G-Protein–Coupled Receptor Mas Is a Physiological Antagonist of the Angiotensin II Type 1 Receptor

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Background—We previously identified the G-protein–coupled receptor Mas, encoded by the Mas proto-oncogene, as an endogenous receptor for the heptapeptide angiotensin-(1-7); however, the receptor is also suggested to be involved in actions of angiotensin II. We therefore tested whether this could be mediated indirectly through an interaction with the angiotensin II type 1 receptor, AT1.

Methods and Results—In transfected mammalian cells, Mas was not activated by angiotensin II; however, AT1 receptor–mediated, angiotensin II–induced production of inositol phosphates and mobilization of intracellular Ca2+ was diminished by 50% after coexpression of Mas, despite a concomitant increase in angiotensin II binding capacity. Mas and the AT1 receptor formed a constitutive hetero-oligomeric complex that was unaffected by the presence of agonists or antagonists of the 2 receptors. In vivo, Mas acts as an antagonist of the AT1 receptor; mice lacking the Mas gene show enhanced angiotensin II–mediated vasoconstriction in mesenteric microvessels.

Conclusions—These results demonstrate that Mas can hetero-oligomerize with the AT1 receptor and by so doing inhibit the actions of angiotensin II. This is a novel demonstration that a G-protein–coupled receptor acts as a physiological antagonist of a previously characterized receptor. Consequently, the AT1-Mas complex could be of great importance as a target for pharmacological intervention in cardiovascular diseases. (Circulation. 2005;111:1806-1813.)

Key Words: angiotensin ■ calcium ■ muscle, smooth ■ vasoconstriction ■ receptors

The Mas proto-oncogene was first detected through its tumorigenic activity in in vivo tumor assays. In mammals, the gene is expressed predominantly in testis and distinct areas of forebrain, including hippocampus and amygdala, and less strongly but at detectable levels in kidney and heart. It encodes a protein with 7 transmembrane domains that contain features characteristic of class I G-protein–coupled receptors (GPCRs), and in early studies, it was suggested to be a receptor for the octapeptide angiotensin II (Ang II), the main effector peptide of the renin-angiotensin system. However, Ambroz et al and Ardaillou later showed that Ang II–induced elevation of intracellular Ca2+ in Mas-transfected cells was only observed in cells that endogenously expressed the Ang II type 1 (AT1) receptor, one of the 2 receptors now known to represent the natural targets for Ang II. Thus, Mas is not per se a receptor for Ang II. Despite this, clear evidence indicates a physiological role for Mas in the function of Ang II. For example, Walther et al demonstrated an alteration of neuronal AT1 receptor signaling after Ang II stimulation in mice in which the Mas proto-oncogene had been inactivated, and these mice also display gender-specific alterations in heart rate and blood pressure regulation. Furthermore, we have recently identified Ang-(1-7), a peptide metabolite generated mainly from angiotensin I and with distinct biological functions, as the endogenous agonist for the Mas receptor. Taken together, our previous findings provide evidence for an in vivo interaction between the Mas and AT1 GPCRs, although the molecular events that govern this interaction remain unknown.

One mechanism of receptor–receptor interaction that has recently gained much prominence in the field of GPCRs is the phenomenon of receptor dimerization/oligomerization. In some cases, oligomerization is a prerequisite for efficient agonist binding and signaling, and in others, it simply occurs constitutively without overt biological consequences. Previous studies have shown that the AT1 receptor can form...
hetero-oligomeric complexes with certain other GPCRs. For example, dimerization between the AT\textsubscript{1} receptor and the bradykinin B\textsubscript{2} receptor results in enhanced function of Ang II and may underlie much of the hypertension associated with the condition of preeclampsia in pregnant women.\textsuperscript{15,16} Interactions between AT\textsubscript{1} and AT\textsubscript{2} receptors have also been demonstrated in vitro, but in such circumstances, the AT\textsubscript{2} receptor appears to functionally antagonize the AT\textsubscript{1} receptor.\textsuperscript{17} To date, however, studies of class I GPCRs have not established the in vivo physiological relevance of heterodimerization, the postulated AT\textsubscript{1}-B\textsubscript{2} receptor interaction being characteristic of a pathophysiological state. Given our strong evidence for a physiological interaction between the Mas and AT\textsubscript{1} receptors, we chose to examine the potential for direct interactions between these 2 receptors as the molecular basis underlying their interactions in vivo.

