# Bioluminescence Resonance Energy Transfer Reveals the Adrenocorticotropin (ACTH)-Induced Conformational Change of the Activated ACTH Receptor Complex in Living Cells

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The melanocortin 2 receptor (MC2R) accessory protein (MRAP) is a small single-transmembrane domain protein that plays a pivotal role in the function of the MC2R. The pituitary hormone, ACTH, acts via this receptor complex to stimulate adrenal steroidogenesis. Using both coimmunoprecipitation and bioluminescence resonance energy transfer (BRET), we show that the MC2R is constitutively homodimerized in cells. Furthermore, consistent with previous data, we also show that MRAP exists as an antiparallel homodimer. ACTH enhanced the BRET signal between MC2R homodimers as well as MC2R-MRAP heterodimers. However, ACTH did not enhance the physical interaction between these dimers as determined by coimmunoprecipitation. Real-time BRET analysis of the MRAP-MC2R interaction revealed two distinct phases of the ACTH-dependent BRET increase, an initial complex series of changes occurring over the first 2 min and a later persistent increase in BRET signal. The slower ACTH-dependent phase was inhibited by the protein kinase A inhibitor KT5720, suggesting that signal transduction was a prerequisite for this later conformational change. The MRAP-MC2R BRET approach provides a unique tool with which to analyze the activation of this receptor. **(*Endocrinology* 152: 495–502, 2011)**

An intact pituitary-adrenal axis is essential for life. The essential regulatory component of this axis is the peptide hormone ACTH, which is secreted by the pituitary corticotroph cells and acts on the adrenal cortex to stimulate steroidogenesis. The receptor mediating this action is the melanocortin 2 receptor (MC2R), a member of the melanocortin receptor family that collectively forms a subfamily of the rhodopsin/$\beta$-adrenergic-like family A of the G protein-coupled receptor (GPCR) superfamily. The MC2R is unique among its family members in that it is highly selective for the 39-residue ACTH peptide and does not bind to related $\alpha$-, $\beta$-, or $\gamma$-MSH peptides (1). Similar to the other four members of the melanocortin receptor family, the MC2R signals primarily via the G protein $G\alpha_s$ to stimulate adenylyl cyclase and mediate steroidogenesis by activating the cAMP/protein kinase A (PKA)-dependent signaling pathway.

Cell surface expression of a functional MC2R is dependent on the presence of a small single-transmembrane domain accessory protein known as the MC2R accessory protein (MRAP) (2). The MC2R is retained at the endoplasmic reticulum (ER) when expressed in cells that are devoid of MRAP. When MC2R is coexpressed with MRAP, however, as in adrenocortical cell lines or in appropriately transfected cells, the MC2R is seen to traffic to the plasma membrane and to generate a signal in response to ACTH (2–5). MRAP therefore acts as an accessory factor by facilitating trafficking of the MC2R to the cell surface. MRAP appears to have an important additional function in assisting ACTH binding and consequently signal transduction (3, 4). Mutations in MRAP result in a syndrome of severe

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Abbreviations: BRET, Bioluminescence resonance energy transfer; EPAC, exchange protein directly activated by cAMP; ER, endoplasmic reticulum; EYFP, enhanced YFP; GPCR, G protein-coupled receptor; HA, hemagglutinin; HEK, human embryonic kidney; MC2R, melanocortin 2 receptor; MRAP, MC2R accessory protein; PKA, protein kinase A; RAMP, receptor activity-modifying protein; YFP, yellow fluorescent protein.

ACTH insensitivity (6, 7), and knockdown of MRAP in an adrenocortical cell line reduces ACTH responsiveness (8).

MRAP functions as a homodimer that is highly resistant to dissociation by sodium dodecyl sulfate and β-mercaptoethanol as shown by coimmunoprecipitation studies (8). Furthermore, Sebag and Hinkle (9) have suggested that this is an antiparallel homodimer, such that one molecule has its N terminus projecting extracellularly, and its partner molecule has its N terminus projecting intracellularly, as shown by immunocytochemical and glycosylation studies. Such a structure is apparently unique in eukaryotic biology and raises interesting questions over the mechanisms of translation and membrane insertion of these proteins.

