Phage Display Selection on Whole Cells Yields a Peptide Specific for Melanocortin Receptor 1*

(Received for publication, June 5, 1997, and in revised form, August 18, 1997)

Michael Szardenings‡§, Susanna Törnroth‡, Felikss Mutulis‡¶, Ruta Muceniece‡**,
Kari Keinänen†‡, Arja Kuusinen†‡§§, and Jarl E. S. Wikberg‡

From the ‡Department of Pharmaceuti cal Pharmacology, Uppsala University, S-75 124 Uppsala, Sweden, the
¶Department of Medicinal Chemistry, Latvian Institute of Organic Synthesis, LV-1006 Riga, Latvia, the **Laboratory of
Pharmaceuticals, Latvian Institute of Organic Synthesis, LV-1006 Riga, Latvia, and the †††VTT Biotechnology and Food
Research, FIN-02044 Espoo, Finland

A phage display system for the selection of peptides binding to heterologously expressed human melanocortin receptor 1 on the surface of insect cells has been established. It could be shown that phage particles displaying the natural ligand α-melanocyte-stimulating hormone bind selectively to cells expressing this receptor and that these phages exhibit biological activity on mouse B16F1 melanoma cells. Insect cells were superior to other cell lines tested and have been used to select binders from a small library, in which critical determinants (Phe7-Arg8-Trp9) were kept, whereas the flanking regions where allowed to vary freely. One peptide displaying little similarity with native hormone was found that binds to the receptor also in its free form with an affinity of 7 nM. It showed a remarkable selectivity for this receptor, because it binds to the other melanocortin receptor subtypes with a maximum affinity of 21 μM. This is the first time phage display has been used successfully with G-protein-coupled receptors lacking an extracellular binding domain.

Phage display techniques, i.e. the display of libraries of peptides, enzymes, antibodies, and other proteins on the surface of bacteriophages and selection of functional sequences thereof, have undergone a rapid development (1–3). Originally used as selection systems to identify peptide epitopes (4, 5), phage display is today in use for almost any kind of problem that involves the interaction of peptides and proteins with other materials. Most of the published literature deals with the development of antibodies (6–8). With the use of this technique one comes close to the power of the human immune system. Other milestones were the display of enzymes (9), enzyme inhibitors (10), hormones (11), and cloning of active protein domains (12), to list only a few applications. Among the numerous publications about the display of hormones there are only a few dealing with the bioactivity of the phage (for examples see Refs. 13 and 14), because in almost all cases purified domains of cell surface receptors are used for the selection of binders from libraries (for examples see Refs. 11 and 15). Only a very few successful experiments are published with receptors displayed on living cells and in all such cases the receptors bind the ligands with extracellular domains (16–18).

The selection of random libraries of small peptides is naturally rendered more difficult when working with impure target proteins. Libraries of larger protein ligands usually contain common structural features directing and restricting binding of phages to the intended target. Especially G-protein-coupled receptors for small ligands that bind the ligands within their transmembrane helices have been the exclusive target for chemical peptide and other compound libraries (19–23). These can be analyzed and deconvoluted by other means than just the affinity of the compounds, as has been shown for example for the MC1 receptor (24), but they lack the complexity of bacteriophage displayed libraries. Because these receptors have not yet been purified in quantities and in a quality sufficient for the standard panning of phages, the selection on isolated membranes or whole cells remains the only possibility.

Good candidates for studying whether phage panning on such receptors is possible in principle against these odds are the recently cloned melanocortin receptors. Some of their ligands, the family of melanocortin peptides, have been known already for a long time for their effects on pigmentation in melanocytes (α-MSH)3 and for regulation of steroid production in the adrenal gland (adrenocorticotropic hormone). Already in early studies it was recognized that they display numerous other effects (25, 26), which is now confirmed and explained by molecular cloning of different receptor subtypes.

The first receptor to be cloned was the melanocortin receptor (MC1) of the melanocytes, soon followed by the identification of the adrenocorticotropic hormone receptor of the adrenal gland (MC2) and three other receptor subtypes (MC3, MC4, and MC5) with initially unknown functions (27–31). The distribution of these receptors in different tissues is known (29, 31–33), but the cellular function of these three subtypes is still not identified. Only the MC4 receptor, which is found in many tissues of the brain (30, 34), has recently been identified to be involved in weight homeostasis (35, 36).