**Methods**

**Cell Culture and Transfection**

CHO-K1 cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, penicillin-streptomycin (10 000 IU/mL to 10 000 μg/mL), and 2 mM/L MHEPES (radioligand binding assays) or basal ISCOVE medium supplemented with 10% fetal bovine serum, penicillin-streptomycin (10 000 IU/mL to 10 000 μg/mL), and 2 mM/L L-glutamine (signaling studies). For radioligand binding assays, CHO-K1 cells at 50% to 80% confluence were transfected transiently with the indicated cDNAs (21 μg of plasmid DNA per 150-cm\textsuperscript{2} tissue culture flask) with Lipofectamine reagent (GIBCO BRL) according to the manufacturer’s instructions. For cell-signaling experiments, 2×10\textsuperscript{5} CHO-K1 cells were seeded into 35-mm dishes. About 24 hours later, cells were transfected transiently at 50% to 80% confluence with the indicated receptor cDNAs (2 or 0.06 μg of total plasmid DNA/35-mm well) with Lipofectamine. In cotransfection experiments, 1 μg of receptor DNA each was used.

**Inositol Phosphate Accumulation Assay**

Inositol phosphate accumulation assays were performed essentially as described previously.\textsuperscript{18}

**Fluorometric Imaging Plate Reader Cell Signaling Assay**

Six to 18 hours after transfection, CHO-K1 cells were seeded into 96-well plates at a density of 50 000 cells per well and cultured for 18 to 24 additional hours until used in a fluorometric imaging plate reader (FLIPR; Molecular Devices) assay. CHO-K1 cells were loaded with 95 μL of HBSS containing 20 mM/L MHEPES, 2.5 mM/L probenecid, 4 μM/L fluorescent calcium indicator dye Fluo4 (Molecular Devices), and 1% fetal bovine serum for 1 hour (37°C, 5% CO\textsubscript{2}). Cells were washed 3 times with PBS containing 1 mM/L MgCl\textsubscript{2}, 1 mM/L EDTA, and 2.5 mM/L probenecid in a Tecan cell washer. After the final wash, 100 μL of residual volume remained on the cells in each 96-well plate. Peptides were dissolved in water or the appropriate solvent as 2-mmol/L stock solutions and diluted at least 1:100 into the washing buffer described above. Peptides were separated into aliquots as 3× solutions into a 96-well plate before the assay. Agonist activity was determined with peak fluorescent counts.

**[\textsuperscript{3}H]Ang II Binding Assay**

Forty-eight hours after transfection, CHO-K1 cells expressing either the AT\textsubscript{1} receptor plus vector, the Mas receptor plus vector, or the AT\textsubscript{1} plus Mas receptors were used for saturation binding assays with [\textsuperscript{tyrosyl-3,5-}\textsuperscript{3}H]Ang II (20 μM; Amersham-Pharmacia-Biotech) as the radioligand. CHO cells were harvested by trypsinization followed by centrifugation (300g, 3 minutes) and resuspension of the pellet in HEPES buffer (in mmol/L: NaCl 110, KCl 5.4, CaCl\textsubscript{2} 1.8, MgSO\textsubscript{4} 1, glucose 25, MHEPES 50, and sucrose 58; pH 7.4), repeated twice. Subsequently, 10\textsuperscript{5} cells/tube were distributed into assay tubes that contained increasing concentrations of [\textsuperscript{3}H]Ang II (0.2 to 50 nmol/L) in a final volume of 1 mL/tube and allowed to equilibrate for 3 hours at 4°C. Nonspecific binding was determined with 100 nmol/L unlabeled Ang II. Reactions were terminated by rapid filtration through ice-cold MHEPES buffer (1 mL/tube) with Whatman GF/C filters. Filters were washed 3 times with 4-mL aliquots of ice-cold buffer and dried before being exposed to 5 mL of scintillation cocktail (Ultima Gold LSC-cocktail; Packard BioScience). Radioactivity was determined via scintillation counting.