Several pieces of data, including fluorescent complementation studies, indicate that the MRAP homodimer forms in the ER and that the dimeric form is essential for the MC2R to be trafficked to the cell surface (10). However, there is no reported data on the dynamics of the ACTH receptor complex. We used a bioluminescence resonance energy transfer (BRET) approach to study the homo/heterodimerization of the MC2R and MRAP in living cells and explore the possibility that receptor activation may influence the complex. These studies reveal an apparent requirement for a dimeric MC2R that undergoes conformational change on ACTH stimulation.

## Materials and Methods

### Reagents

Laboratory reagents and culture media were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated. ACTH(1–39), ACTH(1–24), and ACTH(1–13) were purchased from Bachem/Peninsula Laboratories (St Helen's, Merseyside, UK), and Angiotensin II was purchased from Sigma-Aldrich. The PKA inhibitor KT5720 and the adenylyl cyclase inhibitor SQ22536 were purchased from Calbiochem (Nottingham, UK).

### Plasmid constructions and transfections

MRAP-Rluc, Rluc-MRAP, and MC2R-Rluc were generated by subcloning human MRAP and human MC2R into the codon humanized *Renilla* luciferase vectors pRLuc N1 and pRLuc C3 (PerkinElmer, Cambridgeshire, UK). Enhanced yellow fluorescent protein (EYFP)-MRAP and MC2R-EYFP were generated by cloning MC2R and MRAP into the expression vectors pEYFP N1 and C1 (CLONTECH Laboratories, Inc., Mountain view, CA). AT1R-EYFP was generated by subcloning AT1R into pEYFP-N1. β-Arrestin-Rluc was generated by cloning β-arrestin2 into pEYFP-N1 and replacing EYFP with Rluc. The untagged human MRAP construct was obtained by subcloning human MRAP into pcDNA 3.1(+) vector (Invitrogen Corp., Carlsbad, CA). MRAP × 3Flag construct was generated by subcloning human MRAP into the p3xFlag-CMV-14 vector (Sigma-Aldrich). The human MC2RxFlag construct was generated by subcloning human MC2R sequence into the p3xFlag-CMV-14 vector. The receptor activity modifying protein (RAMP)1-EYFP-myc construct was a kind gift from Professor Michele Bouvier (Department of Biochemistry, University of Montreal, Montreal, Quebec, Canada). The BRET-based exchange protein directly activated by cAMP (EPAC) cAMP biosensor consisting of the human EPAC1 sequence flanked by citrine (an EYFP) and *Renilla* luciferase (11) was kindly provided by Professor Sternweis (University of Texas Southwestern Medical Centre, Dallas, TX). The MC2Rx3 hemagglutinin (HA) construct was obtained from Missouri University of Science and Technology cDNA Resource Centre (www.cDNA.org). Human embryonic kidney (HEK)293A cells were maintained in DMEM containing 10% fetal calf serum and 1% penicillin/streptomycin. Cells were transfected with Lipofectamine 2000 (Invitrogen, Paisley, UK).

### Coimmunoprecipitations and Western blotting

HEK293 cells were transfected with MRAPx3Flag and/or MC2Rx3 HA constructs. For the detection of MC2R homodimerization cells were transfected with MC2Rx3HA and/or MC2Rx3Flag constructs. Some cells were stimulated 24 h after transfection for 20 min with $10^{-6}$ M ACTH. Cells were scraped into lysis buffer containing PBS + 0.1% n-dodecyl-β maltoside and a protease inhibitor cocktail. For coimmunoprecipitations, the lysates were incubated with anti-HA agarose beads and incubated at room temperature for another 2 h. Sodium dodecyl sulfate loading buffer was added to the samples and subjected to Western blotting using a 1:1000 dilution of the antiflag antibody M2. Secondary antibody IRDye 800CW goat antimouse IgG (LI-COR Biotechnology-UK Ltd, Cambridge, UK) was used at a 1:10,000 dilution and imaged using the LI-COR Odyssey Infrared Image System.

### cAMP assay

For cAMP assays HEK293 cells were transfected with MC2Rx3HA along with either MRAPx3Flag, EYFP-MRAP, MRAP-Rluc. or Rluc-MRAP DNA constructs. Cells were stimulated 24 h after transfection with $10^{-6}$ M ACTH for 20 min followed by lysis and assayed using the Tropix cAMP screen ELISA system (Applied Biosystems, Bedford, MA) according to the manufacturer's instructions.