Aside from the MC2 receptor, all of them bind the 13-amino acid-long α-MSH or analogues with nanomolar and subnanomolar affinities (37–40), but ligands highly specific for a single receptor subtype have not been found up to now. Intense studies have been carried out on the characterization and identification of residues involved in binding of the receptors, and the synthesis of numerous natural and unnatural peptides have been tried to achieve peptides with selectivity for a single

---

1 The abbreviations used are: α-MSH, α-melanocyte-stimulating hormone; gpIII, product of bacteriophage fd geneIII; MC, melanocortin; PCR, polymerase chain reaction.

---

* This work was supported by the Swedish Medical Research Council (04X-05975), Swedish National Board for Laboratory Animals, and the
§ Supported by the Academy of Sciences. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed: Dept. of Pharmaceutical Pharmacology, Biomedical Center, Box 591, S-75 124 Uppsala, Sweden. Fax: 46-18-559718; E-mail: msz@bmc.uu.se.
¶ Supported by the Swedish Institute.
§§ Supported by the Academy of Finland.
receptor (24, 41, 42). A common and essential motif of natural MSH peptides is a stretch of four amino acids (His^6^Phe^-Arg^-Trp^9_) believed to be essential for binding, an usually short N terminus preceding this sequence, and a less size-restricted C-terminal sequence (26). These features that should allow the design of phage libraries, where the C terminus is linked to the phage protein. It should also be possible to improve the selection procedure by using only partially randomized sequences with the motif His^6^Phe^-Arg^-Trp^9_ or parts thereof. This should restrict specific phage binding to the receptor protein.

This report shows that phages displaying an α-MSH-gpIII fusion protein bind specifically to cells expressing the MC1 receptor and even retain a biological activity. A selection system based on heterologously expressed receptors on the cell surface was established and was exploited to select novel peptides specific for the MC1 receptor from a phage display library.

MATERIALS AND METHODS

Cloning Procedures—Plasmid pComb3d derived from pComb3 (43) with the light chain cloning sites removed as a pG3H6 (45), a derivative of pHEN (7), were used in this study. The plasmids were cut with EcoRI/SmaI and PstI/XhoI, respectively. PCR reactions were carried out in 100-μl volumes with 5 μM dNTPs and 2 units of Vent - Vent-polymerase (Biolabs) in the buffer supplied with the enzymes. In a typical PCR reaction about 500 ng of template DNA and 20 pmol of each primer were used. The PCR reactions were carried out in a Biometra Trio PCR cycler using the following cycling: three cycles of 94 °C for 30 s, 48 °C for 30 s, and 72 °C for 6 min; seventeen cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 6 min; and then one cycle of 72 °C for 10 min. The primers were kinased with T4 polynucleotide kinase (Boehringer Mannheim) prior to PCR. The oligonucleotides used in this study were MS-4pcr (5'-AACAGTTTACCAAGATGTCCTCATAGTAAGACCGCAATGGCTGGTTTTTGGGTCG-3' and MS-5pcr (5'-VNNVNNVNNN-NNNCAGGAVVNNVNNVNNVNNAGGCGCATCCTGGGCGAGC-3') and MS-3pcr (5'-GCCGAGCTCATGAGTTTGT-3') and MS-1pcr (5'-CCCCGGGGCGCAGACTGTT-3') and CP3-198 (5'-CCCCGGGGGGGAGGT TGCTTCCA-3') partially complementary to the pE/B leader sequence gene in both vectors and cp3-1 (5'-CCCCGGGGCGCAATGACTGTT-3') and CP3-198 (5'-CCCCGGGGGGGAGGT TGCTTCCA-3') partially complementary to the 5' region of gene III in the vectors pG3H6 and pComb3d, respectively.

PCR products were purified by agarose gel electrophoresis (46). Ligation reactions were carried out with T4 ligase at a DNA concentration of 4–6 μg/ml in 500-μl volumes at 15 °C overnight. For standard transformation a simple one-step procedure was used (47). For library construction the ligation products (3 μg) were purified on a silica support (JetPure, Genomed) and used for 12 individual electroporations of XL-1-Blue using Electroporator II (InVitrogen) according to the manufacturer's description.