**Bioluminescence Resonance Energy Transfer**

**Construction of Plasmids**

Enhanced yellow fluorescent protein (eYFP) was appended to the C-terminal tail of the AT\textsubscript{1} receptor from which the stop codon was eliminated, and Mas was modified by C-terminal, in-frame addition of Renilla luciferase. The production of thyrotropin-releasing hormone (TRH) receptor-1-eYFP and demonstration of the improved resolution of energy transfer signal from the emission spectrum of Renilla luciferase that is produced by the use of a variant of the bioluminescence resonance energy transfer (BRET\textsuperscript{2}) have been described elsewhere.\textsuperscript{19}

**Bioluminescence Resonance Energy Transfer**

HEK293 cells were grown to ~60% confluence before transfection with Lipofectamine reagent according to the manufacturer’s instructions. The majority of traditional BRET experiments (eg, Figures 4A and 4C) were performed with a Spex fluorolog spectrofluorimeter (SPEX). Cells were harvested 48 hours after transfection. Media were removed from cell culture dishes, and cells were washed twice with PBS before they were detached to form a suspension. Approximately 3×10\textsuperscript{5} cells in 1.5 mL of PBS buffer were then added to a glass cuvette; an equal volume of PBS containing 10 μM/L h-coelenterazine was added, and the contents of the cuvette were mixed. The emission spectrum (400 to 600 nm) was acquired immediately with the excitation lamp turned off (slit width 10 nm, 2 seconds per increment). For comparisons between experiments, emission spectra were normalized with the peak emission from Renilla luciferase in the region of 480 nm being defined as an intensity of 1.00. Energy transfer signal was calculated by measuring the area under the curve between 500 and 550 nm. Background was taken as the area of this region of the spectrum when we examined emission of cells that expressed only the energy donor, eg, Mas-Renilla luciferase. In a number of cases, BRET (eg, Figure 4B) measurements were performed with a Mithras LB 940 (Berthold Technologies Ltd) because its sensitivity to detect BRET is greater than that of the Spex fluorolog spectrofluorimeter. These experiments were performed in 96-well whitewall microtiter plates, with each treatment being performed in triplicate. To 100 μL of cells expressing appropriate constructs, an equal volume of 10 μM/L coelenterazine was added; this was mixed and immediately placed in the Mithras LB 940. When the cells were treated with drugs, the cells were incubated at 37°C for 5 minutes before addition of coelenterazine. Each well was counted for 1 second, and the data were collected with emission filters at 480 nm (luciferase emission) and 530 nm (energy transfer). In these examples, data are presented as the ratio (530/480 nm) of these values.

To perform BRET\textsuperscript{2} assays, cells were washed 3 times in PBS and then harvested in PBS supplemented with magnesium (0.1 g/L) and glucose (1 g/L). They were then counted on a hemocytometer, and ~700 000 cells per well were dispensed into a 96-well white-walled culture plate (Packard Biosciences). DeepBlueC (Packard Biosciences) reagent was prepared in accordance with the manufacturer’s directions and added to a final concentration of 10 μM/L. BRET\textsuperscript{2} signals were then measured immediately in a Mithras LB940 with a 410-nm filter (band pass 80 nm) to measure light emitted from DeepBlueC and a 515-nm filter (band pass 30 nm) to measure light emitted from a modified green fluorescent protein (GFP). Energy
acceptor to energy donor expression ratios were measured as described previously.19

