Up-regulation of the Angiotensin II Type 1 Receptor by the MAS Proto-oncogene Is Due to Constitutive Activation of Gq/G11 by MAS*

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Coexpression of the MAS proto-oncogene with the angiotensin II type 1 (AT1) receptor in CHO-K1 cells has been reported to increase the number of [3H]angiotensin II-binding sites, although MAS does not bind [3H]angiotensin II. In HEK293 cells stably expressing AT1 receptor-cyan fluorescent protein (CFP), MAS-yellow fluorescent protein (YFP) expression from an inducible locus caused strong up-regulation of AT1 receptor-CFP amounts and [3H]angiotensin II binding levels. The time course of AT1 receptor-CFP up-regulation was also markedly slower than that of induction of MAS expression. These effects were not mimicked by induced expression of I138D MAS-YFP, a slower than that of induction of MAS-yellow fluorescent protein (YFP) expression from an linked GPCRs, the angiotensin II type 1 (AT1) and 2 (AT2) components of the renin-angiotensin system and, as such, plays a major understood (2, 3), important biological functions such as vasoconstric-tor receptor have also been reported (7, 8).

The octapeptide hormone angiotensin (Ang)2 II is one of the key extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase.

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EXPERIMENTAL PROCEDURES

Materials—All materials for tissue culture were from Invitrogen (Paisley, UK). The PKC inhibitor Ro 31-8220, phospholipase Cβ (PLCβ), doxycycline, and Ang II were from Sigma. GF 109203X was from Tocris (Avonmouth, UK), and YM-254890 was the kind gift of Astrellas Pharma Inc. (Osaka, Japan).
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Site-directed Mutagenesis—To introduce amino acid substitutions into the primary structure of the human MAS receptor, site-directed mutagenesis of the encoding nucleotide sequence was performed using the QuikChange® II site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The following primers were designed for the production of the I138D mutation (with the bases underlined): GTCCTTTACCCCGACTGGTACCGATGC (forward) and GCATCGTAGACGGTGGGTAAGGAC (reverse).

Flp-In Constructs—To create MAS receptor constructs N-terminally tagged with the vesicular stomatitis virus G (VSV-G) epitope sequence and fused at the C terminus with yellow fluorescent protein (YFP), the human MAS receptor and I138D MAS were used as PCR templates. An oligonucleotide encoding a BamHI restriction site, the VSV-G epitope sequence, and the first 21 bases of the MAS receptor sequence was used as the forward primer (GGGGATCCATGTACACCGACATCGAATTGACCCCGCTTGGTAAAGGATGGGTCAACACGTGACATCA), and an oligonucleotide containing the last 21 bases of the MAS sequence without its stop codon and a NotI restriction site was employed as the reverse primer (TTTTCTTTTGGCCCGCGGTAACGACATGCACGTTAC). The PCR product was then inserted into vector pCDNA5/FRT/TO (Invitrogen) already containing YFP and previously digested with BamHI and NotI.

