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Evolutionary Aspects in Evaluating Mutations in the Melanocortin 4 Receptor

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More than 70 missense mutations have been identified in the human melanocortin 4 receptor (MC4R), and many of them have been associated with obesity. In a number of cases, the causal link between mutations in MC4R and obesity is controversially discussed. Here, we mined evolution as an additional source of structural information that may help to evaluate the functional relevance of naturally occurring variations in MC4R. The sequence information of more than 60 MC4R orthologs enabled us to identify residues that are important for maintaining receptor function. More than 90% of all inactivating mutations found in obese patients were located at amino acid positions that are highly conserved during 450 million years of MC4R evolution in vertebrates. However, for a reasonable number of MC4R variants, we found no correlation between structural conservation of the mutated position and the reported functional consequence. By reevaluating selected mutations in the MC4R, we demonstrate the usefulness of combining functional and evolutionary approaches. (*Endocrinology* 148: 4642–4648, 2007)

THE MELANOCORTIN 4 receptor (MC4R) belongs to the family of rhodopsin-like G protein-coupled receptors (GPCR). MC4R is activated by MSHs and signals via G_s protein/adenylyl cyclases. The hypothalamic melanocortin system has been implicated in food intake, energy balance, and body weight control (1). Consistently, mutations in MC4R have been made responsible for monogenetic obesity in humans (2, 3) and other mammals (4, 5). To date, more than 80 missense, nonsense, and frame-shifting mutations have been identified or associated with obesity in humans. These mostly heterozygously occurring MC4R mutations are implicated in 1–6% of early-onset or severe adult obesity cases. It has been suggested that already a small decrease in overall MC4R activity can cause obesity (6). Thus, alterations of MC4R function have been reported to be very multiform, ranging from a total suppression of receptor activation in response to agonists to an alteration of the basal activity of the MC4R. This is in contrast to most other GPCR-related diseases where pathway inactivation is usually coupled to prominent functional receptor defects (7). This may highlight the central role of the MC4R for adipostat in humans. However, one has to consider reasonable problems associating the genotype with *in vitro*/*in vivo* phenotype because an obese phenotype can result from a multitude of factors. Classically, the functional relevance of missense mutations is tested *in vitro*, *e.g.* in cAMP assays, radioligand binding studies, and tests for subcellular distribution of mutant receptors. However, missense mutations that have been associated with obesity are also found in nonobese individuals (8). Especially in cases of no correlation, additional information is required to substantiate causal genotype/phenotype relations.

The availability of in-depth genome sequence data for human, chimpanzee, and many other vertebrates as well as of numerous invertebrates has provided the unique opportunity to apply comparative approaches to identify conserved sequences. These new aspects have been implemented in studying structure-function relationships and even three-dimensional receptor models, *e.g.* for melanocortin receptors (9, 10) but may be useful also in evaluating naturally occurring mutations in GPCRs (11).

Herein, we took advantage of the sequence information of over 60 MC4R orthologs (43 were fully or partially cloned for this study) to reevaluate missense mutations in the MC4R that have been associated with obesity. We show that most residues affected by loss-of-function missense mutation are highly conserved during 450 million years of MC4R evolution (conservation also shown by Ref. 12). Consequently, mutations that affect residues highly variable among MC4R orthologs had no significant influence on receptor function. However, we identified and functionally reevaluated several missense mutations that were not compatible with evolutionary conservation data. Our results clearly support the power of large ortholog sequence data sets when used in combination with functional testing.

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Abbreviations: ECL, Extracellular loop; GPCR, G protein-coupled receptor; HA, hemagglutinin; MC4R, melanocortin 4 receptor; TMD, transmembrane domain.

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TABLE 1. Structural comparison of MC4R orthologs

Domain	Length (min/max)	Conservation between mammalian and fish orthologs (% identity)
Full-length	323/339 (41)	67.3 ± 2.3 (25:12)
N terminus	35/49 (41)	29.2 ± 6.8 (25:12)
C terminus	23/31 (41)	54.4 ± 7.8 (25:12)
ECL1	21/22	28.3 ± 5.1 (56:16)
ECL2	12	71.5 ± 8.5 (53:14)
ECL3	12	80.3 ± 6.8 (48:17)
ICL1	11	90.5 ± 3.9 (53:17)
ICL2	20	78.5 ± 3.6 (56:15)
ICL3	21	69.2 ± 7.0 (53:18)
TMD1	24	68.4 ± 3.9 (30:14)
TMD2	23	92.7 ± 3.7 (53:17)
TMD3	23	84.4 ± 3.5 (55:16)
TMD4	24	61.2 ± 4.4 (54:14)
TMD5	22	85.6 ± 5.9 (53:16)
TMD6	24	84.4 ± 3.5 (54:17)
TMD7	23	90.1 ± 3.0 (33:16)

The sequence information of 76 full-length or partially identified MC4R orthologs (see supplemental Table 1) was used to determine global and more distinct structural parameters. Segment lengths (minimum/maximum) and the structural conservation between fish and mammalian orthologs (given as percent identity determined by Clustal W) are shown. For comparing domain conservation of other GPCR, see Ref. 16. Numbers given in *parentheses* indicate the number of orthologs used for analysis. Data are given as means ± SD.