Coimmunoprecipitation of AT1-eYFP and Mas-Gαi1
HEK293T cells were transfected transiently to express AT1-eYFP, Mas-Gαi1, or both. Sixteen hours later, cells were lysed with RIPA buffer in the presence of a cocktail of protein inhibitors (Complete, EDTA free, Roche). The lysates were centrifuged (100 000g, 1 hour) then precleared with protein-G sepharose. Protein content was adjusted to 1 mg/mL, and 500 μL was immunoprecipitated with an anti-GFP antiserum. After 3 washes with RIPA, addition of Laemmli buffer and separation by SDS-PAGE were performed. The proteins were transferred to nitrocellulose and immunoblotted with an anti-Gαi/Gαi1 antisem.

Effects on Vascular Tone of Mesenteric Microvessels
Wild-type and Mas-deficient mice were anesthetized with sodium pentobarbital 70 mg/kg IP and exsanguinated. The mesentery was removed, and third branch mesenteric arteries were removed and placed on a small-vessel myograph20 capable of measuring isometric tension. and containing a physiological solution, as already reported.21 Their passive tension and internal circumference were determined, and the mean internal diameter ranged between 150 and 300 μm, with nonsignificant differences among the different groups of mice. Arteries were then contracted with 125 mmol/L KCl (equimolar substitution of KCl for NaCl in the physiological solution) for 2 minutes. The bath was then washed several times, and a further resting period of 30 minutes was allowed. Afterward, the arteries were subjected to increasing concentrations of Ang II (10 μmol/L to 1 μmol/L) or endothelin-1 (10 μmol/L to 0.1 μmol/L) at 2-minute intervals, either in the absence or presence of 1 μmol/L losartan, according to procedures described previously.22

Statistical Analysis
Radioligand saturation binding data sets were fitted globally via nonlinear regression to mass-action models of both total and non-specific binding with Prism 4.0 (GraphPad Software) to derive estimates of radioligand affinity (Kd) and maximal receptor density (Bmax). All concentration-response data were fitted to a 4-parameter Hill equation with Prism. Statistical evaluation of compound effects on the vascular tone of mesenteric microvessels was performed by deviations from the mean with regard to the curves for Ang II and losartan or endothelin-1 with factorial 2-way ANOVA. Student t test was used in other comparisons (mean±SEM). A value of P<0.05 was considered statistically significant.

Results
Initially, cDNAs encoding the human forms of the Mas and AT1 receptors individually and in combination were expressed in CHO-K1 cells, and mobilization of intracellular calcium ([Ca2+]i) on stimulation with varying concentrations of Ang II was recorded. Expression of Mas alone did not confer responsiveness to Ang II (Figure 1A). In contrast, in cells expressing the AT1 receptor, Ang II potently mediated elevation of [Ca2+]i, with a log EC50 = −9.51 ±0.11 (n=3; Figure 1A, filled squares). Importantly, coexpression of Mas with the AT1 receptor significantly impaired the potency (log EC50 = −8.90 ±0.11; n=3; P<0.05) and the maximal effectiveness of Ang II to enhance [Ca2+]i mobilization (Figure 1A, open squares). To test whether the impairment by Mas of Ang II–mediated [Ca2+]i mobilization via the AT1 receptor is retained at lower expression levels, identical coexpression experiments were performed with 1/30th of the cDNA for transfections. Under these conditions, Ang II mediated elevation of [Ca2+]i, via the AT1 receptor with a log EC50 = −9.40 ±0.13; n=3 (Figure 1A, filled circles). Notably, coexpression of Mas impaired Ang II–mediated [Ca2+]i mobilization to a similar extent (log EC50 = −8.85 ±0.29; n=3; Figure 1A, open circles). Increasing concentrations of the AT1 receptor antagonist losartan induced significant rightward shifts of the Ang II concentration-response curves of cells expressing the AT1 receptor alone (Figure 1B) or the AT1 receptor in combination with Mas (Figure 1C). Schild plots for losartan were linear over a wide concentration range (0.03 to 3.0 μmol/L); the slopes of the plots were not significantly different from unity (P>0.05), and antagonist potency estimates were similar in the absence or presence of Mas. Despite the effect of Mas on responsiveness to Ang II, these results indicate that the AT1 receptor retains its pharmacological characteristics with respect to competitive antagonists, such as losartan. Both Ang II elevation of [Ca2+]i, and its diminution in the presence of Mas were unaffected by the presence of the Ang metabolite Ang-(1-7) (Figure 1D), which has been described recently as an endogenous agonist for Mas.11