c-Myc-AT₁ receptor (AT₁-R)-cyan fluorescent protein (CFP) was obtained using the same strategy as described above with the human AT₁ receptor as a template; a forward primer encoding a HindIII restriction site, the c-Myc epitope sequence, and the first 21 bases of the receptor sequence (CCCAAGCTTATGGAACAAAAACTTTATTTCTGAAGAGATCTCAGTCTCTACTGAAGATTGG), and a reverse primer containing the last 21 bases of the AT₁ receptor without its stop codon and a KpnI restriction site (CCGGGTACCCCTACCCCTCAGGCAGTGGTACGCGATGATG). The PCR product was then inserted in pcDNA3.1 already containing CFP and previously digested with HindIII and KpnI. c-Myc-AT₁-R-YFP in pcDNA5/FRT/TO was subcloned by digesting c-Myc-AT₁ receptor (AT₁-R)-cyan fluorescent protein (CFP) was obtained using the same strategy as described above with the human AT₁ receptor as a template; a forward primer encoding a HindIII restriction site, the c-Myc epitope sequence, and the first 21 bases of the receptor sequence (CCCAAGCTTATGGAACAAAAACTTTATTTCTGAAGAGATCTCAGTCTCTACTGAAGATTGG), and a reverse primer containing the last 21 bases of the AT₁ receptor without its stop codon and a KpnI restriction site (CCGGGTACCCCTACCCCTCAGGCAGTGGTACGCGATGATG). The PCR product was then inserted in pcDNA3.1 already containing CFP and previously digested with HindIII and KpnI. c-Myc-AT₁ receptor (AT₁-R)-cyan fluorescent protein (CFP) was obtained using the same strategy as described above with the human AT₁ receptor as a template; a forward primer encoding a HindIII restriction site, the c-Myc epitope sequence, and the first 21 bases of the receptor sequence (CCCAAGCTTATGGAACAAAAACTTTATTTCTGAAGAGATCTCAGTCTCTACTGAAGATTGG), and a reverse primer containing the last 21 bases of the AT₁ receptor without its stop codon and a KpnI restriction site (CCGGGTACCCCTACCCCTCAGGCAGTGGTACGCGATGATG). The PCR product was then inserted in pcDNA3.1 already containing CFP and previously digested with HindIII and KpnI. c-Myc-Δ²⁵⁵AT₁ receptor (AT₁-R)-cyan fluorescent protein (CFP) was obtained using the same strategy as described above with the human receptor cDNA in the pcDNA5/FRT/TO vector and pOG44 vectors (1:9) using Effectene according to the manufacturer’s instructions. Cell maintenance and selection were as described (16). Resistant clones were screened for receptor expression by both fluorescence and Western blotting. To induce expression of receptors cloned into the Flip-In locus, cells were treated with 0.1 μg/ml doxycycline for varying periods of time. PKC activation and inhibition, as well as Gαq/Gα11, inhibition treatments, were performed 6 h after inducing the receptors for an overnight period.

Live Cell Epifluorescence Microscopy—Cells expressing the appropriate receptors tagged with CFP or YFP were grown on poly-D-lysine-treated coverslips. The coverslips were placed into a microscope chamber containing physiological saline solution (130 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, and 10 mM d-glucose, pH 7.4). Fluorescent images of the cells were acquired using a Nikon TE2000-E inverted microscope (Nikon Instruments, Melville, NY) equipped with a ×40 (numerical aperture = 1.3) oil immersion Plan Fluor lens and a cooled digital CoolSNAP_HQ charge-coupled device camera (Photometrics, Tucson, AZ) (see Ref. 17 for details).

Visualization of the Plasma Membrane—To fluorescently visualize the plasma membrane in live HEK293 cells expressing CFP- or YFP-fused receptors, cells were treated (as specified by the manufacturer) with the reagents in the Image-IT plasma membrane and nuclear labeling kit (Invitrogen), in which the plasma membrane is specifically labeled with wheat germ agglutinin-Alexa Fluor 594, and nuclei are stained simultaneously with Hoechst 33342. CFP and YFP were excited as described above, and Alexa Fluor 594 was excited at 575/12 nm and imaged using the following filter set: dichroic, Q595LP; and emitter, HQ645/75m. Using these filters, no bleed-through was observed, and the resultant sequential 12-bit images were overlaid using MetaMorph software (Version 6.3.5, Universal Imaging Corp., Downingtown, PA).

For three-dimensional imaging, stacks of images (2 × 2 binning, 150–200 ms exposure/image) with a 0.339-μm Z step (20–25 frames/stack) were sequentially acquired for each fluorescent dye.