### BRET assays in live cells

For BRET assay HEK293 cells were grown in six-well plates and transiently transfected as described above. For optimization of BRET conditions, the donor-acceptor ratio was varied. The optimum ratio of 1:1 was used for the subsequent transfections. The expression levels of the donor and acceptor constructs were observed using fluorescence microscopy and luminometry. Cells were washed 24 h after transfection, detached using PBS, and then transferred into 96-well black optiplates (PerkinElmer) using HEPES-buffered Ham's F-12 media. For endpoint assays, 24 h after transfection, cells were stimulated with ligands for 20 min. Coelenterazine h (Invitrogen) was added in HEPES-buffered Ham's F-12 to a final concentration of 5 μM, and readings were collected immediately after this addition using the Polarstar Omega plate reader (BMG Labtech Ltd, Buckinghamshire, UK) that allows simultaneous dual emission detection. For real-time experiments coelenterazine h was added to a final concentration of 5 μM directly before ligand injection.. Readings were taken immediately at 0.2-sec intervals for 40 sec followed by 3-sec intervals for 160 sec and 60-sec intervals for 700 sec. Cells were kept at 37 C throughout the BRET measurements.

The BRET ratio is defined as emission at 530 nm (light emitted by EYFP)/emission at 485 nm (light emitted by Rluc). The normalized BRET ratio was calculated by subtracting the BRET ratio obtained in cells expressing the donor only (Rluc) from cells coexpressing both donor (Rluc)- and acceptor (EYFP)-tagged constructs. To assess the effect of the PKA inhibitor KT5720 or the adenylyl cyclase inhibitor SQ22536, cells were incubated with these reagents for 1 h and stimulated with ACTH(1–39) for 20 min before the addition of coelenterazine h.

### Statistical analysis

The data reported are the mean ± SEM of at least three independent experiments, each performed at least in duplicate. Statistical analysis was performed using ANOVA.

## Results

MRAP constructs with luciferase engineered onto either the N or C terminus and MRAP with EYFP at the N terminus were all capable of supporting the functional expression of the MC2R when transfected individually with MC2R into HEK293 cells (Fig. 1A), indicating that the presence of the luminescent or fluorescent moiety does not impede the trafficking and signaling role of the MRAP molecule. Note that these experiments were performed in the absence of phosphodiesterase inhibitor, as a result of which cAMP accumulation is modest.

Coexpression of MRAP with N terminally located luciferase (Rluc-MRAP) and MRAP with N terminally located EYFP moieties (EYFP-MRAP) gave no BRET signal. In contrast, expression of MRAP with C-terminal luciferase (MRAP-Rluc) in place of the N terminally tagged variety together with N terminal EYFP-MRAP produced a significant BRET response (Fig. 1B). These effects were not enhanced by the presence of ACTH and suggest that an antiparallel homodimer is the only significant form of MRAP found in the absence of MC2R. Cotransfection of MC2R resulted in a small but significant BRET signal in the case of the parallel homodimeric form of MRAP, but a sizeable increase in BRET signal in the case of the antiparallel form. Stimulation with ACTH did not increase this signal. The specificity of the MRAP dimer is illustrated by the finding that no BRET signal is generated between MRAP and RAMP1 (Fig. 1B).

One explanation for these findings is that the presence of receptor may influence the relative conformation or the tightness of the MRAP-MRAP antiparallel dimer and consequently increase the BRET signal. Alternatively, or perhaps in addition, receptor dimerization/oligomerization may bring together additional MRAP dimers so that BRET between different MRAP dimers can take place. This would be the case if each MC2R monomer was accompanied by a single MRAP homodimer, or even if more than one MRAP homodimer became associated with each MC2R monomer. In support of this latter view is the observation that parallel homodimer BRET signals become detectable only in the presence of MC2R.

