Identification of Domains Directing Specificity of Coupling to G-proteins for the Melanocortin MC3 and MC4 Receptors*

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The melanocortin receptors, MC3R and MC4R, are G protein-coupled receptors that are involved in regulating energy homeostasis. Using a luciferase reporter gene under the transcriptional control of a cAMP-responsive element (CRE), the coupling efficiency of the MC4R and MC3R to G-proteins was previously shown to be different. MC4R exhibited only 30–50% of the maximum activity induced by MC3R. To assess the role of the different MC3R and MC4R domains in G-protein coupling, several chimeric MC3R/MC4R receptors were constructed. The relative luciferase activities, which were assessed after transfecting the chimeric receptors into HEK 293T cells, showed that the i3 (3rd intracellular) loop domain has an essential role in the differential signaling of MC3R and MC4R. To reveal which amino acid residue was involved in the MC4R-specific signaling in the i3 loop, a series of mutant MC4Rs was constructed. Reporter gene analysis showed that single mutations of Arg250 to Ala and Thr232 to either Val or Ala increased the relative luciferase activities, which suggests that these specific amino acids, Arg250 and Thr232, in the i3 loop of MC4R play crucial roles in G-protein coupling and the subtype-specific signaling pathways. An examination of the inositol phosphate (IP) levels in the cells transfected with either MC3R or MC4R after being exposed to the melanocortin peptides revealed significant stimulation of IP production by MC3R but no detectable increase in IP production was observed by MC4R. Furthermore, none of the MC4R mutants displayed melanocortin peptide-stimulated IP production. Overall, this study demonstrated that MC3R and MC4R have distinct signaling in either the cAMP- or the inositol phospholipid-mediated pathway with different conformational requirements.

Melanocortins are peptide hormones that are derived from the precursor peptide pro-opiomelanocortin, by a series of proteolytic cleavages (1). The melanocortins are known to have a broad spectrum of physiological actions, which include the regulation of melanocyte pigmentation (2), thermoregulation (3), obesity (4), control of the cardiovascular system (5), and learning and memory (6), and have also been found to have immunomodulatory effects (7). These hormones mediate their effects through G protein-coupled receptors by stimulating adenylate cyclase (8). To date five melanocortin receptor subtypes, with different patterns of tissue expression in the brain and peripheral body, have been cloned and characterized (8–12).

It has been reported that the activation of melanocortin 4 receptor (MC4R) by α-melanocyte-stimulating hormone (MSH) increases energy expenditure and decreases food intake. Moreover, the genetic disruption of MC4R was found to cause obesity in mice (13). Recent experiments in MC3R-null mice indicate that the inactivation of MC3R results in increased fat mass and reduced body mass, despite the fact that the animals were hypophagic and maintained normal metabolic rates (14, 15). These results suggest the nonredundancy of the MC3R and MC4R melanocortin receptors in the regulation of energy homeostasis (14, 15).

In previous studies, we and others have demonstrated that heterologously expressed MC3R and MC4R are coupled to the cAMP pathway. We analyzed several α-MSH analogues upon stimulation of MC3R and MC4R using a CRE (cAMP responsive element)-mediated reporter gene transcription activity assay (16), and were able to show that both MC3R and MC4R, expressed in human cell line HEK 293T, stimulate transcription when stimulated using different analogues of melanocortin at different levels. Our previous studies have shown that MC3R and MC4R may have differential efficiencies and/or modes of signaling in terms of G-protein coupling, in addition to their specific ligand-receptor interactions, which can specify subtype-specific signaling pathways in vivo (16).

The role of the third intracellular (i3) loop in G-protein coupling specificity has been investigated extensively for many seven-transmembrane domain receptors, including adrenergic, serotonergic, muscarinic, and dopaminergic receptors (17–21). For example, swapping experiments performed upon two different G protein-coupled receptors demonstrated the importance of this loop in selective coupling to specific G-protein effector systems (22, 23).

To identify the role(s) of the third intracellular loop of the
MC3R and MC4R receptors in terms of G-protein coupling specificity and receptor activation, several chimeras were constructed and characterized. We used the CRE-luciferase reporter gene assay to score the efficacy of receptor-G proteins coupling (24, 25). In parallel, amino acid mutations were generated in the third intracytoplasmic loop of MC4R to identify the residues that play a role in G-protein coupling. These mutant receptors were examined in terms of their abilities to bind melanocortin receptor-specific ligands and with respect to signal transduction at the cAMP level.