Preparation of Phage—All media used were ISO approved and obtained from Difco or Oxoid. To avoid counterselection due to overexpression of the gpIII fusion protein only freshly transformed bacteria were used. Precultures of XL-1-Blue harboring the phagemid were grown in Antibiotic-Medium-3 with 30 μg/ml tetracycline and 250 μg/ml ampicillin at 37 °C. dYT medium (10 g/liter yeast extract, 16 g/liter BactoTryptone, 5 g/liter NaCl) with ampicillin was inoculated 1:100 with this culture, and library transformed cells were directly added to about 200 ml in a 1-liter erlenmeyer flask. At an A_{550} of 0.5–0.7 the culture was superinfected at a multiplicity of infection of 5 with low titer M13KO7 (48) prepared as in Ref. 49. After 1 h, kanamycin (30 μg/ml) and optionally isopropyl β-d-thiogalactoside (0.5 mM) were added. The culture was shaken an additional 8–12 h, the bacteria were removed carefully in 1–2 centrifugation steps, and phages were precipitated in the cold by addition of 1/4 volume filtered 20% PEG6000, 2.5 M NaCl and centrifugation at 20,000 × g. The resulting phage pellet was resuspended in 1/8 of the original volume 100 mM HEPEs (pH 7.2), centrifuged to remove insoluble debris, precipitated a second time, and finally suspended in 5/8 of the original volume HEPEs buffer. These phage preparations did not disturb the growth of HEK293 or COS-1 cells if diluted 1:500 in cell culture medium. Aliquots of purified libraries were stored at −70 °C with 50% glycerol.

Expression of Melanocortin Receptors—The melanocortin receptors were cloned into pNeoSV (InVitrogen). The resulting vectors were used for transformation of the mammalian cell lines HEK293 (50) and COS-1 (51) using liposomes (52) as described previously (53). Cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum at 7% CO2.

Transfected cell lines were kept under selection of 180 μg/ml, respectively, 250 μg/ml zmo for at least 2–3 days. COS-1 cells could even be distributed two or three times under selection with large loss of receptors. Sf9 (54) and High Five (InVitrogen; Ref. 55) insect cell lines were grown in synthetic medium (SF900III, Life Technologies, Inc.) and infected with baculovirus v508-6 carrying a gene for the hMC1 receptor as described (40) in T80 flasks. 48 h after infection the cells were dissociated by pounding the flasks several times on the table and collecting cells by centrifugation.

Phage Display—Peptides were synthesized on a Pioneer Peptide Synthesis System (PerSeptive Biosystems) and purified by high-pressure liquid chromatography. The molecular weight of the peptides was confirmed by mass spectrometry.

Binding, Competition, and Selection Experiments—All binding and selection experiments with phages as well as radio ligand competition experiments were carried out in buffers and under conditions as described previously for COS-1 (37) and Sf9 (40) cells; in addition, 1 mM 2-mercaptoethanol was added to protect the free sulphydryl groups of some peptides. Phage binding experiments were usually carried out with total phage titers of 1 × 10^9 colony-forming units/ml. For competition experiments with pGEM7 (Promega) phagemid solution usually contained about 2% pMS4-23 or pMS4-26. 2.5 × 10^9 insect cells or 1 × 10^6 COS-1 were incubated at 37 °C gently shaken in a water bath in 4 ml of phage suspension in binding buffer (Ref. 37, minimal essential medium with proteinase inhibitors) for 30 min. The cells were washed four times with 4 ml of binding buffer after centrifugation at a maximum of 1000 × g for 10 min. Finally only 1 ml of binding buffer was added, and the incubation was extended to 30 min. Cells were harvested by centrifugation at 5000 × g, and aliquots of the cell pellet and this final supernatant were used to infect cell lines in smaller vectors. Two constructs were made originally, could be a useful approach for the generation of peptide libraries. Most recently other groups recently applied a similar approach for codon mutagenesis (58) and deletions (59), indicating that this still is a useful approach for the generation of peptide libraries. In this study an aliquot was plated on agar plates containing 250 μg/ml X-Gal. Blue/white colonies were counted after 20 h of incubation in the dark at 37 °C. In library panning the entire cell pellet was used to infect 100 ml of XL-1-blue at A_{600} = 0.4–0.6 and grown as described above. For library analysis an aliquot was plated on agar plates containing 250 μg/ml ampicillin 1–2 h after the infection.