We next investigated whether impairment by Mas of Ang II–mediated activation of the AT1 receptor is also detectable at the level of inositol phosphate production, for which less signal amplification exists than with Ca2+ mobilization (Figure 1E). Evidently, coexpression of Mas impaired the potency and efficiency of Ang II–mediated generation of inositol phosphates in a manner similar to that observed in the Ca2+ mobilization assays (Figure 1A).

To explore the specificity of the effect of Mas on AT1 receptor–mediated mobilization of [Ca2+]i, the AT1 receptor was coexpressed with a series of other class I GPCRs. In contrast to the effects of Mas, Ang II mobilization of [Ca2+]i, was unaffected by the presence of the Gq/11-coupled sphingosine-1-phosphate [S1P3], urotensin II, or muscarinic M1 acetylcholine receptors (Figure 2). Specific ligands of each receptor stimulated [Ca2+]i, in AT1-cotransfected cells in a manner identical to that observed with each receptor alone, and Mas did not interfere with [Ca2+]i, mobilization of the bona fide Gq-coupled urotensin II receptor (data not shown). Mas thus appeared to act specifically as a functional antagonist of the AT1 receptor, and it can be excluded that AT1 is, in general, an indiscriminate coupling partner for other GPCRs or that Mas represents an indiscriminate inhibitor.

Because it was possible that the antagonist effect of Mas on AT1 receptor signaling was due to a reduction in AT1 receptor expression, we performed [3H]Ang II binding experiments to directly determine Bmax and Kd for the radioligand to the AT1 receptor in the absence and presence of Mas. In contrast to the effects observed on Ang II–mediated signaling, coexpression of Mas resulted in a significant enhancement (235%) of the [3H]Ang II Bmax value; there was no significant effect on radioligand affinity (Figure 3). The paradoxical effect of Mas expression on [3H]Ang II binding capacity was not due to radioligand binding to Mas, because there was no specific binding observed in cells transfected with Mas plus vector alone (Figure 3 inset). One possible explanation for the enhancement in [3H]Ang II Bmax is an alteration in steady state cell-surface expression due to a
change in AT1 receptor trafficking properties, as has been demonstrated for other GPCR heterodimers.23

The differential effects on AT1-mediated Ang II binding capacity on the one hand and signaling on the other by the coexpression of Mas suggested that the AT1 receptor was physically altered by Mas such that the enhanced cell-surface expression of the AT1 receptor was offset by reduced receptor functionality, most likely due to a conformational constraint on AT1 signaling within the hetero-oligomer. The potential for such direct interactions between Mas and the AT1 receptor was thus investigated with various forms of BRET.19,24,25

eYFP was appended to the C-terminal tail of the AT1 receptor from which the stop codon was eliminated. When this construct was expressed transiently in HEK293 cells, fluorescence microscopy demonstrated it to be present largely at the cell surface (data not shown). Such eYFP-tagged constructs act as appropriate energy acceptors of light generated from the oxidation of coelenterazine by the luciferase from Renilla reniformis if the luciferase and eYFP are within 50 to 100 Å.24,25 Mas was modified by the in-frame addition of

