R expression; in initial experiments we were unable to detect significant amounts of ACTH-R expression in the 293 cell line. We have not yet determined EC_{50} values for adenylyl cyclase activation by the ACTH-R using the Cloudman cell line because the endogenous MSH-R is activated at concentrations of ACTH above 2 nM.

The coding regions of the murine MSH-R, the human MSH-R, and the human ACTH-R were sequenced, and the predicted amino acid sequences were aligned with three representative G protein–coupled receptors: the cannabinoid receptor (*19*), the bombesin receptor (*20*), and the β_2-adrenergic receptor (*21*) (Fig. 2). The human MSH-R amino acid sequence was 76% identical and colinear with the murine MSH-R cDNA sequence, whereas the human ACTH-R was ~39% identical and nearly colinear with the human MSH-R (Fig. 2). A comparison of the MSH-R and ACTH-R amino acid sequences with the cannabinoid (~32% identity), β_2-adrener-

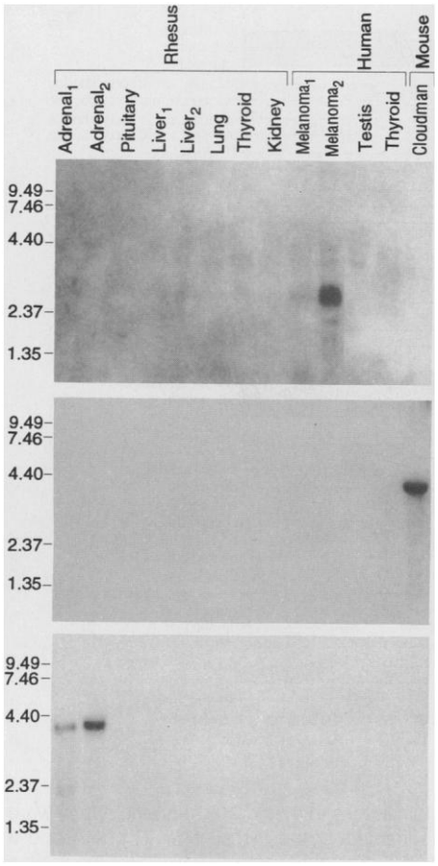


Fig. 3. Distribution of expression of mRNAs for the murine MSH-R, the human MSH-R, and the human ACTH-R by Northern blot analysis (*33*). We successively hybridized a single nitrocellulose filter with human MSH-R (top), murine MSH-R (middle), or human ACTH-R (bottom) probes at moderate stringency to allow for cross-reaction between human and rhesus sequences. Numbers on the left indicate the migration of RNA size markers in kilobases (BRL). Tissues used are as indicated. $Adrenal_1$ and $Liver_1$ were from an 11-year-old rhesus macaque, whereas $Adrenal_2$ and $Liver_2$ were from a female rhesus macaque, 1.5 years old. Human $Melanoma_2$ was the melanoma sample used for the initial PCR amplification of the MSH-R and ACTH-R receptor sequences.

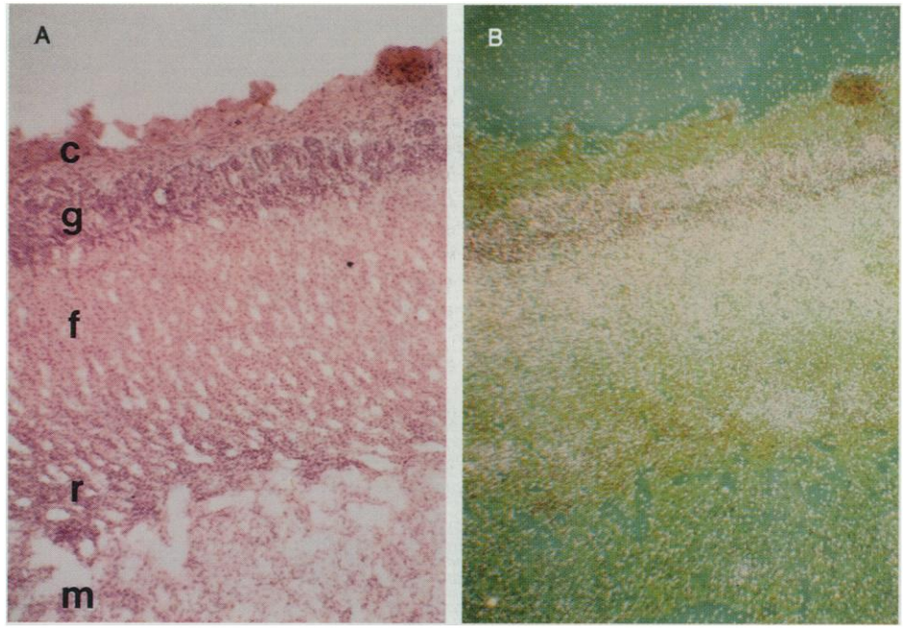


Fig. 4. Localization of the ACTH-R mRNA to the adrenal cortex. Light-field (**A**) and dark-field (**B**) micrographs (×60 magnification) of a section of the adrenal gland from the rhesus macaque *Macaca mulatta* after hybridization of a human ACTH-R probe (*34*). Bright spots indicate areas of hybridization. Letters indicate capsule (c), zona glomerulosa (g), zona fasciculata (f), zona reticulata (r), and medulla (m).