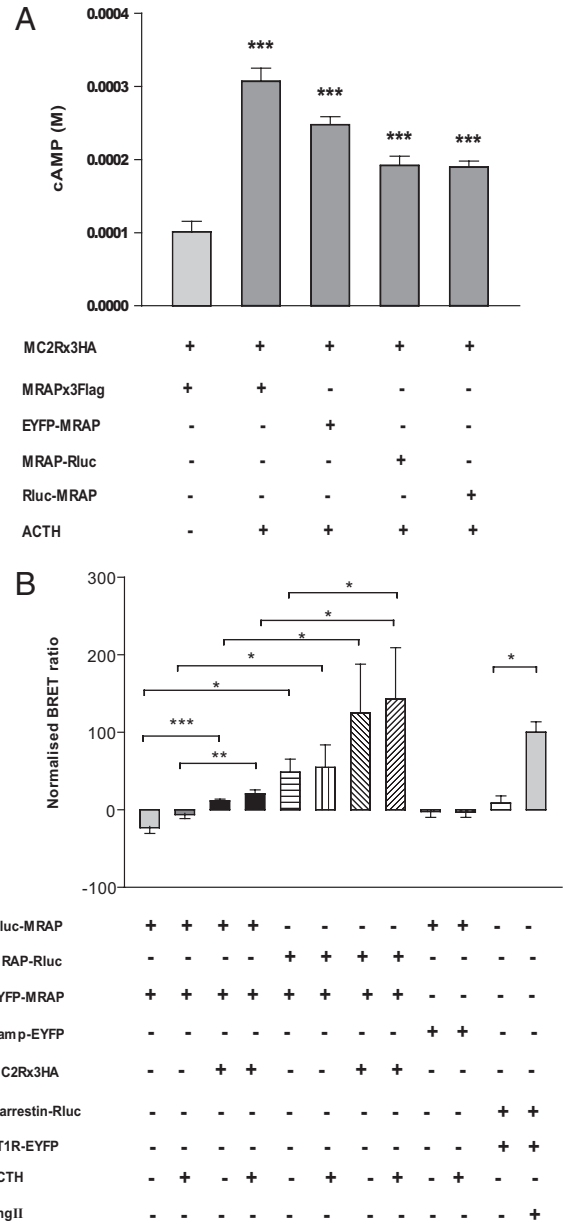


**FIG. 1.** MRAP homodimerization. A, Function of MRAP constructs used for BRET. HEK293 cells were transfected with MC2Rx3HA along with either MRAPx3Flag, EYFP-MRAP, MRAP-Rluc, or Rluc-MRAP. Cells were stimulated 24 h after transfection with $10^{-6}$ M ACTH (without the addition of isobutylmethylxanthine) and assayed for cAMP (n = 3 independent experiments). ***, $P < 0.001$ compared with unstimulated. B, HEK293 cells were transfected with EYFP-tagged MRAP and MRAP tagged with Rluc at the C or N termini. Some cells were also transfected with a MC2Rx3HA construct. Controls included cells transfected with RAMP1-YFP and Rluc-MRAP, or $\beta$-arrestin-Rluc and AT1R-YFP (positive control). Cells were stimulated with ACTH or angiotensin II (AngII) for 20 min at $10^{-6}$M (n = 4 independent experiments). *, $P < 0.05$; ***, $P < 0.001$.

A number of studies have suggested that several melanocortin receptors can form homo/heterodimeric structures (10, 13). We investigated the effect of MRAP and ACTH on the homodimerization of the MC2R using coimmunoprecipitation. Coexpression of MC2Rx3Flag and MC2Rx3HA followed by Flag immunoprecipitation and immunoblotting for HA provides evidence for MC2R homodimerization (Fig. 2). The presence of MRAP does not influence MC2R dimerization, implying that it occurs in the ER, because the MC2R does not escape the ER in the absence of MRAP. The reverse coimmunoprecipitations produced similar results (data not shown). ACTH does not appear to influence the efficiency of this interaction.

To seek confirmation of this finding, MC2R was tagged with luciferase or EYFP at the C terminus, and a small BRET signal was detected in the absence of MRAP. ACTH was without influence as would be predicted because it is likely that the MC2R would be located in the ER and inaccessible to agonist. However, when MRAP was cotransfected with MC2R-Rluc and MC2R-EYFP and cells were stimulated with ACTH, a significant increase in BRET signal was seen. No BRET between MC2R-Rluc and AT1-yellow fluorescent protein (YFP) was detected, supporting the notion that the MC2R dimer was specific (Fig. 3)

The striking effect of ACTH in this model would most simply be explained by a conformational change in the receptor in response to agonist stimulation such that the two C-terminal tails of the receptor were drawn into closer proximity. If this were the case, similar changes might be predicted in the interaction between MRAP and the receptor. Figure 4A demonstrates that this is the case when N terminally tagged EYFP-MRAP and MC2R-Rluc show a marked increase in BRET signal in response to agonist. No BRET signal was seen in cells expressing MC2R-Rluc and RAMP1-YFP in the absence or presence of ACTH.