EXPERIMENTAL PROCEDURES

Construction of Chimeric Receptors—Chimeric receptors were made by using the polymerase chain reaction with sequence substitution of the I3 and carboxyl-terminal domain in rat MC3R (GenBank™ accession number X70667) and human MC4R (GenBank™ accession number S77415). Overlapping primers were designed from the junction of I3 and the carboxyl-terminal domain in MC3R and MC4R (Table I). To construct the chimeras, fragments originating from each receptor were first amplified in separate reactions with the indicated primers. The nucleotides of mutant amino acid residues were confirmed by full double stranded sequencing. Fragments of plasmid DNAs were sequenced entirely and all amino sequences were extracted from the NCBI data base and aligned using the ClustalW and GENE DOC programs. Primers were designed from the sequences in the I3 loop domain of MC4R using a recommended protocol (QuikChange™ Site-directed mutagenesis Kit, Stratagene) (Table II). Briefly, denatured double stranded plasmid DNA was annealed to a mutagenic primer pair of forward and reverse oligonucleotides and the new strand of DNA so obtained was synthesized with pfu DNA polymerase (Pfu Turbo DNA polymerase, Stratagene) by polymerase chain reaction (95°C for 1 ha t3 7 min for 16 cycles). Methylated template DNA was digested with DpnI enzyme for1ha t3 7°C for 30 s, 55°C for 1 min, and 68°C for 12 min). The reaction mixtures were then used to transform competent XL-1 blue E. coli and the plasmid DNA was prepared. Inserts of plasmid DNAs were sequenced entirely and all amino acid changes were confirmed by full double stranded sequencing.

Expression of Melanocortin Receptors and Luciferase Reporter Gene Assay—Rat MC3R and human MC4R cDNA, kindly provided by Dr. Roger D. Cone, were cloned into pSV expression vector (16, 25). For receptor expression, HEK 293T cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and transfected with pSV-rMC3R and pSV-hMC4R, respectively, using FuGENE 6 transfection reagent (Roche Molecular Biochemicals) using the procedure recommended by the manufacturer. Briefly, 5–7 × 10^5 cells were plated per six-well culture dish and transfected with 1 µg of pCRE-luc (Stratagene), 0.5 µg of plasmid pCH11O carrying the β-galactosidase gene, and 1 µg of each chimeric receptor or MC4R mutant plasmid DNA. After 6 h, the transfection mixture was replaced with fresh growth medium. Twenty-four hours after transfection, HEK 293T cells were treated for 3 h with various concentrations of α-MSH-ND or NDP-MSH in Dulbecco's modified Eagle's medium supplemented with 0.5% bovine serum albumin. After treatment, the cells were lysed and assayed for luciferase activity using the luciferase assay system (Promega), and luminescence was measured using a 96-well luminometer (Microtuml;
EG & G Berthold, Bad Vilbad, Germany). The expression of the reporter gene was normalized (26) using β-galactosidase activity (27). Transfection in the control group was performed under the transfection conditions described above with 1 µg of pCRE-luc, 0.5 µg of plasmid pCH110 carrying the β-galactosidase gene, and 1 µg of chimeric receptor or MC4R mutant plasmid DNA, but without stimulating by the melanocortin peptides. Results are expressed as the ratio of luciferase activity of the transfected cells to that of the unstimulated controls. The mean values of the data obtained were fitted to a sigmoid curve with a variable slope factor using nonlinear squares regression in GraphPad Prism software. EC50 values (nM) are described as mean ± S.E. from at least three independent experiments.

**RESULTS**

MC3R and MC4R share 60% overall amino acid identity and 76% similarity. The transmembrane regions show the highest degree of homology, whereas the intra- and extracellular loops show the lowest. As depicted in Fig. 1, four types of MC3R/MC4R chimeric receptors were constructed to investigate the role of the different domains of MC3R and MC4R in G-protein coupling. These chimeric receptors were designed to determine the contribution made by the i3 domain to G-protein coupling efficiency. Thus, the i3 loop and the remainder of the COOH-terminal domain of MC3R were swapped with the corresponding domain of the MC4R (named chimera 1), and in chimera 2, only the i3 loop of MC3R was swapped with that of MC4R. In chimera 3, the i3 loop and the cytoplasmic COOH-terminal loop of MC3R were swapped with that of MC4R, and in chimera 4, only the cytoplasmic loop of MC3R (named chimera 3 or 4, respectively) was swapped with the corresponding region of the MC4R.