RESULTS

Investigations on the Selection System—To display α-MSH in an active form on bacteriophages constructs were made by long inverse PCR amplification of the entire plasmids. Although we are still not satisfied with the quality of the DNA obtained, other groups recently applied a similar approach for codon mutagenesis (58) and deletions (59), indicating that this still could be a useful approach for the generation of peptide libraries in smaller vectors. Two constructs were made originally, based on the vectors pG3H6 (45) and a pComb3d derivative (43, 44) fusing the sequence of human α-MSH to the N terminus (pMS4-23 from pG3H6) and amino acid 198 (pMS4-26 from pComb3d) of bacteriophage fd gene III as outlined in Fig. 1. Cyclizing the PCR product with DNA ligase resulted in vectors containing no additional sequences above those needed for the phage display. pMS4-23 behaved similarly to pMS4-26, but we did not study this construct in great detail, because it turned out to be difficult to obtain clean phage preparations due to lysis of the bacterial host. Both constructs required induction by isopropyl β-d-thiogalactoside, otherwise no enrichment on
cells expressing the MC1 receptor was found (data not shown).

The phagomids were obtained for specific binding to the hMC1 receptor heterologously expressed in the mammalian cell lines HEK293 and COS-1, as well as baculovirus-infected insect cells. We tried to improve the amounts of receptor generally used in our laboratory in mammalian cell culture using the vector pZEO-SV (Invitrogen). COS-1 cells expressing the large SV40 antigen maintained this plasmid due to its SV40 origin and could be replated at least three times under selection without loss of receptor expression, reducing the experimental effort involved in the transfection of cells, but these procedures did not improve the number of binding sites significantly above levels published earlier (40). Insect cells infected with baculovirus v508-6 (40) turned out to express at least the same number of receptors per cell, although they are substantially smaller in size.2

Because panning with adherently growing cells failed, gentle centrifugation of detached cells had to be used instead. HEK293 cells lysed during these procedures. COS-1 cells were more stable and used for more intensive studies together with different insect cell lines. The binding experiments were carried out with pMS4-26 in direct competition with a 50-fold excess of phagemids derived from pGEM7, which allowed easy evaluation of the binding experiments after infecting HEK293 cells lysed during these procedures. COS-1 cells expressing the MC1 receptor was found (data not shown). This is not unusual for this receptor, because the complexes of the MC1 receptor with natural and synthetic ligands also exhibit high stability and low dissociation constants (60). In general only a very weak enrichment of pMS4-26 phage particles over pGEM7 on COS-1 expressing the receptor could be seen. This may be caused by a high unspecific binding of phage particles to the membranes under the conditions used for selection, and a similar tendency had been found earlier (17). Recently published data about peptides selected on insect cells revealed a strong preference for sequences containing tryptophan and arginine as present in all displayed peptides (18). There seemed to be a slight enrichment of pMS4-26 on insect cells without the MC1 receptor, but the statistics of these data were weak and seemed not to rectify alternating library selection on insect cells and mammalian cells as had been necessary to obtain ligands for the urokinase receptor (17). The difference of binding on SF9 and High Five cells may simply be due to a slightly higher expression level found with the latter. These expression levels vary even within our lab, and the conclusion that those cells are in general better should not be drawn from these data.

Aside from evaluating specific binding, the bioactivity of the α-MSH displaying phages was tested by investigating cAMP stimulation in B16F1 (56) cells (Fig. 2). There is about one active peptide displayed per two or three phages according to these results, if one assumes a stimulatory efficacy close to that of α-MSH. These results were further substantiated by Western blotting experiments with phagemid supernatant. Using a monoclonal antibody against gpIII (61), the blots showed an estimated amount of about one fusion protein per ten full length gpIII for pMS4-26 phagemids (Fig. 3). Western blots with polyclonal antibodies against α-MSH showed a strong band for pMS4-26 and only a very weak response for pMS4-23, above an overall high background (not shown).

Library Construction and Selection—A small library (about 105 individual clones) was constructed to test this phage dis-
play system using a partially randomized oligonucleotide (MS-5pcr) that kept the MSH-core sequence Phe-Arg-Trp, which is believed to be most essential for receptor binding, to direct phage binding toward the receptors. The Ser at the junction with the pelB leader sequence was also maintained to reduce the amount of sequences that may not be processed by the leader peptidase. We observed problems in the specificity of PCR reactions, low transformation efficiencies of the PCR derived vector DNA as well as truncated sequences in the resulting libraries. These were partially abolished by using exo'-Vent polymerase, indicating mismapping and partial digestion of the degenerated sequence by exonuclease activities during the PCR reaction. Three rounds of selection were run on infected High Five insect cells. Blue/white colony screening experiments were carried out in parallel by mixing library phages with pGEM7 phages to evaluate the binding properties of the entire library (Table II). After three rounds of selection, a significant enrichment of cell surface bound phages could be found.