[^35]GTPγS Binding—[^35]GTPγS binding experiments were initiated by the addition of membranes to assay buffer (20 mM HEPES, pH 7.4, 3 mM MgCl₂, 100 mM NaCl, 1 μM GDP, 0.2 mM ascorbic acid, and 100 nM of[^35]GTPγS) containing the indicated concentrations of receptor ligands. Nonspecific binding was determined under the same conditions but in the presence of 100 μM GTPγS. Reactions were incubated for 30 min at 30°C and terminated by the addition of 0.5 ml of ice-cold buffer containing 20 mM HEPES, pH 7.4, 3 mM MgCl₂, 100 mM NaCl, and 0.2 mM ascorbic acid. The samples were centrifuged at 16,000 × g for 10 min at 4°C, and the resulting pellets were resuspended in solubilization buffer (100 mM Tris, 200 mM NaCl, 1 mM EDTA, and 1.25% Nonidet P-40) plus 0.2% SDS. Samples were prewashed with Pan sorbin (Calbiochem, Nottingham, UK), followed by immunoprecipitation with antisemur CQ (18). Finally, the immunocomplexes were washed twice with solubilization buffer, and bound[^35]GTPγS was measured by liquid scintillation spectrometry.

Cell-surface Receptor Measurement and Enzyme-linked Immunosorbent Assay—Cells were grown in 96-well poly-d-lysine-coated plates and induced with different concentrations of doxycycline for 24 h. Afterward, cell-surface receptors were labeled with anti-VSV-G antibody (1:1000) in growth medium for 30 min at 37°C. The cells were then washed once with 20 mM HEPES and Dulbecco’s modified Eagle’s medium and then incubated for another 30 min at 37°C in growth medium supplemented with horseradish peroxidase-conjugated anti-rabbit IgG as the secondary antibody and 1 μM Hoechst nuclear stain (Sigma) to determine the number of cells in each well. The cells were...
was mixed with scintillation mixture and counted (Packard 2000 CA scintillation counter) to measure bound 

RESULTS

We recently reported that coexpression of the GPCR MAS with the AT\textsubscript{1} receptor in CHO-K1 cells results in a decreased capacity of Ang II to increase intracellular [Ca\textsuperscript{2+}] and inositol phosphate accumulation (14). Paradoxically, however, this is associated with an increase in 

Generation of Flp-In T-Rex HEK293 Cell Lines—A Flp-In T-Rex HEK293 clonal cell line was established in which a form of the human AT\textsubscript{1} receptor C-terminally tagged with CFP and N-terminally tagged with the c-Myc epitope sequence (c-Myc-AT\textsubscript{1}, R-CPFP) was expressed stably and constitutively. These cells also harbored, at the Flp-In locus, a form of human MAS C-terminally tagged with YFP and N-terminally tagged with the VSV-G epitope sequence (VSV-G-MAS-YFP). The Flp-In locus ensures a single defined site of chromosomal integration; and in the T-REx form of these cells, expression from this locus is controlled in a Tet-on-inducible fashion by the addition of either tetracycline or the related antibiotic doxycycline (16). In the absence of doxycycline, expression of c-Myc-AT\textsubscript{1}, R-CPFP and VSV-G-MAS-YFP is negligible. Fluorescence corresponding to YFP (A\textsubscript{1} and B\textsubscript{1}) and CFP (A\textsubscript{2} and B\textsubscript{2}) was then imaged.

FIGURE 1. Induced expression of MAS results in up-regulation of a coexpressed AT\textsubscript{1} receptor. Flp-In T-Rex HEK293 cells harboring VSV-G-MAS-YFP at the Flp-In locus (A\textsubscript{1} and B\textsubscript{1}) and constitutively expressing c-Myc-AT\textsubscript{1}, R-CPFP (A\textsubscript{2} and B\textsubscript{2}) were left untreated (A\textsubscript{1} and A\textsubscript{2}) or were treated with doxycycline (0.1 μg/ml) for 24 h (B\textsubscript{1} and B\textsubscript{2}) to induce VSV-G-MAS-YFP expression. Fluorescence corresponding to YFP (A\textsubscript{1} and B\textsubscript{1}) and CFP (A\textsubscript{2} and B\textsubscript{2}) was then imaged.
AT1R-CFP was markedly increased (Fig. 1B), with much of the signal apparently inside the cell.