gic (~30% identity), and bombesin (~25% identity) receptors allows us to define the melanocortin receptors as a subfamily of the major G protein–coupled receptor gene family. No primary amino acid sequence homology was detected between the melanocortin receptors and the glutamate (*22*) or parathyroid hormone–calcitonin (*23*) receptor gene families. The melanocortin receptors are the smallest G protein–coupled receptors identified to date (297 to 317 amino acids) because they have a short NH_2-terminal extracellular domain, a short COOH-terminal intracellular domain, unusually short fourth and fifth transmembrane-spanning domains, and a small second extracellular loop. The melanocortin receptors lack several amino acid residues present in most G protein–coupled receptors (*12*). These include the proline residues in the fourth and fifth transmembrane domains, which are thought to introduce a bend in the α-helical structure of the transmembrane domains and to participate in the formation of the binding pocket (*24*), and one or both of the cysteine residues thought to form a disulfide bond between the first and second extracellular loops (*25, 26*). Multiple sequence alignment of the MSH-R and ACTH-R with 37 other G protein–coupled receptors with a method that groups sequences according to the least difference between each pair-wise combination (*27*) suggests that the melanocortin receptors are not closely related to the other G protein–coupled receptors that recognize peptide ligands, such as the receptors for bombesin (*20*) or substance K (*28*). They are instead most closely related to the receptor for Δ9-tetrahydrocannabinol (*19*) and form a subfamily distinct from the peptide and amine receptors (Fig. 2). The cannabinoid receptor also lacks the conserved proline in transmembrane region V and the cysteine in the first extracellular loop.

The MSH-R mRNA was expressed in melanocytes, and the ACTH-R mRNA was expressed in adrenal tissue (Fig. 3). The murine MSH-R is encoded predominantly by a single mRNA species of 4 kb (Fig. 3), whereas the human MSH-R was encoded predominantly by a 3-kb species in two melanoma samples (Fig. 3). Both the 3- and 4-kb mRNA species were seen at approximately equal stoichiometry in two primary human melanocyte samples (*18*). Several human melanoma samples and two primary human melanocyte samples examined contained small amounts of MSH-R mRNA (*18*). In contrast, the amount of MSH-R mRNA was increased in some melanoma samples tested (*18*) (Fig. 3).

Northern analysis of tissues from the rhesus macaque performed under high stringency conditions demonstrated the existence of a cross-reacting 4-kb ACTH-R mRNA species specific to the rhesus adrenal gland (Fig. 3). The human ACTH-R was encoded predominantly by a single mRNA species of approximately 4 kb in the human adrenal gland although several minor species were present as well (*18*). In situ hybridization of a fragment of the human ACTH-R to sections of rhesus adrenal gland localized the expression of this receptor exclusively to the cortex; no hybridization to the medulla or capsule was apparent (Fig. 4). In the cortex, ACTH-R expression was found primarily across the zona fasciculata, the site of glucocorticoid production, and in the cortical half of the zona glomerulosa, the site of aldosterone synthesis. A small band of hybridization was also detected adjacent to the medulla in what is probably the zona reticulata.

Southern (DNA) analysis of human genomic DNA hybridized at low stringency to the human MSH-R probe indicated the

existence of as many as five or six members of the melanocortin receptor gene family (*18*). The isolation of the MSH-R, the ACTH-R, and perhaps additional melanocortin receptor genes should provide a more detailed understanding of the control of melanocyte and adrenal cortical function and help explain the actions and pharmacology of melanocortins in the brain and other tissues.