Materials and Methods

MC4R ortholog identification and site-directed mutagenesis

To analyze the sequence of MC4R orthologs, genomic DNA samples were prepared from tissue of various species (sources are given in supplemental Table S1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Tissue samples were digested in lysis buffer [50 mM Tris/HCl (pH 7.5), 100 mM EDTA, 100 mM NaCl, 1% SDS, 0.5 mg/ml proteinase K] and incubated at 55 C for 18 h. DNA was purified by phenol/chloroform extraction and ethanol precipitation. Degenerated primer pairs (supplemental Table S2) were applied to amplify MC4R-specific sequences. PCR were performed with *Taq* polymerase under variable annealing and elongation conditions. Conditions of a standard PCR were as follows: genomic DNA (100 ng) used in PCR (50 μl) with primers (1.5 μM each), ThermoPol reaction buffer (1×), dNTP (250 μM, each), and *Taq* polymerase (1 U; NEB, Frankfurt, Germany). The reactions were initiated with a denaturation at 94 C for 1 min followed by 35 cycles of denaturation at 94 C for 30 sec, annealing at 60 C for 30 sec, and elongation at 72 C for 1 min. A final extension step was performed at 72 C for 10 min. Specific PCR products were directly sequenced and/or subcloned into the pCR2.1-TOPO vector (Invitrogen, La Jolla, CA) for sequencing. In case of heterozygosity, allelic separation was performed by subcloning and subsequent sequencing. Sequencing reactions were performed on PCR products with a dye-terminator cycle sequencing kit and applied on an ABI 3700 automated sequencer (Applied Biosystems, Foster City, CA).

The full-length human MC4R was inserted into the mammalian expression vector pcDps and epitope-tagged with an N-terminal hemagglutinin (HA) epitope by a PCR-based overlapping fragment mutagenesis approach (13). MC4R mutations were introduced into the tagged version of MC4R using a PCR-based site-directed mutagenesis and restriction fragment replacement strategy. The identity of the various constructs and the correctness of all PCR-derived sequences were confirmed by restriction analysis and sequencing.

Cell culture and functional assays

COS-7 cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 C in a humidified 7% CO_2 incubator. Lipofectamine 2000 (Invitrogen, Groningen, The Netherlands) was used for transient transfection of COS-7 cells.

[^{3}H]cAMP assay. Cells were split into 12-well plates (1.5×10^5 cells per well) and transfected with a total amount of 1 μg plasmid DNA/well. Labeling with 2 μCi/ml [^{3}H]adenine (31.7 Ci/mmol; Pharmacia,

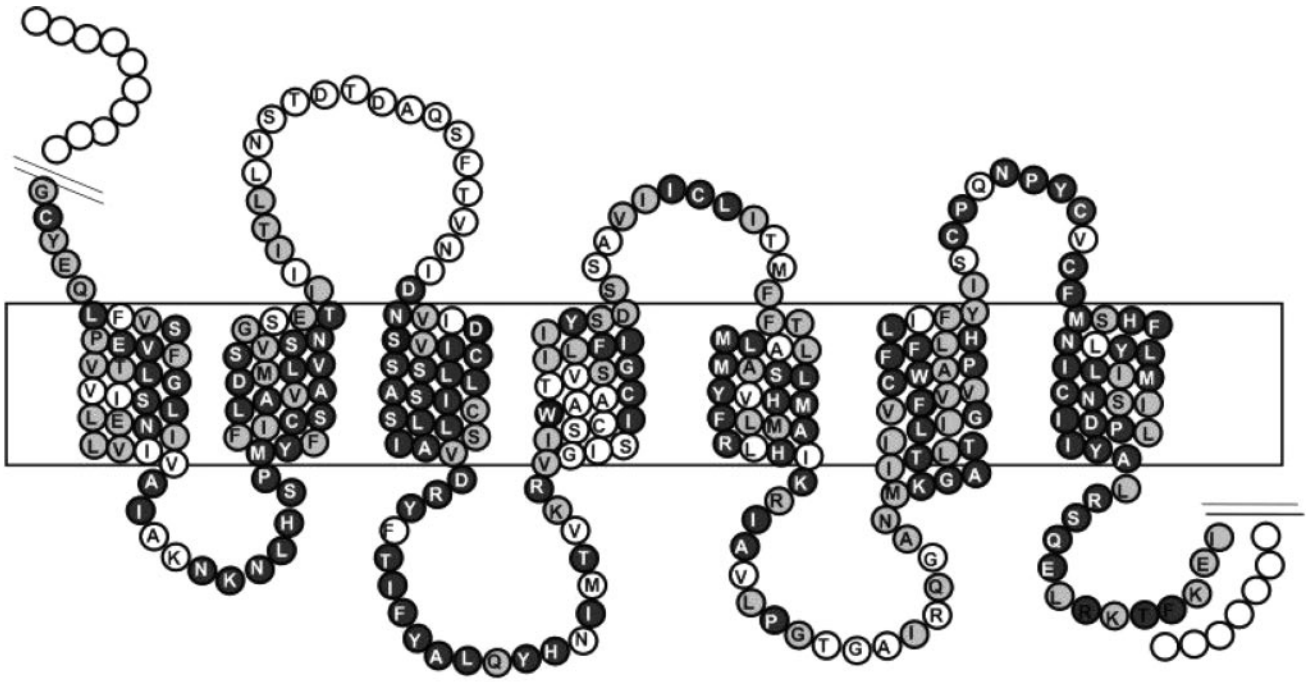

FIG. 1. Structural conservation of amino acid positions in MC4R orthologs. The amino acid sequence of the human MC4R is shown. Positions conserved in vertebrate MC4R orthologs are depicted in *black*. Positions that vary only by two amino acids are shown in *gray*. Positions given in *white* are not preserved during MC4R evolution.