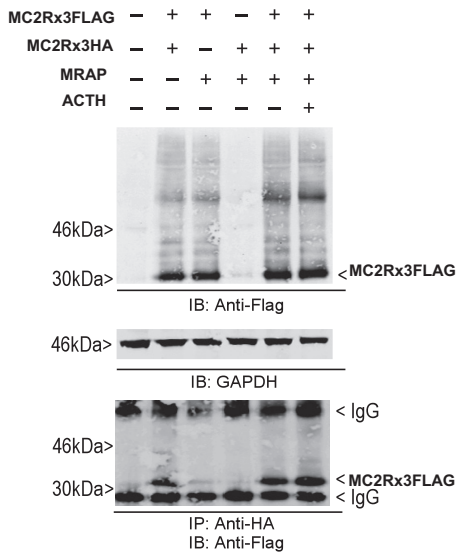


**FIG. 2.** MC2R homodimerization demonstrated by coimmunoprecipitation: MC2Rx3HA, MC2Rx3Flag, and MRAP were coexpressed in HEK293 cells without and with 20 min of ACTH treatment. Immunoblot (IB) with anti-Flag (*top panel*) demonstrates expression of MC2Rx3Flag (indicated by *arrowhead*). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) immunoblotting (*middle panel*) shows equal loading. Coimmunoprecipitation of these samples with anti-HA agarose beads and immunoblotting with anti-Flag reveals the presence of dimeric MC2R (*lower panel*). IP, Immunoprecipitation.

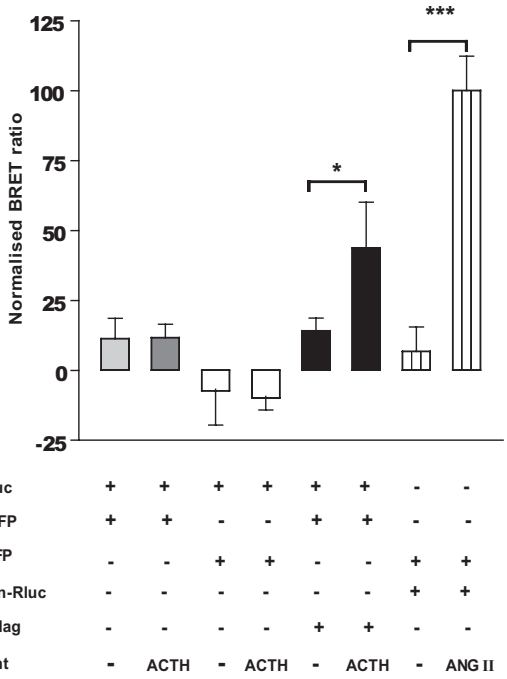


**FIG. 3.** MC2R homodimerization demonstrated using BRET. HEK293 cells were transfected with MC2R-Rluc and MC2R-EYFP constructs. Some cells were also transfected with a MRAPx3Flag construct. As controls for the assay, cells were transfected with MC2R-Rluc and AT1R-EYFP or $\beta$-arrestin-Rluc and AT1R-EYFP (positive control). ACTH and angiotensin II (ANGII) were used at $10^{-6}$ M concentration for 20 min (n = 3 independent experiments). *, $P < 0.05$; ***, $P < 0.001$.

The ACTH(1–39)-induced BRET increase was not observed when the ACTH competitive antagonist, ACTH (11–24), which binds the receptor but fails to generate a signal, was used (12). This finding implies that signal generation is required for this presumed conformational change. In support of this, stimulation of the receptor with ACTH in the presence of the PKA inhibitor KT5720 inhibited generation of a BRET signal (Fig. 4A). The use of the adenylyl cyclase inhibitor SQ 22536 also resulted in a significant reduction in the ACTH-induced BRET response (Fig 4A). An alternative possibility, that agonist stimulation led to additional recruitment of MRAP into the receptor complex, was investigated by coimmunoprecipitation studies, but no evidence for this occurrence was found (Fig. 4B). The four bands that are represented by the *arrows* in this figure are typical of the appearance of human MRAP on immunoblotting that we have reported previously (3). The predicted molecular mass of the hu-