**Binding and CRE-mediated Reporter Gene Activity of Chimeric Receptors—Wild type MC3R, MC4R, and the chimeric MC3/MC4R receptors were transiently transfected into HEK 293T cells. MC3R, MC4R, and all chimeric receptors all bound [125I]NDP-MSH with high affinity, as shown in Table III. The affinities of the expressed MC3R and MC4R receptors for [125I]NDP-MSH were 3.775 ± 0.4906 nM, respectively, and the Bmax values estimated for the two receptors were very similar (361.7 ± 20.47 and 375.9 ± 40.07 fmol/mg of protein for MC3R and MC4R, respectively, Table III). The respective chimeric receptors were found to have affinities that were similar to those of the parent receptors. These data indicate that substitution of the third intracellular loop had no significant conformational influence on ligand-binding domains, which are localized mainly within the transmembrane domain regions.

In parallel, wild type and chimeric MC3/MC4R receptors were transfected with the CRE-luciferase reporter gene into HEK 293T cells, as described earlier (16). In our previous study (16), using a luciferase reporter plasmid containing a CRE in its promoter (pCRE-Luc), we were able to show that it is pos-

**TABLE III**

**[125I]Labeled NDP-MSH binding to MC3R and MC4R, and to chimeric receptors**

|         | Bmax (fmol/mg protein) | KD (nM) |
|---------|------------------------|---------|
| MC3R    | 361.7 ± 20.47          | 3.775 ± 0.4906 |
| MC4R    | 375.9 ± 40.07          | 4.269 ± 1.0690 |
| Chimera 1 | 329.2 ± 21.56          | 3.310 ± 0.5175 |
| Chimera 2 | 329.3 ± 18.82          | 3.558 ± 0.4753 |
| Chimera 3 | 298.7 ± 23.34          | 3.164 ± 0.5977 |
| Chimera 4 | 361.2 ± 53.52          | 4.298 ± 1.3350 |

supplemented with myo-[3H]inositol (1 µCi/ml, 25 mCi/mmol) (DuPont Biotechnology Systems). The cells were then pretreated with Dulbecco’s modified Eagle’s medium supplemented with 20 mM LiCl for 30 min, and then incubated in the absence or presence of different concentrations of α-MSH for 5 s to 45 min. The reaction was stopped by the addition of perchloric acid to a final concentration of 5% (w/v). Cells were extracted and [3H]inositol polyphosphates were analyzed by anion-exchange high performance liquid chromatography, using a Partisphere SAX column (Whatman), as described previously (28, 29). All experiments were performed in triplicate.

**Statistical Analysis—**Cellular responses to the various peptides were compared using one-way analysis of variance and the Student’s t test with Instat software (GraphPad).

**Determination of Total Inositol Phosphate—**HEK 293T cells were plated at a density of 3 × 10⁵ cells/well in six-well plates and allowed to recover for 24 h. The cells were then transfected with pSV-MC3R and pSV-hMC4R, respectively, using the FuGENE 6 transfection reagent (Roche Molecular Biochemicals) as described above, after which 2 ml of Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum was added to each well and the cells were incubated for an additional 24 h. The medium was then aspirated, and cells were incubated for 16 h in myo-inositol-free Dulbecco’s modified Eagle’s medium.
sible to monitor variations in intracellular cAMP levels induced by transient transfections of MC3 and MC4 receptors. When increasing concentrations of α-MSH analogues were added, a typical dose-dependent and saturable induction of luciferase activity was observed (Fig. 2). However, we detected no significant effect of the α-MSH analogues on cells transfected with the reporter plasmid alone, which rules out the possibility of the presence of endogenous melanocortin receptors in the cell lines used for transfection, as the presence of such endogenous species might have affected the measured luciferase activity levels (data not shown). Another control transfection, using a luciferase reporter vector devoid of a CRE sequence, showed no significant response, demonstrating that the luciferase activity measured was dependent on the presence of the CRE site in the reporter plasmid (data not shown). These experiments demonstrate that the measured CRE-luciferase activity levels reflect the effects elicited by the receptors on changes in the intracellular cAMP levels, as represented by changes in cAMP-mediated gene expression, and thus provided evidence of receptor-G protein-effector interaction. Alterations in the abilities of different chimeric receptors to induce luciferase reporter gene activity were measured.