Freiburg, Germany) was performed 48 h after transfection. One day later, cAMP accumulation assays were performed. Cells were washed once and incubated in serum-free DMEM containing 1 mM 3-isobutyl-1-methylxanthine (Sigma, Munich, Germany) in the absence or in increasing amounts of agonists NDP-α-MSH (Sigma), α-MSH, β-MSH, γ-MSH, or ACTH (kindly provided by Dr. P. Henklein, Charité Universitätsmedizin Berlin, Institute of Biochemistry) for 1 h at 37 C. The reactions were stopped by aspiration of medium, and intracellular cAMP was released by the incubation with 1 ml 5% trichloric acid and measured by anion-exchange chromatography as described previously (14).

ALPHAScreen cAMP assay. As a second test, cAMP content of cell extracts was determined by a nonradioactive cAMP assay based on the ALPHAScreen technology (Perkin-Elmer Life Science, Inc., Boston, MA) (11). Thus, cells were split into 50-ml cell culture flasks (1×10^6 cells per flask) and transfected with a total amount of 4 μg plasmid. One day after transfection, cells were seeded in 48-well plates (5×10^4 cells per well). Stimulation of cells with agonists was performed 48 h after transfection as described above. The reactions were stopped by aspiration of media, and cells were lysed in 50 μl lysis buffer (see ALPHAScreen manual) containing 1 mM 3-isobutyl-1-methylxanthine. From each well, 5 μl lysate were transferred to a 384-well plate. Acceptor beads (in stimulation buffer without 3-isobutyl-1-methylxanthine) and donor beads were added according to the manufacturers' protocol. cAMP accumulation data were analyzed by the GraphPad Prism program (GraphPad Software, San Diego, CA).

Binding assay. One day after transfection, cells were seeded in 48-well plates for assay. The number of cells was chosen to obtain a 5–10% binding of the radioligand. Cells were assayed 48 h after tranfection in competition binding assays using [^{125}I]NDP-α-MSH as tracer. Radioligand was bound in a buffer composed of 0.5 ml 50 mM HEPES buffer (pH 7.2) supplemented with 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.1% BSA and displaced in a dose-dependent manner by unlabeled ligands. The assay was performed in duplicate for 3 h at 25 C and stopped by washing twice in the buffer. Cell-associated, receptor-bound radioligands were determined by the addition of lysis buffer (48% urea, 2% Nonidet P-40 in 3 M acetic acid).

An indirect cellular ELISA was used to ligand-independently estimate cell surface expression of receptors carrying an N-terminal HA tag (15).

Results and Discussion

Structural and functional conservation of MC4R orthologs in vertebrates

Public sequence databases were mined for full-length fish and mammalian MC4R orthologs. The overall identity between full-length mammalian (25 species) and fish (12 species) is 67.3 ± 2.3% (see Table 1). During 450 million years of evolution, 173 (~52%) of all amino acid residues in the MC4R remained unchanged between these selected species (supplemental Fig. S1). In comparison, rhodopsin presents an overall identity of 77.4 ± 2.2% between fish and mammalian orthologs (16). However, the overall structural conservation of MC4R is still remarkably high when compared with large ortholog data sets of other GPCR such as the *lyso*-phosphatidylserine receptor GPR34 (16).

We further increased the sequence information by fully or partially cloning additional MC4R orthologs (see supplemental Table S1). The sequence information of 76 full-length or partially identified MC4R orthologs enabled us to determine global and more distinct structural parameters of MC4R that are relevant for maintaining the receptor structure and its agonist binding and signal transduction abilities. First, we found no length variations within the extracellular and intracellular loops except extracellular loop 1 (ECL1). In platypus and all fish orthologs, ECL1 contains one additional amino acid. ECL1 is the most diverse region between mammalian and fish MC4Rs (10). Second, the N terminus is most diverse between vertebrate orthologs (see Table 1), clearly indicating less specific relevance in ligand binding and receptor activity. Third, the C terminus contains at least one Cys residue that allows anchoring via palmitoylation; however, a proposed di-leucine motive equivalent (Ile^{316}/Ile^{317} in human MC4R) (17) is less preserved during evolution. Ile^{316} was found substituted by Met and Ile^{317} by Met, Cys, Phe, and Val in vertebrate MC4R orthologs. Fourth, transmembrane domain 1 (TMD1) and TMD4 display the lowest conservation (see Table 1 and Fig. 1), whereas the other TMDs are almost equally conserved at levels of more than 82%. This picture of conservation is very similar to rhodopsin but differs significantly from other GPCR, *e.g.* the *lyso*-phosphatidylserine receptor GPR34, where only TMD3 and TMD7 show high conservation (16). This indicates that proper MC4R function requires a reasonably higher number of determinants and intramolecular constraints than other GPCR. Fifth, it is of interest to note that all five extracellular Cys residues (Cys40, Cys196, Cys271, Cys277, and Cys279) are conserved during vertebrate MC4R evolution. This indicates importance even for Cys40 and Cys279, although experimental evidence in support of their functional relevance is not available yet (13).