**MAS Constitutively Activates Gα11**—Transient transfection of MAS into HEK293 cells along with the phospholipase Cβ-linked G protein Gα11 resulted in loading of ~4-fold greater amounts of [35S]GTPγS onto the G protein than observed in the absence of MAS, consistent with the substantial ligand-independent, constitutive activity of this GPCR (Fig. 2). We have demonstrated previously that mutation of key hydrophobic residues in the second intracellular loop of many rhodopsin-like class A GPCRs ablates their capacity to activate cognate G proteins (18, 19). Conversion of lle138 to Asp in MAS (I138D MAS) and expression of this form of the receptor with Gα11 failed to enhance [35S]GTPγS binding to the G protein (Fig. 2).

**I138D MAS Is Delivered to the Cell Surface but Does Not Up-regulate the AT1 Receptor**—Based on the ability of the I138D mutation to eliminate constitutive MAS-induced loading of [35S]GTPγS onto Gα11, we generated additional Flp-In T-REx HEK293 cell lines that constitutively expressed c-Myc-AT1R-CFP but also harbored VSV-G-I138D MAS-YFP at the Flp-in locus (Fig. 3A). Induction of VSV-G-I138D MAS-YFP expression by treatment with doxycycline resulted in a completely different distribution pattern compared to MAS, but not I138D MAS, causes constitutive activation of Gα11. HEK293 cells were transfected transiently to express Gα11, MAS, and Gα11, or I138D MAS and Gα11, and membranes were prepared. Following incubation with [35S]GTPγS, samples were immunoprecipitated with anti-Gα11 antiserum (18) and counted. Data represent the means ± S.E. (n = 3).

**FIGURE 2. MAS Up-regulates the AT1 Receptor**

**FIGURE 3. I138D MAS, but not MAS, is located predominantly at the cell surface.** A and B, Flp-In T-REx HEK293 cells harboring VSV-G-I138D MAS-YFP at the Flp-in locus (left panels) and constitutively expressing c-Myc-AT1R-CFP (right panels) were left untreated (A) or were treated with doxycycline (0.1 µg/ml) for 24 h (B) to induce VSV-G-I138D MAS-YFP expression. Fluorescence corresponding to YFP (left panels) and CFP (right panels) was then imaged. C, intact cell anti-VSV-G enzyme-linked immunosorbent assays were performed on Flp-In T-REx HEK293 cells harboring VSV-G-MAS-YFP (open bars) or VSV-G-I138D MAS-YFP (closed bars) after treatment of the cells for 24 h with various concentrations of doxycycline (Dox). Abs, absorbance. D, cells as in C, harboring VSV-G-MAS-YFP (panels i and ii) or VSV-G-I138D MAS-YFP (panels iii and iv), were left untreated (panels i and iii) or were treated with doxycycline (0.1 µg/ml) for 24 h (panels ii and iv) and stained with Image-iT membrane dye (red).
with VSV-G-MAS-YFP. The I138D MAS construct appeared to be located largely at the plasma membrane (Fig. 3B). Expression of VSV-G-I138D MAS-YFP neither up-regulated c-Myc-AT, or CF nor markedly altered its cellular distribution (Fig. 3B). To confirm substantially more effective plasma membrane localization of VSV-G-I138D MAS-YFP, we performed a series of intact cell anti-VSV-G enzyme-linked immunosorbent assays. Although induction of VSV-G-MAS-YFP expression with increasing concentrations of doxycycline resulted in little increase in anti-VSV-G cell-surface immuno-reactivity (Fig. 3C), a clear, doxycycline concentration-dependent