REFERENCES AND NOTES

1. E. Hanneman *et al.*, in *Peptide Hormones as Prohormones: Processing, Biological Activity, Pharmacology*, J. Martinez, Ed. (Horwood, Chichester, United Kingdom, 1989), pp. 53–82.
2. J. B. Tatro, *Brain Res.* **536**, 124 (1990).
3. D. DeWied and J. Jolles, *Physiol. Rev.* **62**, 977 (1982).
4. M. T. Murphy *et al.*, *Science* **221**, 192 (1983).
5. E. Ellerkmann, G. M. Nagy, L. S. Frawley, *Endocrinology* **130**, 133 (1992).
6. J. G. Cannon, J. B. Tatro, S. Reichlin, C. A. Dinarello, *J. Immunol.* **137**, 2232 (1986).
7. J. B. Tatro *et al.*, *Cancer Res.* **50**, 1237 (1990).
8. D. I. Buckley and J. Ramachandran, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7431 (1981).
9. D. G. Grahame-Smith *et al.*, *J. Biol. Chem.* **242**, 5535 (1967).
10. L. M. Mertz and K. J. Catt, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8525 (1991).
11. J. Pawelek, *J. Invest. Dermatol.* **66**, 210 (1976).
12. W. C. Probst, L. A. Snyder, D. I. Schuster, J. Brosius, S. C. Sealfon, *DNA Cell Biol.* **11**, 1 (1992).
13. R. K. Saiki *et al.*, *Science* **230**, 1350 (1985).
14. J. B. Tatro *et al.*, *J. Clin. Invest.* **85**, 1825 (1990).
15. J. M. Pawelek, *Yale J. Biol. Med.* **58**, 571 (1985).
16. T. K. Sawyer *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5754 (1980). 4-Norleucine, 7-D-phenylalanine α-melanocyte–stimulating hormone (NDP-MSH) is an extremely potent synthetic α-MSH analog that is resistant to enzymatic degradation.
17. A. Slominski, R. Costantino, J. Wortsman, R. Paus, N. Ling, *Life Sci.* **50**, 1103 (1992).
18. K. G. Mountjoy and R. D. Cone, unpublished data.
19. C. Gerard *et al.*, *Nucleic Acids Res.* **18**, 7142 (1990); L. A. Matsuda *et al.*, *Nature* **346**, 561 (1990).
20. E. R. Spindel, E. Giladi, P. Brehm, R. H. Goodman, T. P. Segerson, *Mol. Endocrinol.* **4**, 1956 (1990).
21. B. K. Kobilka *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 46 (1987).
22. M. Masu *et al.*, *Nature* **349**, 760 (1991).
23. H. Y. Lin *et al.*, *Science* **254**, 1022 (1991); H. Jüppner *et al.*, *ibid.*, p. 1024.
24. M. L. Applebury and P. A. Hargrave, *Vision Res.* **26**, 1881 (1986).
25. R. A. F. Dixon *et al.*, *EMBO J.* **6**, 3269 (1987).
26. S. S. Karnik, T. P. Sakmar, H. B. Chen, H. G. Khorana, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8459 (1988).
27. M. Devereux, U. Haeberli, O. Smithies, *Nucleic Acids Res.* **12**, 387 (1984).
28. Y. Masu *et al.*, *Nature* **329**, 836 (1987).
29. C. Chen and H. Okayama, *Mol. Cell. Biol.* **7**, 2745 (1987).
30. A. G. Gilman, *Proc. Natl. Acad. Sci. U.S.A.* **67**, 305 (1970).
31. Sequences of the human MSH-R and ACTH-R were originally obtained by PCR with human melanoma cDNA as a template and degenerate oligonucleotides from transmembrane domains III and VI as primers [Q.-Y. Zhou *et al.*, *Nature* **347**, 76 (1990)]. The transmembrane domain III oligonucleotide was 5′-GAGTCGACCTGTG[CT]G[CT][CG]AT[CT][AG]CIIT[GT]GAC[AC]G[CG]TAC-3′, and the transmembrane domain VI oligonucleotide was 5′-CAGAATTCAG[AT]AGGGCAIC-CAGCAGAI[CG][AG][CT]GAA-3′. Brackets indicate variable nucleotide positions containing one of the species indicated; I represents positions containing an inoside nucleotide. We then labeled a 300-bp Eco RI–Sal I PCR fragment by random priming and used it to screen a Cloudman S91 melanoma line cDNA library constructed in the λZAP vector (Stratagene). Eight independent clones were isolated after hybridization at moderate stringency [40% formamide, 1 M NaCl, 50 mM tris (pH 7.5), sodium pyrophosphate (0.1%), SDS (0.2%), salmon sperm DNA (100 μg/ml), and ×10 Denhardt's] at 42°C for 18 hours. We determined that the clones were derived from the same gene by restriction mapping and partial sequencing. Clone A was sequenced with the dideoxy method [F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)]. Gene sequences encoding the human MSH-R and ACTH-R were isolated from a human genomic DNA library in the EMBL3 vector by random primer labeling of both PCR fragments and hybridization at high stringency (50% formamide at 42°C).
32. J. E. Gerst, J. Sole, E. Hazum, Y. Salomon, *Endocrinology* **123**, 1792 (1988); F. Solca, W. Siegrist, R. Dorzdz, J. Girard, A. N. Eberle, *J. Biol. Chem.* **264**, 14277 (1989).
33. RNA was prepared from each of the tissues shown with the guanidinium thiocyanate procedure [J. M. Chirgwin, A. E. Przbyla, R. J. MacDonald, W. J. Rutter, *Biochemistry* **18**, 5924 (1979)]. Equivalent amounts of total RNA (20 μg) were analyzed by electrophoresis on a 2.2 M formaldehyde–1.2% agarose gel, transferred to a Nytran filter (Schleicher and Schuell, Keene, NH), and hybridized with fragments spanning transmembrane domains III to VI of the human MSH-R, the murine MSH-R, or the human ACTH-R under moderately stringent conditions [45% formamide, 1 M NaCl, 50 mM tris (pH 7.5), sodium pyrophosphate (0.1%), SDS (0.2%), 100 μg/ml salmon sperm DNA, and ×10 Denhardt's] at 42°C for 18 hours. Before rehybridization, the probe was removed by incubation of the filter in 50% formamide, 10 mM tris (pH 8), 1 mM EDTA, and 0.1% SDS at 68°C for 1 hour. Ethidium bromide staining of ribosomal RNAs confirmed that equivalent amounts of intact RNA were loaded in each lane.
34. Adrenal tissue from a 1-year-old rhesus macaque was fixed for 24 hours in formalin (10%) in phosphate-buffered saline (PBS) and then incubated for 24 hours in sucrose (30%) in PBS. Sections (20 μm) were prepared and hybridized with a 648-nucleotide ^{35}S-labeled RNA antisense probe complementary to the coding sequence spanning transmembrane domains I through VI of the human ACTH-R. Sections were hybridized in 65% formamide in 0.26 M NaCl, ×1.3 Denhardt's, 13 mM tris (pH 8), 1.3 mM EDTA, and dextran sulfate (13%) at 65°C for 18 hours.