Evaluation of naturally occurring mutations in the human MC4R

To date, about 70 missense variants (60 amino acid positions) of the human MC4R have been described (supplemental Table S3). About 46% missense variants were reported to have a partial or complete loss of function, about 44% displayed no differences to the wild-type receptor when studied in *in vitro* tests, and about 10% are not tested yet (Table 2). Taking advantage of this information, we analyzed the correlation of *in vitro* assay results and structural con-

TABLE 2. Function-conservation relationship of natural occurring mutations in MC4R

Conservation of the position	Wild-type function	Partial or loss of function
>2 Amino acids	18[a]	3
2 Amino acids	8	9
1 Amino acid	5	24[b]

More than 70 missense mutations at 60 positions within the human MC4R have been described. About 90% of all mutations were functionally tested. The table gives a correlation between the functional consequence of individual mutations and the evolutionary conservation of the mutated position. The complete list of missense mutations included in the analysis and the references are given in supplemental Table S3. For several mutations, different functional consequences were reported (see supplemental Table S3). Therefore, we considered all functional phenotypes by including and counting them individually. The evolutionary conservation of each mutated position was determined by aligning all MC4R orthologs, and the variability of the position was categorized in three groups from no variation (one amino acid) to high variation (more than two amino acids) in vertebrate orthologs. *P* values were determined by Fisher exact test.

[a] $P < 0.05$.

[b] $P < 0.001$.

servation of the positions found to be mutated in a human individual.

However, conservation as well as variability of distinct positions can only be properly interpreted in the light of functional data of different orthologs. Therefore, a number of mammalian and fish MC4R orthologs were stimulated with the natural agonists α-MSH, β-MSH, γ-MSH, and ACTH and functionally analyzed in cAMP assays (ALPHAScreen cAMP assay, see *Materials and Methods*). As shown in Table 3, we found only minor differences in basal activity. For control purposes, the human MC1R was included, which is known for high basal activity. Although there were obviously some differences in agonist potencies between the orthologs, *e.g.* lower potency of α-MSH at sea lion and bat orthologs (Table 3), we consider these differences as minor and probably physiologically not relevant (potency differences less than 10-fold). Several differences become relative when ratios are calculated (see Table 3). γ-MSH appears to be an exception, at least at the halibut ortholog. However, the potency of γ-MSH is always the lowest among the tested MC4R orthologs. To verify these results, we performed [^{3}H]cAMP accumulation assays with the same MC4R orthologs and α-MSH, β-MSH, and NDP-α-MSH as agonists, which yielded essentially similar results (see supplemental Table S4). Finally, radioligand displacement assays were performed. Consistent with the functional data, only minor differences [less than 10-fold differences in IC_{50} values, except Fat sandrat/human (ACTH), ~15-fold)] were found (Table 4). These data indicate that structural differences have no major effects on receptor function, at least for these orthologs.

Previous studies with the V2 vasopressin receptor have shown that 88% of inactivating (disease-causing) missense mutations hit fully or almost fully conserved positions (7). There were only a few missense mutations, mainly to Cys mutations in the ECL (11), which cause receptor inactivation at positions with low conservation. As summarized in Table 2, 92% of all partial and complete loss-of-function mutations occur at positions that are highly conserved during evolution, and 58% of all mutations with no functional effect are found on positions that vary in more than two amino acids between orthologs. However, there are a reasonable number of mutants with wild-type receptor properties carrying substitutions at 100% conserved residues (Val^{50}Met, AL^{154}Asp, Ile^{226}Thr, Asn^{274}Ser). One may speculate that the number of ortholog sequences is still insufficient to detect even rare functionally tolerated substitutions in wild-type MC4R orthologs. On the other hand there are several substitutions that have been claimed to be inactivating but naturally occur in other vertebrate MC4R orthologs (Val^{95}Ile, Ala^{175}Thr, Asn^{240}Ser, Val^{253}Ile). One may speculate that a reduced MC4R activity may be of advantage in the affected species or that the inactivating substitution is functionally rescued in the sequence context of the specific ortholog. Furthermore, one cannot exclude errors in *in vitro* testing of the mutant receptors, and careful reevaluation is needed. One candidate for reevaluation is Val^{95}Ile. This mutation in TMD2 was identified in an obese patient and proposed to cause MC4R inactivation by two independent groups (18, 19).