To our surprise numerous mutated vectors were found to be enriched in the final library that carried deletions in the cloning region. 50 clones were analyzed, 43 had changes that could be easily detected by restriction analysis, and only 4 could be sequenced unambiguously (Table III). All peptides were synthesized as amides to avoid an extra negative C-terminal charge, and affinities to the hMC1 receptor were characterized in standard ligand binding assays with both the insect cell and the COS-1 expressed receptor (Table IV and Fig. 4). Values obtained for both cell types were identical as could be expected from earlier studies (40) and are therefore not listed separately. The only high affinity ligand MS-04 was further tested for its selectivity for the hMC1 receptor compared with the other MSH binding receptors (Table V). To rule out possible oxidation or dimerization caused by Cys, an analogue with Ala (MS-4ala), was also synthesized and tested; it behaved similar but had an overall lower affinity.

The peptide MS-04 was also compared with \( \alpha \)-MSH and [Nle\(^4\), d-Phe\(^7\)]-\( \alpha \)-MSH in a cAMP stimulation experiment on B16F1 cells. MS-04 exhibited very weak agonistic activity (Fig. 5).

**DISCUSSION**

The data presented here prove that panning of phage displayed peptide libraries on whole cells expressing the MC1 receptor using partially randomized libraries can yield specific high affinity ligands. This opens new ways to find leads for the receptor using partially randomized libraries can yield specific high affinity ligands. This opens new ways to find leads for the receptor, and affinity to the hMC1 receptor were characterized in standard ligand binding assays with both the insect cell and the COS-1 expressed receptor (Table IV and Fig. 4). Values obtained for both cell types were identical as could be expected from earlier studies (40) and are therefore not listed separately. The only high affinity ligand MS-04 was further tested for its selectivity for the hMC1 receptor compared with the other MSH binding receptors (Table V). To rule out possible oxidation or dimerization caused by Cys, an analogue with Ala (MS-4ala), was also synthesized and tested; it behaved similar but had an overall lower affinity.

The peptide MS-04 was also compared with \( \alpha \)-MSH and [Nle\(^4\), d-Phe\(^7\)]-\( \alpha \)-MSH in a cAMP stimulation experiment on B16F1 cells. MS-04 exhibited very weak agonistic activity (Fig. 5).

**TABLE II**

**Analysis of the library selection experiments**

| Round of selection | Wash | Pellet |
|--------------------|------|--------|
| 1                  | 1.9  | 0.7    |
| 2                  | 32.0 | 34.0   |
| 3                  | 19.0 | 33.0   |

**TABLE III**

**Comparison of phage display-derived synthesized peptides with \( \alpha \)-MSH and the primer defined sequence**

Underlined and double underlined sequences in the \( \alpha \)-MSH sequence are taken from Ref. 63; substitution by alanine in these positions resulted in loss of affinity by a factor of more than 10 and 100 times, respectively.

| MS4pcr                 | Ser***************************Phe-Arg-Trp***************************ProGly... |
|------------------------|-------------------------------------------------------------------|
| MS-01                  | Ser-Ser-Leu-Glu-Cys-Ser-Phe-Arg-Trp-Gly-Pro-Glu-His-NH₂           |
| MS-02                  | Ser-Val-Thr-Val-Val-Pro-Phe-Arg-Trp-Tyr-Ser-Cys-Ser-NH₂           |
| MS-03                  | Ser-Leu-Asp-Phe-Asn-Ser-Phe-Arg-Trp-Cys-Ser-Ala-Leu-NH₂           |
| MS-04                  | Ser-Ser-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Leu-Cys-Asp-NH₂           |
| MS-04ala               | Ser-Ser-Ile-Ile-Ser-His-Phe-Arg-Trp-Gly-Leu-Ala-Asp-NH₂           |
| \( \alpha \)-MSH        | Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH₂         |
library (62).

The deletion problems observed among the selected clones are of minor importance for the final results, because the size of the library would anyway not allow real statistical analysis of multiple clones. These truncated clones may have been selected by some affinity to other surface proteins of the insect cells. At a first glance the variable regions of the four sequences determined in the end do not very much resemble each other or the peptide, aside from Glu, which might be favored as a charge compensating amino acid.