FIGURE 4. AT1 receptor glycosylation, biotinylation, and up-regulation studies. A, shown is the cell-surface biotinylation of Flp-In T-REx HEK293 cells harboring VSV-G-MAS-YFP or VSV-G-I138D MAS-YFP at the Flp-In locus. Cells were left untreated or were treated with doxycycline (Dox; 0.1 µg/ml) for 24 h to induce expression of the forms of MAS-YFP, and cell-surface receptors were biotinylated and pulled down with streptavidin-agarose beads. Total lysates (l) and precipitated samples (b) were then resolved by SDS-PAGE and immunoblotted with anti-VSV-G antibody, β, membranes of cells induced to coexpress VSV-G-MAS-YFP and c-Myc-AT,R-CFP were treated with or without peptide N-glycosidase F (NGaseF), resolved by SDS-PAGE, and immunoblotted with anti-c-Myc antibody to identify forms of c-Myc-AT,R-CFP. C, shown is the cell-surface biotinylation of HEK293 cells expressing c-Myc-AT,R. Samples were resolved by SDS-PAGE and immunoblotted with anti-c-Myc antibody, ub, unbound; l, total lysate; b, cell-surface receptor. D, Flp-In T-REx HEK293 cells harboring VSV-G-MAS-YFP or VSV-G-I138D MAS-YFP at the Flp-In locus and constitutively expressing c-Myc-AT,R-CFP were left untreated or were treated with doxycycline (0.1 µg/ml) for 24 h to induce expression of the forms of MAS-YFP. Lysates of these cells as well as from cells expressing only VSV-G-MAS-YFP in an inducible fashion were then resolved by SDS-PAGE and immunoblotted with anti-c-Myc antibody. E, shown are the results from Western blot densitometry of cell lysates of Flp-In T-REx HEK293 cells harboring VSV-G-MAS-YFP at the Flp-In locus and constitutively expressing c-Myc-AT,R-CFP. A constant dose of 0.1 µg/ml doxycycline was used for different periods of induction. F, Flp-In T-REx HEK293 cells harboring VSV-G-MAS-YFP (upper panel) or VSV-G-I138D MAS-YFP (lower panel) at the Flp-In locus and constitutively expressing c-Myc-AT,R-CFP were treated (●) or not (□) with doxycycline and used to perform intact cell binding of [3H]Ang II. prot, protein.
increase in signal was obtained when VSV-G-I138D MAS-YFP expression was induced (Fig. 3C). Furthermore, staining and image overlay of cells induced to express either VSV-G-MAS-YFP or VSV-G-I138D MAS-YFP with Image-iT membrane dye confirmed effective plasma membrane delivery of VSV-G-I138D MAS-YFP, but not VSV-G-MAS-YFP (Fig. 3D). Cell-surface biotinylation studies also confirmed cell-surface delivery of VSV-G-I138D MAS-YFP, but not VSV-G-MAS-YFP (Fig. 4A).

Only the Core Glycosylated Form of the AT1 Receptor Is Delivered to the Cell Surface—Many GPCRs are produced as immature forms that require final core glycosylation prior to effective plasma membrane delivery and insertion. Membranes of cells induced to coexpress VSV-G-MAS-YFP and c-Myc-AT1R-CFP were treated with or without peptide N-glycosidase F, resolved by SDS-PAGE and immunoblotted with anti-c-Myc antibody to identify forms of c-Myc-AT1R-CFP and to further validate the 60-kDa polypeptide, although immunoblots of total cell lysates indicated that the 60-kDa species was present at similar levels (Fig. 4B). In the untreated samples, polypeptides with apparent masses of 60 and 90 kDa were detected as well as those with higher apparent molecular mass/lower mobility, which may represent dimeric or oligomeric complexes. Treatment with peptide N-glycosidase F resulted in the appearance of a predominant band at an apparent molecular mass of 50 kDa with substantially lower amounts of bands at ~60 kDa. These results suggest that both the 60- and 90-kDa forms are N-glycosylated and that the 90-kDa polypeptide is likely to represent the mature, core glycosylated receptor monomer. To confirm the concept that the higher molecular mass species was the mature form, we performed cell-surface biotinylation assays. In cells expressing the c-Myc-AT1R-CFP construct, we reasoned that the time course of AT1 receptor up-regulation would be slower than that of induction of MAS expres-