TABLE 3. Functional characterization of selected MC4R orthologs

Ortholog	ALPHAScreen cAMP assay																Ratio, α-MSH:β-MSH:γ-MSH:ACTH
	α-MSH			β-MSH			γ-MSH			ACTH							
	Basal cAMP (fold over hMC4R WT basal)	E_{max} (fold over hMC4R WT basal)	EC_{50} (nM)	Basal cAMP (fold over hMC4R WT basal)	E_{max} (fold over hMC4R WT basal)	EC_{50} (nM)	Basal cAMP (fold over hMC4R WT basal)	E_{max} (fold over hMC4R WT basal)	EC_{50} (nM)	Basal cAMP (fold over hMC4R WT basal)	E_{max} (fold over hMC4R WT basal)	EC_{50} (nM)				
Human	1	11.2 ± 1.1	9.9 ± 2.1 (11)	1	27.2 ± 6.7	30.4 ± 6.0 (4)	1	23.8 ± 5.0	355.7 ± 90.7 (4)	1	23.9 ± 5.3	17.6 ± 5.3 (4)					1:3.1:35.9:1.8
Mink whale	0.5 ± 0.1	10.8 ± 1.2	13.7 ± 3.0 (3)	1.0 ± 0.1	15.7 ± 2.8	20.3 ± 7.8 (3)	0.8 ± 0.1	15.8 ± 3.4	320.7 ± 18.6 (3)	1.1 ± 0.3	23.5 ± 3.5	18.6 ± 4.3 (3)					1:1.5:23.4:1.4
Sea lion	0.5 ± 0.1	10.8 ± 1.1	5.5 ± 3.6 (2)	0.8 ± 0.1	17.0 ± 1.9	19.0 ± 2.5 (2)	1.0 ± 0.1	18.0 ± 0.2	532.2 ± 105.5 (2)	1.0 ± 0.2	27.2 ± 15.2	15.3 ± 4.3 (2)					1:3.5:96.8:2.8
Sloth	0.7 ± 0.2	13.2 ± 0.9	11.8 ± 8.6 (3)	1.1 ± 0.6	27.3 ± 3.6	24.4 ± 3.7 (3)	1.3 ± 0.4	26.3 ± 2.3	200.1 ± 54.5 (3)	0.9 ± 0.2	18.8 ± 6.6	5.3 ± 0.7 (2)					1:2.1:17.0:0.4
Bat	0.5 ± 0.1	10.5 ± 0.9	1.6 ± 0.5 (3)	0.8 ± 0.1	18.5 ± 4.6	14.6 ± 2.8 (4)	1.1 ± 0.2	18.9 ± 2.9	51.7 ± 20.2 (4)	0.6 ± 0.3	21.2 ± 5.2	4.5 ± 0.5 (4)					1:9.1:32.3:2.8
Hyrax	0.7 ± 0.2	9.2 ± 1.5	18.5 ± 8.0 (4)	0.7 ± 0.3	22.6 ± 0.7	18.5 ± 1.8 (3)	0.9 ± 0.3	20.3 ± 3.0	352.9 ± 66.4 (3)	0.6 ± 0.1	17.0 ± 3.6	13.3 ± 3.9 (4)					1:1.0:19.1:0.7
Fat sandrat	1.1 ± 0.2	11.3 ± 2.2	8.4 ± 4.1 (4)	1.2 ± 0.2	25.5 ± 4.7	11.6 ± 0.6 (3)	1.6 ± 0.2	23.7 ± 2.4	140.8 ± 64.5 (4)	1.2 ± 0.2	24.1 ± 5.3	10.3 ± 1.5 (4)					1:1.4:16.8:1.2
Brown bear	0.6 ± 0.1	7.7 ± 1.0	14.6 ± 5.0 (9)	1.0 ± 0.3	20.3 ± 1.7	31.9 ± 3.5 (4)	1.0 ± 0.4	18.9 ± 0.5	264.5 ± 57.7 (4)	0.4 ± 0.1	17.6 ± 3.8	20.2 ± 5.3 (4)					1:2.2:18.1:1.4
Polar bear	0.5 ± 0.1	7.2 ± 1.0	11.8 ± 2.7 (7)	0.9 ± 0.3	16.4 ± 2.5	39.2 ± 1.7 (4)	0.9 ± 0.2	15.5 ± 3.1	318.6 ± 89.8 (4)	0.3 ± 0.1	12.0 ± 3.8	13.9 ± 4.3 (3)					1:3.3:27.0:1.2
Halibut	0.7 ± 0.3	9.0 ± 1.5	12.2 ± 4.7 (4)	0.5 ± 0.1	17.9 ± 5.4	18.7 ± 6.9 (3)	1.0 ± 0.4	22.9 ± 7.0	60.9 ± 19.4 (3)	0.4 ± 0.1	25.2 ± 0.8	16.8 ± 6.9 (3)					1:1.5:5.0:1.4
Human MC1R	2.2 ± 0.2	7.7 ± 0.1	4.1 ± 1.1 (2)														

For functional characterization, COS-7 cells were transiently transfected with MC4R constructs, and nonradioactive cAMP assays were performed as described in *Materials and Methods*. E_{max} and EC_{50} values were determined from concentration-response curves of agonists (agonists 10 pM to 1 μM) using GraphPad Prism. Data are presented as means ± SEM of independent experiments (number indicated in parentheses), each carried out in duplicate. cAMP levels (58.2 ± 8.8 amol/cell) of nonstimulated human MC4R wild type served as basal values. Ratios were determined. EC_{50} for α-MSH for each ortholog was set as 1. WT, Wild type.