Table IV lists the Ki values for the different peptides on the MC1 receptor. All values are in nM rounded to the first three digits. std. dev., standard deviation; n.o.e., number of experiments.

| Substance | MS-01 | MS-02 | MS-03 | MS-04 | MS-04ala |
|-----------|-------|-------|-------|-------|---------|
| Kᵢ mean  | 3700.0 | 43000.0 | 15900.0 | 7.6   | 29.5    |
| Kᵢ std. dev. | 580.0 | 12600.0 | 4070.0 | 2.4   | 14.4    |
| n.o.e.    | 4     | 4     | 4     | 10    | 12      |

The deletion problems observed among the selected clones are of minor importance for the final results, because the size of the library would anyway not allow real statistical analysis of multiple clones. These truncated clones may have been selected by some affinity to other surface proteins of the insect cells. At a first glance the variable regions of the four sequences determined in the end do not very much resemble each other or the peptide, aside from Glu, which might be favored as a charge compensating amino acid.

Table IV lists the Ki values for the different peptides on the MC1 receptor. All values are in nM rounded to the first three digits. std. dev., standard deviation; n.o.e., number of experiments.

| Substance | MS-01 | MS-02 | MS-03 | MS-04 | MS-04ala |
|-----------|-------|-------|-------|-------|---------|
| Kᵢ mean  | 3700.0 | 43000.0 | 15900.0 | 7.6   | 29.5    |
| Kᵢ std. dev. | 580.0 | 12600.0 | 4070.0 | 2.4   | 14.4    |
| n.o.e.    | 4     | 4     | 4     | 10    | 12      |

The deletion problems observed among the selected clones are of minor importance for the final results, because the size of the library would anyway not allow real statistical analysis of multiple clones. These truncated clones may have been selected by some affinity to other surface proteins of the insect cells. At a first glance the variable regions of the four sequences determined in the end do not very much resemble each other or the peptide, aside from Glu, which might be favored as a charge compensating amino acid.

Table IV lists the Ki values for the different peptides on the MC1 receptor. All values are in nM rounded to the first three digits. std. dev., standard deviation; n.o.e., number of experiments.

| Substance | MS-01 | MS-02 | MS-03 | MS-04 | MS-04ala |
|-----------|-------|-------|-------|-------|---------|
| Kᵢ mean  | 3700.0 | 43000.0 | 15900.0 | 7.6   | 29.5    |
| Kᵢ std. dev. | 580.0 | 12600.0 | 4070.0 | 2.4   | 14.4    |
| n.o.e.    | 4     | 4     | 4     | 10    | 12      |

The deletion problems observed among the selected clones are of minor importance for the final results, because the size of the library would anyway not allow real statistical analysis of multiple clones. These truncated clones may have been selected by some affinity to other surface proteins of the insect cells. At a first glance the variable regions of the four sequences determined in the end do not very much resemble each other or the peptide, aside from Glu, which might be favored as a charge compensating amino acid.

Table IV lists the Ki values for the different peptides on the MC1 receptor. All values are in nM rounded to the first three digits. std. dev., standard deviation; n.o.e., number of experiments.

| Substance | MS-01 | MS-02 | MS-03 | MS-04 | MS-04ala |
|-----------|-------|-------|-------|-------|---------|
| Kᵢ mean  | 3700.0 | 43000.0 | 15900.0 | 7.6   | 29.5    |
| Kᵢ std. dev. | 580.0 | 12600.0 | 4070.0 | 2.4   | 14.4    |
| n.o.e.    | 4     | 4     | 4     | 10    | 12      |

The deletion problems observed among the selected clones are of minor importance for the final results, because the size of the library would anyway not allow real statistical analysis of multiple clones. These truncated clones may have been selected by some affinity to other surface proteins of the insect cells. At a first glance the variable regions of the four sequences determined in the end do not very much resemble each other or the peptide, aside from Glu, which might be favored as a charge compensating amino acid.