As described previously the luciferase activity induced by MC4R was found to be about 30–50% of the level induced by MC3R. Interestingly, chimera 1, where the i3 loop with the rest of the COOH-terminal domain of the MC3R were swapped with the corresponding domain of the MC4R, was similar to native MC3R in terms of its maximal reporter gene activity. Chimera 4 showed a slight increase in maximal reporter gene activity. However, the abilities of chimeras 2 and 3 to induce luciferase reporter gene activity were significantly affected, and decreased by 60 and 45% in maximal reporter gene activity, respectively (Fig. 2, A and B). The EC50 calculated from the dose-response curves was in general highly reproducible over several experiments, results are summarized in Table IV. The ability to induce the luciferase reporter gene activity of chimeras 2 receptors upon stimulation with α-MSH-ND was significantly affected by 8.7-fold (EC50 = 11.37 nM) versus MC3R (EC50 = 1.3 nM), whereas the EC50 values of other chimeric receptors were not significantly changed (Table IV). Therefore, our analysis of the four chimeras suggests that the dominant loss of G-protein coupling efficiency was associated mainly with the 3rd intracytoplasmic loop of the MC3R and MC4R receptors.

**Binding and CRE-mediated Reporter Gene Transcription Activity of Mutant MC4R**—To assess the role of the i3 loop and to establish whether mutations in the i3 loop of MC4R alter the coupling profiles of the receptor, amino acid mutations were generated in the i3 loop of MC4R (Fig. 3). Charged amino acids, such as, arginine 220, lysine 224, arginine 225, and arginine 236 were substituted with alanine (R220A, K224A, R225A, and R236A respectively), which lacks an amino acid chain beyond the carbon and also avoids the introduction of steric hindrance or unwanted ionic interactions. We also substituted threonine 232 with valine (T232V), a nonpolar amino acid, or
with alanine (T232A). In addition, isoleucine 235 and arginine 236 in MC4R were substituted with proline and glutamine, respectively (I235P and R236Q), which are native amino acids located at these positions in MC3R (Fig. 3). Other MC4R-specific amino acids in the i3 loop, such as glycine 238, alanine 239, and asparagine 240 were also substituted with the amino acids present at the same position in the i3 loop of MC3R, respectively (G238H, A239S, and N240C). These mutant receptors were examined in terms of their abilities to bind melanocortin receptor-specific ligands and for their abilities to transduce signals at the cAMP level.

Table 5 summarizes ligand affinities for MC3R, MC4R, and the 11 mutant MC4 receptors. All mutant receptors were found to bind [125I]NDP-MSH, demonstrating that they were all expressed on the plasma membrane. In general, no significant differences in the binding of [125I]NDP-MSH to the mutant receptors were observed.

To assess whether the G-protein coupling efficiencies of the mutant receptors differed from that of MC4R, we measured their abilities to induce CRE-luciferase reporter gene activity. Mutants R220A, T232V, and T232A showed enhanced reporter gene activity, which was very near that of MC3R, whereas the other mutant receptors had CRE-luciferase reporter gene activities similar to that of MC4R (Fig. 4, Table VI). The EC_{50} value of R220A was 12-fold lower than that of MC4R, and the EC_{50} value of T232V and T232A was 6.5- and 5.9-fold, respectively, lower than that of MC4R. The mutant receptors K224A (2.1-fold), R225A (3.5-fold), R236A (3.9-fold), and R236Q (3.5-fold) showed enhanced reporter gene activity similar to that of MC3R, whereas the other mutant receptors had CRE-luciferase reporter gene activities similar to that of MC4R (Fig. 4, Table VI). The mutant receptors K224A (2.1-fold), R225A (3.5-fold), R236A (3.9-fold), and R236Q (3.5-fold) showed enhanced reporter gene activity similar to that of MC3R, whereas the other mutant receptors had CRE-luciferase reporter gene activities similar to that of MC4R (Fig. 4, Table VI).