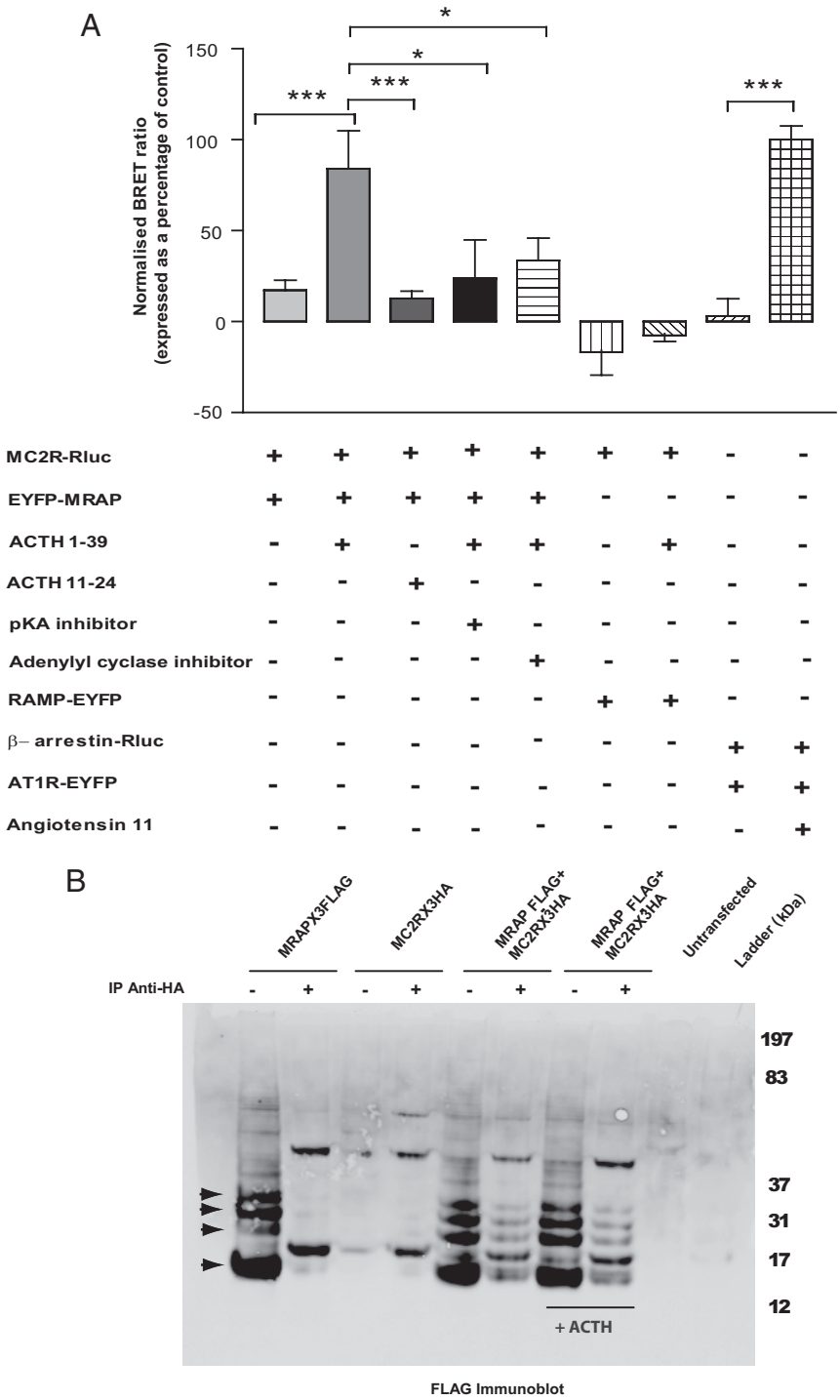


**FIG. 4.** MC2R-MRAP heterodimerization. A, HEK293 cells were transfected with MC2R-RLuc and EYFP-MRAP. Some cells were incubated with the PKA inhibitor (KT5720; $10^{-7}$ M) or the adenylyl cyclase inhibitor SQ22536 ($10^{-6}$ M)for 1 h before stimulation with $10^{-6}$ M ACTH(1–39). Some cells were also exposed to ACTH(11–24) antagonist ($10^{-6}$ M). As controls for the assay, cells were transfected with MC2R-RLuc and RAMP1-EYFP or β-arrestin-Rluc and AT1R-EYFP (positive control). Angiotensin II was used at $10^{-6}$ M concentration (n = 3 independent experiments). *, $P < 0.05$; ***, $P < 0.001$. B, Influence of ACTH on the heterodimerization between MRAP and MC2R using coimmunoprecipitation. HEK293 cells were transfected with MC2Rx3HA or MRAPx3Flag or coexpressed with both constructs. Cells coexpressing both proteins were stimulated with media alone or $10^{-6}$ M ACTH for 20 min. Lysates were immunoprecipitated (IP) with the anti-HA antibody and immunoblotted using anti-Flag antibody. Human MRAPx3Flag is depicted by the *arrowheads*.

man MRAP Flag protein is 24 kDa, and the multiple bands may correspond to posttranslationally modified MRAP or cleavage products.