Figure 1. Coexpression with Mas reduces AT1 receptor–mediated elevation of intracellular calcium and inositol phosphate generation. Mas and AT1 receptor were expressed in CHO-K1 cells either alone or in combination. pcDNA3.1 vector was used to equalize amounts of total DNA used in each transfection. A, Elevation of \([Ca^{2+}]_i\) in response to varying concentrations of Ang II was recorded. Squares represent those curves performed after transfection of 2 μg of total DNA per 35-mm dish, and circles display curves recorded after transfection of 0.06 μg of total DNA per 35-mm dish. B and C, Ang II concentration-response curve in presence of various concentrations of specific AT1 receptor antagonist losartan in CHO cells transiently transfected with AT1 receptor (B) or AT1 receptor and Mas (C). Insets in B and C, Transformation of dose ratios into Schild plots for losartan. D, Concentration-response curve for Ang II in AT1 versus AT1/Mas-transfected cells in absence and presence of Mas agonist Ang-(1-7). A, Representative experiment performed in triplicate. B and C, Representative dose-response curves performed in duplicate; in Schild plots, each point is mean of 4 independent experiments. D, Values are percentage of maximal Ang II–induced stimulation of \([Ca^{2+}]_i\) measured in AT1 receptor–expressing cells. Data are mean ± SD of at least 3 independent experiments performed in duplicate. E, Elevation of inositol phosphates (IP) in response to varying concentrations of Ang II in IP accumulation assays. Values are expressed as percentage of maximal Ang II–mediated IP production by AT1 receptor. Data are mean ± SD of 3 independent experiments performed in duplicate. Where not shown, error bars lie within dimensions of symbols. FIU indicates fluorescence intensity units; DR, dose ratio.
Renilla luciferase to the C-terminal tail of this polypeptide. Expression of Mas–Renilla luciferase in HEK293 cells followed by the addition of coelenterazine resulted in the generation of luminescence with a single peak centered at 480 nm (Figure 4A). By contrast, addition of coelenterazine to HEK293 cells transfected to express a BRET-positive control, consisting of a fusion protein between Renilla luciferase and eYFP, resulted in both the peak at 480 nm and a second peak at 527 nm that reflects energy transfer (Figure 4B).20,22 Coexpression of Mas–Renilla luciferase and AT1–eYFP followed by the addition of coelenterazine also generated a spectrum consistent with strong energy transfer between the luciferase and eYFP (Figure 4A). Such results are consistent with the hypothesis that these 2 GPCRs are able to form an oligomeric complex.14,26 This interaction was constitutive, because it was not altered significantly by addition of receptor ligands that act as agonists at the AT1 receptor but not at Mas (Ang II), at Mas but not at the AT1 receptor [Ang-(1-7)], or as an antagonist at the AT1 receptor (losartan; Figures 4A and 4B).11

Coexpressed GPCRs frequently display a propensity to interact and can often be coimmunoprecipitated.21 It was important to examine whether the interaction between Mas–Renilla luciferase and AT1–eYFP was selective. We therefore coexpressed Mas–Renilla luciferase with a C-terminal eYFP-tagged form of the TRH receptor-1.19 We have previously shown this receptor construct to interact with only low affinity with the KOP opioid receptor.19 TRH receptor-1–eYFP was expressed at similar levels as AT1–eYFP as monitored directly by the fluorescence that corresponded to eYFP. No significant BRET signal was obtained (Figure 4C), which suggests there was little or no interaction between Mas and TRH receptor-1.

We also wished to explore direct protein–protein interactions between the AT1 receptor and Mas with coimmunoprecipitation. Because available antisera against the native receptors were unable to immunoprecipitate with high efficiency (data not shown), we used the AT1–eYFP construct in conjunction with a form of Mas tagged at the C-terminus with the G protein Goα11. Immunoprecipitation with an anti-GFP antiserum of transfected HEK293 cells resulted in coimmunoprecipitation of Mas–Goα11 when the 2 receptor constructs were coexpressed but, importantly, not when lysates of cells that individually expressed either AT1–eYFP or Mas–Goα11 were mixed before immunoprecipitation (Figure 4D). In addition to polypeptides in the region of 75 to 80 kDa that were consistent with the anticipated size of Mas–Goα11, a band of some 60 kDa was detected in the coimmunoprecipitation studies and may represent a proteolytic fragment.