Table IV lists the Ki values for the different peptides on the MC1 receptor. All values are in nM rounded to the first three digits. std. dev., standard deviation; n.o.e., number of experiments.

| Substance | MS-01 | MS-02 | MS-03 | MS-04 | MS-04ala |
|-----------|-------|-------|-------|-------|---------|
| Kᵢ mean  | 3700.0 | 43000.0 | 15900.0 | 7.6   | 29.5    |
| Kᵢ std. dev. | 580.0 | 12600.0 | 4070.0 | 2.4   | 14.4    |
| n.o.e.    | 4     | 4     | 4     | 10    | 12      |

The deletion problems observed among the selected clones are of minor importance for the final results, because the size of the library would anyway not allow real statistical analysis of multiple clones. These truncated clones may have been selected by some affinity to other surface proteins of the insect cells. At a first glance the variable regions of the four sequences determined in the end do not very much resemble each other or the peptide, aside from Glu, which might be favored as a charge compensating amino acid.

Table IV lists the Ki values for the different peptides on the MC1 receptor. All values are in nM rounded to the first three digits. std. dev., standard deviation; n.o.e., number of experiments.

| Substance | MS-01 | MS-02 | MS-03 | MS-04 | MS-04ala |
|-----------|-------|-------|-------|-------|---------|
| Kᵢ mean  | 3700.0 | 43000.0 | 15900.0 | 7.6   | 29.5    |
| Kᵢ std. dev. | 580.0 | 12600.0 | 4070.0 | 2.4   | 14.4    |
| n.o.e.    | 4     | 4     | 4     | 10    | 12      |

The deletion problems observed among the selected clones are of minor importance for the final results, because the size of the library would anyway not allow real statistical analysis of multiple clones. These truncated clones may have been selected by some affinity to other surface proteins of the insect cells. At a first glance the variable regions of the four sequences determined in the end do not very much resemble each other or the peptide, aside from Glu, which might be favored as a charge compensating amino acid.

Table IV lists the Ki values for the different peptides on the MC1 receptor. All values are in nM rounded to the first three digits. std. dev., standard deviation; n.o.e., number of experiments.

| Substance | MS-01 | MS-02 | MS-03 | MS-04 | MS-04ala |
|-----------|-------|-------|-------|-------|---------|
| Kᵢ mean  | 3700.0 | 43000.0 | 15900.0 | 7.6   | 29.5    |
| Kᵢ std. dev. | 580.0 | 12600.0 | 4070.0 | 2.4   | 14.4    |
| n.o.e.    | 4     | 4     | 4     | 10    | 12      |
ligand for the MC1 receptor described so far. Now more efforts are needed to create larger libraries and select them also on the other melanocortin receptor subtypes. The door is wide open for new specific peptides to examine the role of these receptors in vivo.

Acknowledgments—We thank Dr. M. Widersten (University of Uppsala) and Dr. L. Frykberg (Swedish Agricultural University, Uppsala) for the plasmids. Monoclonal antibodies against gp112 were a gift from Dr. M. Tesar (Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany).

REFERENCES
1. Kay, B., Winter, J., and McCafferty, J. (1996) Phage Display of Peptides and Proteins: A Laboratory Manual, Academic Press, San Diego
2. Cortese, R., Monaci, P., Nicosia, A., Luzzago, A., Felici, F., Galloro, G., Pessi, A., Tramontano, A., and Sollazzo, M. (1995) Curr. Opin. Biotechnol. 6, 73–80
3. O’Neil, L., deGruyter, W. P., Mousea, S. A., Ramachandran, N., and Hoess, R. H. (1994) Methods Enzymol. 245, 370–386
4. Smith, G. P. (1985) Science 228, 1315–1317
5. Parmley, S. P., and Smith, G. P. (1989) Adv. Exp. Med. Biol. 251, 215–218
6. Clackson, T., Hoogenboom, H. R., Griffiths, A. D., and Winter, G. (1991) Nature 352, 624–628
7. Hoogenboom, H. R., Griffiths, A. D., Johnson, K. S., Chiswell, D. J., Hudson, P., and Winter, G. (1991) Nucleic Acids Res. 19, 4133–4137
8. Marks, J. D., Hoogenboom, H. R., Bonnett, T. P., McCafferty, J., Griffiths, A. D., and Winter, G. (1991) J. Mol. Biol. 222, 581–597
9. McCafferty, J., Jackson, R. H., and Chiswell, D. J. (1991) Protein Eng. 4, 955–961
10. Roberts, B. L., Markland, W., Siranosian, K., Saxena, M. J., Guterman, S. K., and Ladner, R. C. (1992) Gene 1248–1251