All these observations were made at a single 20-min time point, but as we were interested in the time course of these changes and their relationship to signal transduction, the MRAP-MC2R BRET response was studied in real time. Unexpectedly, this revealed the existence of two distinct phases. Phase 1 begins immediately on addition of ACTH and consists of a reduction in BRET signal, followed within a few seconds by a recovery of signal strength by 30–60 sec and a final decline in BRET signal by about 120 sec.

Phase 2 becomes apparent after about 2 min and shows steadily increasing BRET signal, which persists for up to 25 min (data not shown). cAMP generation in real time was followed using the EPAC BRET reporter, which showed that cAMP began accumulating within seconds of agonist addition, and this increased steadily thereafter (Fig. 5). In the presence of KT5720, the first-phase response is attenuated and the second phase is absent, supporting the notion that PKA signaling is important for both components. ACTH(1–13), which is unable to bind to the MC2R, induced no BRET response (data not shown).

## Discussion

An emerging theme in GPCR biology is the requirement of some receptors for accessory proteins that assist trafficking and ligand binding. The RAMP proteins, which classically associate with the calcitonin receptor and calcitonin-like receptor, provide the prototype for this class of proteins, and features such as the association with receptor in the ER, trafficking to the plasma membrane, and contribution to ligand binding and signaling are also identified with the receptor transporting proteins, receptor expression enhancing protein and MRAP proteins (2, 16–18). MRAP is unusual in

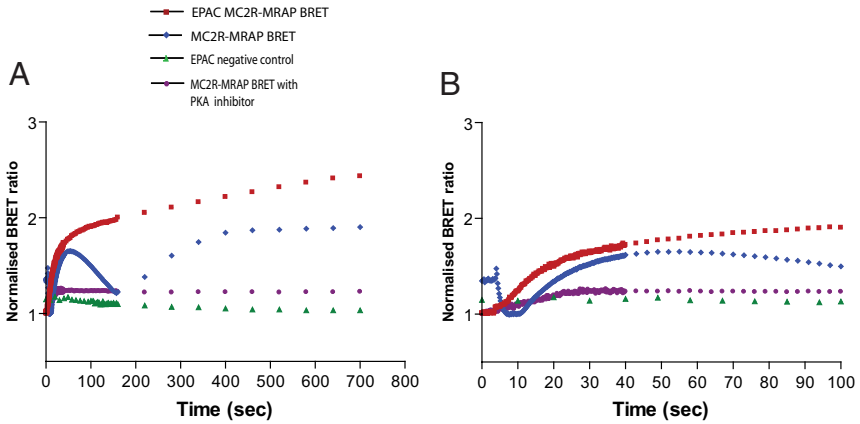


**FIG. 5.** Real-time BRET analysis on the MRAP-MC2R interaction. HEK293 cells were transfected with EYFP-MRAP and MC2R-RLuc. A, Cells were stimulated with ACTH(1–39) in the absence (*blue solid diamond*), and in the presence of the PKA inhibitor KT5720 (*magenta solid diamond*). The production of cAMP in real time was monitored by transfecting cells with MC2Rx3HA, MRAPx3 Flag, and EPAC constructs (*red solid square*). Cells expressing EPAC alone were used to monitor background levels of cAMP (*green solid triangle*). B, The same data as in panel A shown on an expanded scale over the first 100 sec.

that it appears to function as a dimer, and elegant studies from Sebag and Hinkle (9, 10) have made a strong case that it adopts a unique antiparallel conformation. In the present work we sought to test this model using BRET. This technique has the advantage that it enables studies in live cells and the influence of ligand or other modulators can be studied in real time. Our data suggest that the antiparallel conformation is the preferred conformation adopted by MRAP. The magnitude of the BRET signal from this antiparallel dimer is greatly enhanced in the presence of the MC2R. Two factors may account for this. First, the binding of the MRAP dimer to the receptor may lead to a conformational change between the MRAP molecules such that the luciferase and YFP moieties are brought into closer proximity. Second, dimerization of receptor molecules, each with an associated MRAP dimmer, may lead to BRET generation between MRAP dimers in addition to from within a single MRAP dimer.