In “saturation” BRET assays, the ratio of the energy acceptor to energy donor is varied over a significant range. With increasing ratio of acceptor to donor, it is expected that a maximal BRET signal will be reached when all donor molecules interact with an acceptor.22 A hyperbolic saturation curve should thus be generated for pairs of interacting proteins in contrast with noninteracting proteins. Recent developments in BRET technology have improved the signal to background noise ratio that can be obtained by introduction of a novel luciferase substrate that, on oxidation, emits light at shorter wavelengths. This results in less spillover into the region of the spectrum used to measure energy transfer. So-called BRET2 was thus used in saturation BRET assays.19,22 The ratio of coexpression of Mas–Renilla luciferase and the AT1 receptor linked to the modified fluorescent protein GFP2 was varied in populations of HEK293 cells and calculated as in Methods. Addition of deep blue coelenterazine (DeepBlueC) as substrate resulted in energy transfer that approached saturation with increasing energy acceptor (AT1–GFP2) to energy donor (Mas–Renilla luciferase) ratios (Figure 5A). As a control, Mas–Renilla luciferase and the isolated

Figure 2. AT1 receptor elevation of intracellular [Ca2+]i is not affected by coexpression of range of GPCRs. AT1 receptor was coexpressed with pCDNA3.1 vector DNA (control) or series of G-protein–coupled receptors (Mas, sphingosine-1-phosphate [S1P], muscarinic M3, and urotensin II receptor) in CHO-K1 cells, and Ang II–mediated mobilization of intracellular calcium was recorded in functional FLIPR assay as described in Figure 1. Only coexpression with Mas significantly reduced functional response of Ang II. Values are percentage of maximal Ang II–induced stimulation of [Ca2+]i measured in AT1 receptor–expressing cells. Data are mean±SD of at least 3 independent experiments performed in duplicate.

Figure 3. Coexpression with Mas does not reduce expression of AT1 receptor. Saturation binding of [3H]Ang II to CHO-K1 cells transiently cotransfected with human AT1 receptors together with empty pcDNA3.1 vector (●) or with vector containing human Mas gene (○). Nonlinear regression analysis yielded negative log dissociation constants of 8.13±0.13 and 8.45±0.08 (n=3), respectively, for control and Mas-transfected cells. Owing to variations in transfection efficiency, data are expressed in terms of percentage of maximal binding (ca. 480 fmol per 10^5 cells) observed in absence of Mas cotransfection. Inset, Lack of binding of 2 nmol/L [3H]Ang II in CHO cells transfected with Mas alone. DPM indicates disintegrations per minute.
GFP2 were coexpressed in varying ratios. The BRET signal was almost undetectable. It was much smaller at equal energy acceptor to energy donor ratios and did not saturate but simply increased in a linear fashion with increasing GFP2 to Mas-Renilla luciferase expression ratios (Figure 5A). Isolated GFP2 is a soluble polypeptide and thus is not anticipated to be in the same cellular compartment as Mas-Renilla luciferase. The lack of energy transfer between coexpressed Mas-Renilla luciferase and GFP2 also confirms that the energy transfer interactions generated by the Mas-AT1 pairing reflect interactions between the GPCRs and not between the luciferase and GFP2. To investigate the functional consequences that varying levels of Mas exert on AT1 receptor signaling, Ang II–mediated Ca2+/H11001 elevation was measured in CHO cells transiently cotransfected with a fixed amount of AT1 and increasing amounts of Mas cDNA (Figure 5B). Notably, impairment by Mas of Ang II–mediated Ca2+/H11001 release via AT1 was inversely correlated to the amounts of Mas cDNA used in the cotransfection experiments. These data imply that AT1 receptor signaling in vivo may be regulated in a cell- and tissue-specific manner by the level of Mas expression.