TABLE 4. [^{125}I]NDP-α-MSH displacement binding for selected MC4R orthologs

Ortholog	[^{125}I]NDP-α-MSH displacement binding, IC_{50} (nM)				Ratio, α-:β-:γ-MSH:ACTH	Cell surface expression (% of hMC4R)
	α-MSH	β-MSH	γ-MSH	ACTH		
Human	154.3 ± 10.6 (3)	276.2 ± 64.3 (5)	915.3 ± 235.6 (3)	293.3 ± 5.3 (2)	1:1.8:5.9:2.5	100 (8)
Mink whale	130.8 ± 17.4 (3)	350.9 ± 72.2 (5)	1261.6 ± 410.4 (3)	155.2 ± 95.3 (2)	1:2.7:9.6:2.8	111 ± 16 (3)
Sea lion	85.1 ± 1.0 (3)	216.7 ± 34.8 (5)	589.4 ± 209.8 (3)	95.5 ± 42.4 (2)	1:2.5:6.9:2.9	95 ± 3 (3)
Sloth	99.8 ± 9.0 (3)	243.3 ± 64.2 (5)	804.2 ± 287.9 (3)	106.2 ± 44.2 (2)	1:2.4:8.1:1.4	92 ± 7 (3)
Bat	82.4 ± 7.1 (3)	233.8 ± 52.0 (5)	231.5 ± 76.4 (3)	82.3 ± 13.6 (2)	1:2.8:2.8:1.8	101 ± 8 (3)
Hyrax	97.2 ± 23.9 (3)	209.4 ± 60.9 (5)	746.5 ± 412.4 (3)	129.8 ± 10.3 (2)	1:2.2:7.7:0.3	101 ± 8 (3)
Fat sandrat	82.4 ± 4.9 (3)	153.0 ± 30.8 (5)	379.2 ± 121.3 (3)	20.7 ± 4.6 (2)	1:1.9:4.6:1.2	88 ± 11 (3)
Polar bear	177.8 ± 16.4 (3)	277.0 ± 77.0 (5)	1332.0 ± 459.2 (3)	263.7 ± 98.8 (2)	1:1.6:7.5:0.8	108 ± 8 (8)
Halibut	141.5 ± 11.0 (3)	291.8 ± 38.4 (5)	1351.8 ± 199.8 (3)	284.0 ± 108.4 (2)	1:2.0:9.3:2.1	106 ± 16 (3)

For functional characterization, COS-7 cells were transiently transfected with MC4R constructs, and [^{125}I]NDP-α-MSH displacement binding assays were performed as described in *Materials and Methods*. IC_{50} values were determined from concentration-response curves of agonists (agonists 10 pM to 1 μM) using GraphPad Prism. Data are presented as means ± SEM of independent experiments (number indicated in *parentheses*), each carried out in duplicate. Ratios were determined. IC_{50} for α-MSH for each ortholog was set as 1. Cell surface expression levels of MC4R orthologs were measured by a cell surface ELISA. Specific OD readings (OD value of HA-tagged construct minus OD value of GFP-transfected cells) are given as a percentage of wild-type HA-tagged hMC4R. The nonspecific OD value (GFP) was 0.195 ± 0.078 (set as 0%), and the OD value of the wild-type HA-tagged hMC4R was 0.895 ± 0.258 (set as 100%). The number of independent experiments, each carried out in quadruplicate, is given in *parentheses*.

Evaluation of mutant MC4R on the basis of evolutionary and functional data

In most other vertebrates, Val^{95} is preserved, and only armadillo and polar bear MC4R contain Ile^{95}. It is of interest because the next relatives of the polar bear all have only Val^{95} (Fig. 2). Polar bears and brown bears split about 300,000 yr ago from a common ancestor (20), and the Ile^{95} variant became highly frequent (six individuals analyzed all contain Ile^{95}) during this evolutionary short time in the polar bear linage. Reflecting the loss of functionality of the Val^{95}Ile variant in the human MC4R (18, 19), one may ask for advantages the polar bear would have from an inactive MC4R. As a maritime mammal, increase of body fat would be advantageous with respect to temperature isolation and buoyancy in water. Furthermore, the energy demand is increased in a polar climate, and one may speculate that the energy storage in time of food surplus may be improved when MC4R is less active.

As already tested in both cAMP assays (Table 3 and supplemental Table S4) the polar bear MC4R showed a robust agonist-dependent cAMP formation, and comparison with the brown bear ortholog (carrying the human Val^{95}) revealed no differences in functional assays. Except for the slightly lower E_{max} value of polar and brown bear orthologs (Table 5), no differences were obvious when compared with human MC4R. Similar results were obtained in transiently transfected HEK-293 and CHO-K1 cells (data not shown). Furthermore, no differences were seen in ligand-independent measurements of receptor cell surface expression levels (ELISA, Table 5). To analyze whether the Val^{95}Ile substitution has a functional consequence at all, we generated the *vice versa* mutants in human MC4R (Val^{95}Ile) and polar bear MC4R (Ile^{95}Val) and compared the function with the respective wild-type receptor orthologs. As shown in Fig. 3 and Table 5, the mutants were indistinguishable from their parent wild-type receptors. In sum, in the experimental settings used in the present study, we did not obtain evidence for a functional difference between Val^{95} and Ile^{95} variants.