35. We thank S. Reichlin and J. Tatro for human melanoma samples, C. Passavant for the human genomic DNA library, R. Halaban for primary murine and human melanocytes and helpful discussions, P. Stenzel for human adrenal samples, the Oregon Regional Primate Center, Beaverton, for rhesus macaque tissue samples, and J. Shiigi and J. Tasnady for technical assistance in the preparation of the manuscript. Supported by NIH grant R01 DK43859-02 (R.D.C.).

28 April 1992; accepted 1 July 1992

Target Enzyme Recognition by Calmodulin: 2.4 Å Structure of a Calmodulin-Peptide Complex

William E. Meador, Anthony R. Means, Florante A. Quiocho*

The crystal structure of calcium-bound calmodulin (Ca^{2+}-CaM) bound to a peptide analog of the CaM-binding region of chicken smooth muscle myosin light chain kinase has been determined and refined to a resolution of 2.4 angstroms (Å). The structure is compact and has the shape of an ellipsoid (axial ratio ~2:1). The bound CaM forms a tunnel diagonal to its long axis that engulfs the helical peptide, with the hydrophobic regions of CaM melded into a single area that closely covers the hydrophobic side of the peptide. There is a remarkably high pseudo-twofold symmetry between the closely associated domains. The central helix of the native CaM is unwound and expanded into a bend between residues 73 and 77. About 185 contacts (<4 Å) are formed between CaM and the peptide, with van der Waals contacts comprising ~80% of this total.

Calmodulin (CaM) is a 148–amino acid protein present in all eukaryotic cells that serves as the primary receptor for intracellular Ca^{2+} (*1*). The three-dimensional structure of native CaM with four bound Ca^{2+} showed a dumbbell-shaped molecule in which two structurally similar globular domains, each containing a pair of Ca^{2+}-binding sites, were separated by an eight-turn solvent-exposed central α helix (*2*). Whereas the presence of this central helix in solution has been questioned (*3*), several lines of evidence suggested that some amino acids within this region, as well as in both hydrophobic pockets formed within the globular domains, were critical for target interaction (*1*, *4*). Calmodulin activates more than 20 enzymes in eukaryotic cells, and the CaM-binding domain has been identified in several enzymes. In each case, the CaM-binding regions consist of 18 amino acids in linear sequence, and, whereas they show considerable sequence diversity, most are predicted to form amphipathic helices (*5*). Consequently, synthetic peptide analogs of CaM-binding regions have been used to study the interaction with CaM by a variety of biophysical and biochemical techniques.

One of the most intensely studied CaM-dependent enzymes is the smooth muscle form of myosin light chain kinase (smMLCK).

W. E. Meador and F. A. Quiocho, Howard Hughes Medical Institute and Department of Biochemistry, Baylor College of Medicine, Houston, TX 77030.
A. R. Means, Department of Pharmacology, Duke University Medical Center, Durham, NC 27710.

*To whom correspondence should be addressed.