**Table IV**

**Summary of estimated EC\textsubscript{50} values of the melanocortin agonist α-MSH-NDP for MC3R, MC4R and chimeric receptors**

| Receptor | EC\textsubscript{50} (nM) |
|----------|--------------------------|
| MC3R     | 1.309 ± 0.367            |
| MC4R     | 1.007 ± 0.564            |
| Chimera 1| 3.303 ± 0.782            |
| Chimera 2| 11.372 ± 6.128           |
| Chimera 3| 3.163 ± 1.475            |
| Chimera 4| 2.988 ± 0.725            |

* p < 0.01 significantly different from corresponding MC3R value.
**TABLE V**

| Mutant receptor | EC<sub>50</sub> (nM) |
|-----------------|---------------------|
| MC3R            | 4.86 ± 1.18         |
| MC4R            | 3.94 ± 0.472        |
| R220A           | 0.405 ± 0.0539<sup>a</sup> |
| R224A           | 2.36 ± 1.760        |
| R225A           | 1.39 ± 0.7258       |
| T232A           | 0.870 ± 0.201<sup>a</sup> |
| T232V           | 0.744 ± 0.4138<sup>a</sup> |
| I235P           | 3.61 ± 0.815        |
| R236A           | 1.24 ± 0.5365       |
| R236Q           | 1.26 ± 0.9031       |
| G238H           | 2.44 ± 1.205        |
| A239S           | 4.783 ± 0.6933      |
| N240C           | 4.363 ± 1.478       |

<sup>a</sup> *p < 0.05, significantly different from the EC<sub>50</sub> values of MC4R.

**DISCUSSION**

MC3R and MC4R are closely related melanocortin receptor subtypes, which share 60% overall sequence homology (30, 31). However, these two MCR subtypes are biologically distinguishable in a number of respects, for example, they show distinctive agonist and antagonist binding affinities. Although both subtypes couple to G<sub>s</sub> and activate adenylyl cyclase on agonist stimulation, the G<sub>s</sub> coupling efficacy of MC4R is different from that of MC3R (16). Moreover, MC3R and MC4R display distinctive tissue distribution patterns, suggesting that they have different subtype-specific physiological roles. Gene "knock-out" mouse models (13–15) have been established for MC3R and MC4R resulting in different physiological outcomes. Recent experiments in MC3R-null mice indicate that the inactivation of MC3R results in an increased fat mass and a reduced body mass despite being the fact that the animals were hypophagic and maintained normal metabolic rates. This suggests the non-redundancy of MC3R and MC4R in the regulation of energy homeostasis (14, 15). A comparison of MC3R- and MC4R-null mouse phenotypes supports the idea that this melanocortin receptor subtype-specific mediated regulation of feeding behavior appears to be controlled in a finely tuned manner. For example, it has been suggested that in contrast to MC4R, which mainly controls food intake, MC3R might regulate fat stores by some specific metabolic pathway (31, 32).
In our previous study, we compared the CRE-mediated reporter gene activity of MC3R versus MC4R and found that MC4R showed 30–50% of the maximum activity induced by MC3R. Moreover, this was not because of a difference in receptor expression, as was shown by a receptor binding assay. Differential CRE-mediated reporter gene transcription by MC3R and MC4R suggests that MC3R and MC4R may have different signaling efficiencies, in terms of G-protein coupling, in addition to their specific ligand-receptor interactions, which can specify a subtype-specific signaling pathway in vivo.

It has been shown that the third intracellular loop of several G protein-coupled receptors is an important site for G-protein coupling and specificity (10). Moreover, highly charged regions of the third loop are strikingly conserved between the many different seven-transmembrane receptors, and point mutations or deletions affecting these regions disrupt normal signal transduction by these receptors by altering their binding to G-proteins (17–21). In the present study, by chimeric MC3R/MC4R receptor study, we have demonstrated that the i3 loop in MC3R and MC4R plays a pivotal role in G-protein coupling specificity. Indeed, the i3 loop appears to be critical for full activation of G protein for at least MC3R. Replacement of the entire i3 loop of MC3R by MC4R resulted in a ~60% decrease in maximal reporter gene activity, which is similar to the activity induced by MC4R. These findings indicate that the i3 loop is a critical structural determinant for the G-protein coupling property in MC3R and MC4R.