We extended our reevaluation to other MC4R mutations that have been found in obese patients. Asn^{274} is fully conserved among vertebrate MC4R, and mutation to Ser was found in obese patients (21). Previous functional studies revealed wild-type function for this mutation (19, 22). The position Asn^{274} is not conserved only within MC4R orthologs but also in all other members of the melanocortin receptor family except for mammalian MC1R where Asn^{274} is substituted by His. In our functional tests, the basal cAMP values and EC_{50} values for α-MSH and NDP-α-MSH were unchanged when compared with the wild-type MC4R. Whereas the reduction in the E_{max} values was insignificant, cell surface expression level of Asn^{274}Ser was significantly reduced (Table 5). These results were independently verified with the [^{3}H]cAMP accumulation assay (Tarnow, P., H. Brumm, and H. Biebermann, supplemental Table S5). Similarly, substitution of Asn^{274} by Cys displayed unchanged potency and affinity and only a minor reduction in E_{max} values. However, the B_{max} value was reduced to 61% of the wild-type MC4R

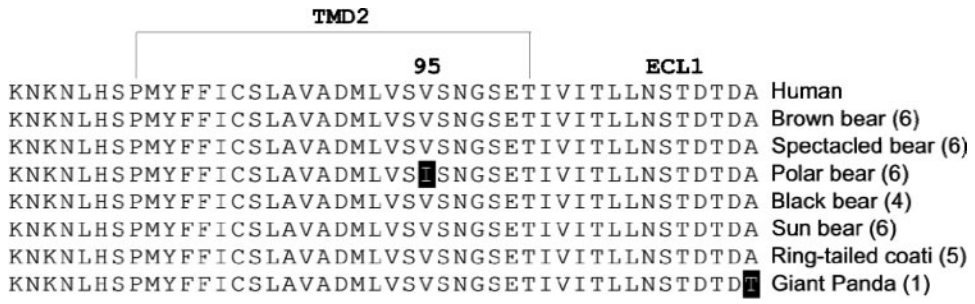

FIG. 2. Amino acid alignment of TMD2 of bears. The amino acid sequence of the human and bear MC4R orthologs are shown. Positions not conserved in the given orthologs are depicted in *black*. The number of individuals sequenced is given in *parentheses*.

TABLE 5. Functional characterization of human and bear MC4R orthologs/mutants

MC4R ortholog/mutant	ALPHAScreen cAMP assay						Cell surface expression (% of hMC4R)
	α-MSH			NDP-α-MSH			
	Basal cAMP (fold over hMC4R WT basal)	E_{max} (fold over hMC4R WT basal)	EC_{50} (nM)	Basal cAMP (fold over hMC4R WT basal)	E_{max} (fold over hMC4R WT basal)	EC_{50} (nM)	
Human	1	11.2 ± 1.1	9.9 ± 2.1 (11)	1	16.8 ± 3.3	0.9 ± 0.3 (7)	100 (8)
Human $Val^{95}Ile$	0.9 ± 0.1	10.8 ± 2.8	6.7 ± 1.1 (7)	1.1 ± 0.1	15.2 ± 4.6	1.5 ± 0.4 (6)	96 ± 7 (6)
Polar bear	0.5 ± 0.1	7.2 ± 1.0	11.8 ± 2.7 (7)	0.5 ± 0.1	9.5 ± 2.9	0.8 ± 0.2 (3)	108 ± 8 (8)
Polar bear $Ile^{95}Val$	0.5 ± 0.1	7.1 ± 1.3	7.2 ± 1.7 (4)	0.8 ± 0.1	8.7 ± 1.9	0.4 ± 0.1 (4)	105 ± 12 (6)
Brown bear	0.6 ± 0.1	7.7 ± 1.0	14.6 ± 5.0 (9)	0.9 ± 0.2	8.8 ± 3.6	1.2 ± 0.6 (4)	97 ± 3 (5)
$Ala^{175}Thr$	1.2 ± 0.2	12.2 ± 4.5	5.4 ± 1.1 (9)	1.6 ± 0.2	16.0 ± 3.8	0.9 ± 0.2 (7)	103 ± 6 (6)
$Phe^{202}Leu$	0.7 ± 0.1	9.2 ± 1.7	6.4 ± 1.7 (9)	1.2 ± 0.1	13.9 ± 3.3	0.9 ± 0.3 (6)	76 ± 4 (6)
$Asn^{240}Ser$	1.1 ± 0.2	7.7 ± 1.3	8.1 ± 3.4 (10)	1.5 ± 0.2	14.6 ± 2.1	1.4 ± 0.5 (7)	92 ± 1 (2)
$Asn^{274}Ser$	1.1 ± 0.1	8.8 ± 1.5	4.5 ± 0.6 (10)	1.4 ± 0.1	12.6 ± 2.0	0.9 ± 0.4 (7)	61 ± 6 (5)

For functional characterization, COS-7 cells were transiently transfected with MC4R constructs, and nonradioactive cAMP assays were performed as described in *Materials and Methods*. E_{max} and EC_{50} values were determined from concentration-response curves of agonists (agonists 10 pM to 10 μM) using GraphPad Prism. Data are presented as means ± SEM of independent experiments (number indicated in parentheses), each carried out in duplicate. cAMP levels (58.2 ± 8.8 amol/cell) of nonstimulated human MC4R wild type served as basal values. Cell surface expression levels of the wild-type and mutant MC4R were measured by a cell surface ELISA. Specific OD readings (OD value of HA-tagged construct minus OD value of GFP-transfected cells) are given as a percentage of wild-type HA-tagged hMC4R. The nonspecific OD value (GFP) was 0.195 ± 0.078 (set as 0%), and the OD value of the wild-type HA-tagged hMC4R was 0.895 ± 0.258 (set as 100%). The number of independent experiments, each carried out in quadruplicate, is given in *parentheses*. WT, Wild type.