The demonstration that MC2R homodimerizes is based on coimmunoprecipitation and BRET data. This is not especially surprising because the majority of GPCRs studied (including some of the melanocortin receptors) have demonstrated some evidence of homodimer formation (13–15, 19). Some authors have suggested that agonist activation of GPCRs induces the formation of dimers of GPCRs that are otherwise monomeric in their inactive state. This is largely based on the low BRET or fluorescence resonance energy transfer signals observed for these GPCRs, which are greatly enhanced after exposure to agonist (20–22). The second model, which is consistent with the findings reported here for the MC2R, suggests that GPCRs are constitutively dimerized at the level of the ER and ligand-induced changes in BRET/fluorescence resonance energy transfer result from conformational changes in the preexisting dimer (23–29).

The interaction of MRAP and MC2R, shown previously using coimmunoprecipitation in cell lysates, is confirmed using BRET in living cells. The finding that ACTH significantly enhanced this signal was a surprise. The increase in BRET signal in the presence of ACTH initially suggested that ACTH may promote the dimerization between these two proteins. However, the inhibition of this signal by both a competitive ACTH antagonist, a PKA inhibitor, and an adenylyl cyclase inhibitor suggests that signal generation is required. Thus it is more likely that a consequence of PKA activation, *e.g.* receptor phosphorylation, induces a conformational change in the receptor and/or MRAP that brings the luciferase and YFP molecules into closer proximity, resulting in a greater BRET signal.

Real-time analysis of the MC2R-MRAP interaction revealed the presence of a complex series of conformational changes that appear to comprise two distinct phases. The first phase was a transient short-term response that was virtually synchronous with cAMP generation. The second phase was a slower persistent BRET increase that is the signal measured in the initial single time point studies (Fig 4), and this signal was blocked by the PKA antagonist. It is tempting to speculate that this latter conformational change correlates with desensitization of the receptor, which we have previously shown to have a similar time course and to be PKA dependent (30). Against this, mutagenesis of serine 208 in the MC2R, which was previously found to be a target of PKA in the mouse MC2R (30), failed to influence BRET signal generation (data not shown). This may indicate that the influence of PKA on the human MC2R is more complex than on the mouse receptor.

The $\beta$2 adrenergic receptor is one of the most extensively studied GPCRs and has been used as a model for understanding agonist binding of GPCRs. Several studies including fluorescence spectroscopy have shown that $\beta$2 adrenoceptor ligand binding and activation is a kinetically and conformationally intricate process with evidence to show the existence of a sequence of conformational changes linking the inactive receptor to a fully active receptor (31, 32). Studies have suggested that the slow and rapid conformational states generated during agonist binding may have unique functional properties including activation of signaling cascades via cAMP production as well as promoting internalization of the receptor.

Further investigations of other GPCRs, including time-resolved peptide binding studies of the neurokinin receptor showed that neurokinin binds and activates its receptor in a biphasic manner. The rapid binding component of neurokinin A to tachykinin receptors was associated with a cellular calcium response. The rate of calcium elevation was found to correlate with the extent of receptor occupancy. The slow component was required for cAMP accumulation and signaling (33) and was detected after 1.5 min and reached a plateau at about 15–30 min. In the case of the $\beta$-2 adrenergic receptor, the rapid conformational change was suggested to be sufficient for activating Gs, whereas the slow conformational change was required for efficient agonist-induced internalization (32).

The combination of coimmunoprecipitation and BRET studies reported here, together with information already published on the MRAP/MC2R interaction, enable us to construct a model for formation and trafficking of this complex. The key elements of this model are:-

1. MRAP forms an antiparallel homodimer in the ER.
2. MC2R forms a homodimer in the ER.
3. Either concomitantly or sequentially a complex consisting (at a minimum) of one MC2R homodimer and two antiparallel MRAP homodimers forms in the ER and may then traffic to the plasma membrane.
4. At the cell surface, one or possibly two molecules of ACTH bind to the hexameric receptor complex. Ligand binding induces a rapid conformational change and subsequent relaxation of the complex.
5. PKA activation by cAMP leads directly or indirectly to a modification of the receptor complex, possibly by a phosphorylation step, which may initiate receptor desensitization.

This model provides several testable steps and may identify actions that may be susceptible to pharmacological intervention with potential therapeutic benefit. Arguably, the existence of MRAP as an accessory protein for this receptor provides a unique tool for study of GPCR activation, which may have implications beyond the melanocortin subfamily of receptors.

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