Impaired G coupled cAMP responsiveness was observed in experiments with chimeric MC3R/MC4R receptor constructs, especially in chimera 2, which had a reduced maximal response to melanocortin. In addition its dose-response curve (EC50) for melanocortin was reduced, and its dose-response curve (EC50) for melanocortin. In addition its maximal cAMP response to melanocortin. In addition its maximal response in MC3R and MC4R, and the different mutants receptors at 45 min after stimulation by the 10−8 M α-MSH in the HEK 293T cells.

In many cases of G protein-coupled receptors, such as the rhodopsin/β-adrenergic receptor subfamily, the i3 loop has been identified as an important structural domain, the residues of which couple to G-proteins and determine the specificity of receptor-G protein interactions (33–35). Our present results show that the replacement of the i3 loop of MC3R, with that of MC4R, suppresses its G-protein coupling efficiency, as evidenced by the reduced CRE-luciferase activity.

The analysis of the amino acid composition of the third loop of the known seven-transmembrane receptors shows the presence of highly charged residues in the loop. It is known that an alteration of hydrophobic/hydrophilic amino acids can influence the secondary structure of proteins (36–38). More refined analysis of the sequences of the i3 loop in different melanocortin receptors has revealed that homologies are shared by MC3R, MC4R, and MC5R. In the present study, the effects of replacing charged amino acids, namely, Arg220, Lys224, and Arg235, which are conserved in MC3R, MC4R, and MC5R, were examined. A mutation introduced by replacing Arg235 with alanine enhanced the EC50 12.9-fold versus native MC4R. Similarly, replacing Thr232, which is specific to MC4R, with valine or alanine also greatly enhanced CRE-luciferase activity by 7.6-fold (Table VI). These data indicate that these mutants acquired an increased G-protein coupling efficiency. However, when Ile235 or Arg236 were substituted with proline or glutamine, the amino acid residues natively present in MC3R, respectively, no significant changes in CRE-luciferase activity versus MC4R were observed. The mutants I235P, A239S, and N240C, where MC4R-specific amino acids were substituted by the amino acids present at the same position in MC3R, did not display significant changes in the CRE-luciferase activity by the mutation. Therefore, the amino acid residues in the i3 loop seem to be involved in the generation of structure or the modification of the environment of the protein, which confers G-
protein coupling selectivity. On the other hand, Thr232 might represent a candidate site for phosphorylation, for example, the phosphorylation involved in desensitization of the G protein-coupled receptor.

To determine the basis of the differential CRE-luciferase activity mediated by MC3R and MC4R more closely, we assessed whether or not these receptors were coupled to other signaling pathways, for example, the pathway involving phospholipase C activation. It has been reported that MC3R is also able to couple to G_q (39). An examination of the IP levels in the cells transfected with either MC3R or MC4R following exposure to the melancortin peptides revealed a rapid stimulation of IP production by MC3R but no detectable increase in IP production was observed by MC4R stimulation, as presented in Fig. 5. Therefore, it is suggested that this differential inositol phospholipid signaling by MC3R and MC4R may also contribute to the differential CRE-luciferase activity mediated by these two receptors. To a certain extent, because a CRE-receptor to transduce a precise effect of melanocortin. Furthermore, this suggests the relevance of the constitutive activity of MC4R.

This aspect would contribute to the distinct signaling pathway mediated by MC3R and MC4R that was observed. Furthermore, this suggests that MC3R and MC4R signaling via the G_q would be more complex and regulated in a fine way.

In conclusion, our study demonstrates that the activation of different signaling pathways by the MC3R and MC4R receptors have distinct conformational requirements. The third intracellular loop of these receptors plays a crucial role in the acquisition of these conformations, inasmuch as, depending on the mutation in this region, it is possible to modify the selectivity of the coupling and to selectively impair the ability of the receptor to transduce a precise effect of melancortin. Furthermore, our results suggest that MC3R and MC4R have distinct signaling in either the cAMP- or the inositol phospholipid-mediated pathway, and this appears to be regulated in a fine tuned way, possibly to adapt the complex and tonic signaling requirements for their physiological role in fuel homeostasis.

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