(13). Our data indicate that mutation of $Asn^{274}Ser$ disturbs proper cell surface trafficking.

$Ala^{175}Thr$ (23–25), $Phe^{202}Leu$ (26), and $Asn^{240}Ser$ (26) contain substitutions that naturally occur in fish (Thr^{175}, Ser^{240}) and bat (Leu^{202}) MC4R orthologs. As shown in Table 5, basal cAMP and EC_{50} values of the mutants were indistinguishable from wild-type MC4R. $Phe^{202}Leu$ and $Asn^{240}Ser$ showed small reductions in E_{max} values and cell surface expression levels.

Conclusion

Over 450 million years of vertebrate evolution, the MC4R shows high structural and functional conservation. Its physiological importance is further reflected by the fact that no pseudogenes are identified yet. Large sets of ortholog sequence data are helpful for the interpretation of clinically relevant mutations. We clearly show that there is a strong correlation between positional conservation and the func-

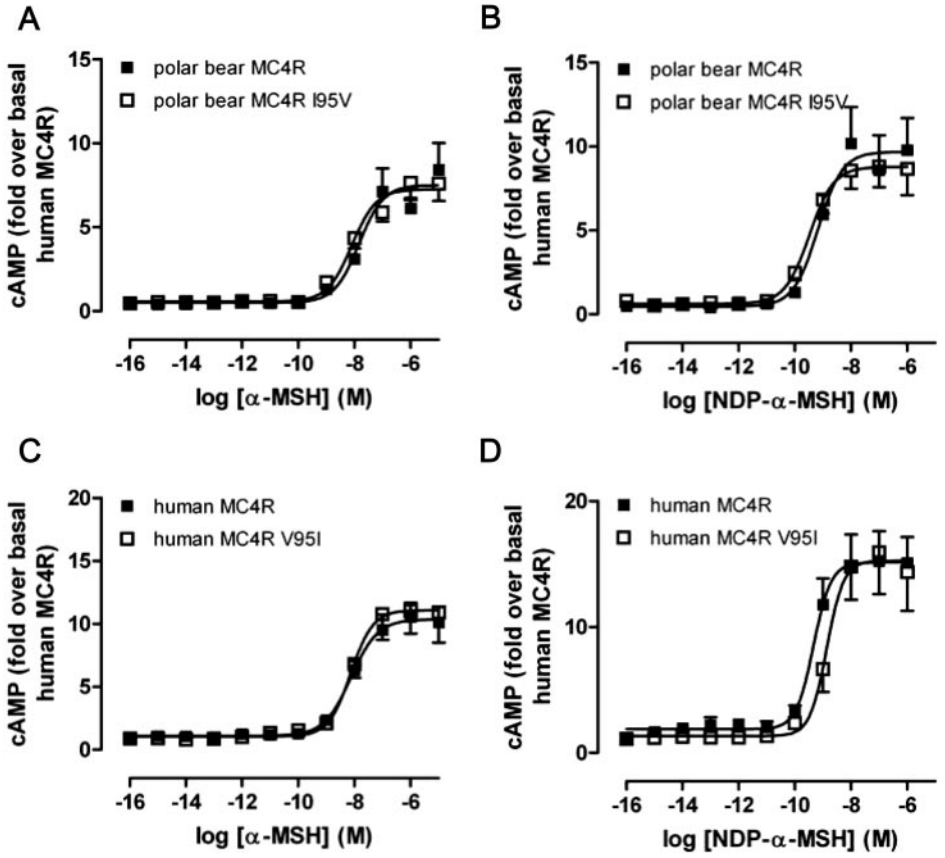

FIG. 3. Functional characterization of mutations at position 95 in human and polar bear MC4R. For functional characterization of the wild-type (human and polar bear) and of mutant MC4R constructs, cDNAs were cloned into the expression vector pcDps and tested for agonist-induced cAMP accumulation. Forty-eight hours after transfection, cells were stimulated with increasing concentrations of α-MSH and NDP-α-MSH. Intracellular cAMP was measured with AlphaScreen cAMP assay (see *Materials and Methods*). Data are given as mean ± SEM of three to seven experiments (see Table 5), each performed in duplicate.

tional relevance of mutation affecting a distinct position. Because of the manifold factors causing obesity, phenotype-genotype correlations are difficult to perform for mutations in MC4R. Evaluation of naturally occurring substitutions in MC4R on the basis of only *in vitro* functional tests has a limited power. The combination of functional and evolutionary data provides several advantages in interpreting mutations as shown by our reevaluation of selected MC4R mutants.

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The sequences reported in this paper have been deposited in the GenBank database (accession nos. EF384226–EF384268; supplemental Table S1).

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