The physiological relevance of hetero-oligomerization between the AT1 receptor and Mas was further investigated with mesenteric microvessels of wild-type mice, which express both receptor mRNAs as demonstrated by reverse transcription–polymerase chain reaction (data not shown), in comparison to Mas-knockout mice. Specifically, although Ang II produced robust contraction of vessels from wild-type mice in its own right, this effect was markedly enhanced in vessels from the Mas-knockout animals at all concentrations of Ang II (Figure 6A). In both types of mice, these contractions were abolished by the AT1 antagonist losartan (1 μmol/L). The specificity of the effect of genetic ablation of Mas on Ang II–mediated contractions was further confirmed by measuring

Figure 4. Hetero-oligomeric interactions after coexpression of Mas and AT1 receptor. Mas-Renilla luciferase was expressed in isolation in HEK293 cells (A, purple; B, Mas) or in combination with AT1-eYFP (A, all other colors; B, MAS/AT). In certain experiments, fusion protein between Renilla luciferase and eYFP was expressed (B, LUC/eYFP). Coexpressions of Mas-Renilla luciferase with AT1-eYFP (MAS/AT) and Mas-Renilla luciferase with TRH receptor-1–eYFP (MAS/TRH) were also performed (C). Coelenterazine and either no ligand (A, purple; B, light gray), 100 nmol/L Ang II (A, yellow; B, dark gray), 100 nmol/L losartan (A, blue; B, white), or 100 nmol/L Ang-(1-7) (A, red; B, black) were added. Emission spectra were then collected on Spex fluorolog spectrofluorimeter (A and C), or BRET data (B) were measured with Mithras LB 940 as detailed in Methods. D, HEK293T cells were mock-transfected (1) or transfected to express AT1-eYFP (2), Mas-G11 (3), or both AT1-eYFP and Mas-G11 (4). In (5), cells expressing either AT1-eYFP or Mas-G11 were mixed. Lysates from these cells were immunoprecipitated with anti-eYFP antibody, and after resolution by SDS-PAGE, immunoblot was performed with anti-G11/G11 antisera. c.p.s. indicates counts per second.
the effect of another vasoconstrictor peptide, endothelin-1, in Mas-deficient microvessels. Although the absolute response to endothelin-1 was greater than that observed for Ang II in microvessels of both genotypes, the pattern of the contractile response in the absence versus the presence of Mas was different for one vasoconstrictor peptide relative to the other. Mas-deficient microvessels did not show a general overreactivity but rather a less pronounced contractile response to higher endothelin-1 concentrations compared with wild-type vessels (Figure 6B).

Taken together, our findings demonstrate that the protein encoded by the Mas proto-oncogene exhibits direct antagonistic properties on the AT1 receptor in vitro and that this oligomeric interaction may represent a natural state for these receptors in vivo in some tissues. Specifically, we have shown that the expression of Mas interferes with functional activity of the AT1 receptor in transfected mammalian cell lines in vitro, even though it enhances maximal cell-surface expression of the AT1 receptor, and that the mechanism is most likely a consequence of the constitutive physical association between the 2 receptors ex vivo. Native tissue data strongly support the cell-line findings, because the absence of the Mas receptor in Mas-knockout animals significantly enhanced the vasoactive properties of Ang II but showed a different pattern of effect on those induced by endothelin-1. The fact that the increased response to Ang II was abolished by the AT1 receptor antagonist losartan, with pharmacology comparable to that of wild-type–derived vessels (Figure 6A), clearly indicates an AT1-specific effect in the native tissues. In conjunction with the oligomerization demonstrated in the BRET studies, the present findings in native tissues suggest that the Mas receptor can act as an in vivo functional antagonist of the AT1 receptor owing to formation of a hetero-oligomeric complex. Given that recent evidence has also shown that the AT2 receptor can act as an antagonist of...
AT₁ function, at least in vitro, it is likely that these collective findings are indicative of a novel property of GPCR signaling, namely, the direct regulation of signal transduction via one GPCR by the physical presence of another.¹⁷

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