**MAS Expression Precedes AT1 Receptor Up-regulation**—If the presence of active MAS were required to cause up-regulation of the AT1 receptor construct, we reasoned that the time course of AT1 receptor up-regulation would be slower than that of induction of MAS expres-
sion. This was confirmed via a series of Western blot studies (Fig. 4E), in which the presence of VSV-G-MAS-YFP could be detected within 6 h of doxycycline addition, whereas significant up-regulation of c-Myc–AT1R-CFP required 10–18 h. To confirm the previous observations, cells constitutively expressing c-Myc–AT1R-CFP were induced or not to express VSV-G-MAS-YFP or VSV-G-I138D MAS-YFP, and the specific binding of increasing concentrations of [3H]Ang II was measured (Fig. 4F). As anticipated from the foregoing, the $B_{max}$ of [3H]Ang II binding was increased (0.2 ± 0.03 to 0.6 ± 0.06 pmol/mg of protein) by coexpression of VSV-G-MAS-YFP, but not VSV-G-I138D MAS-YFP (0.2 ± 0.02 versus 0.2 ± 0.06 pmol/mg of protein; means ± S.E., n = 3), whereas the affinity of [3H]Ang II ($K_a$ = 0.9–1.7 nM in individual experiments) was unaltered.

**Regulation of PKC Activity Modulates AT1 Receptor Levels**—Because MAS is able to constitutively activate $G_q/G_{11}$ (Fig. 2) and hence presumably activate PKC, we added the PKC inhibitor Ro 31-8220 to cells constitutively expressing c-Myc–AT1R-CFP with and without doxycycline to induce expression of VSV-G-MAS-YFP. Although without effect on [3H]Ang II binding in cells not induced to express VSV-G-MAS-YFP, Ro 31-8220 fully inhibited the increase in [3H]Ang II binding produced by expression of MAS (Fig. 5A). Ro 31-8220 was also without effect on [3H]Ang II binding in cells induced to express VSV-G-I138D MAS-YFP (Fig. 5A). Parallel immunoblots confirmed the effect of Ro 31-8220 (Fig. 5B), whereas equivalent immunoblots of total ERK1 and ERK2 MAPKs amounts confirmed equal sample loading. A second PKC inhibitor, GF 109203X, produced similar results (Fig. 5C). We reasoned that activation of PKC in the absence of MAS induction should also up-regulate c-Myc–AT1R, R-CFP levels. Treatment of cells harboring VSV-G-MAS-YFP with PMA produced a high level of c-Myc–AT1R, R-CFP up-regulation without induction of VSV-G-MAS-YFP expression (Fig. 5C). The effect of PMA was also blocked by the co-addition of Ro 31-8220 and was not increased further by induction of VSV-G-MAS-YFP expression (Fig. 5C). Simple quantitation of the levels of CFP fluorescence in living cells (Fig. 5D) confirmed the immunoblot results. As anticipated, PMA treatment of cells expressing c-Myc–AT1R, R-CFP and induced to express VSV-G-I138D MAS-YFP also resulted in marked up-regulation of c-Myc–AT1R, R-CFP, which was again blocked by the co-addition of Ro 31-8220 (Fig. 6).

**A Novel Gq/G11 Inhibitor Prevents MAS-induced Up-regulation of the AT1 Receptor**—Because $G_q$ and $G_{11}$ are upstream of PKC, we added the PKC inhibitor Ro 31-8220 to cells constitutively expressing c-Myc–AT1R, R-CFP with and without doxycycline to induce expression of VSV-G-MAS-YFP. Although without effect on [3H]Ang II binding in cells not induced to express VSV-G-MAS-YFP, Ro 31-8220 fully inhibited the increase in [3H]Ang II binding produced by expression of MAS (Fig. 5A). Ro 31-8220 was also without effect on [3H]Ang II binding in cells induced to express VSV-G-I138D MAS-YFP (Fig. 5A). Parallel immunoblots confirmed the effect of Ro 31-8220 (Fig. 5B), whereas equivalent immunoblots of total ERK1 and ERK2 MAPKs amounts confirmed equal sample loading. A second PKC inhibitor, GF 109203X, produced similar results (Fig. 5C). We reasoned that activation of PKC in the absence of MAS induction should also up-regulate c-Myc–AT1R, R-CFP levels. Treatment of cells harboring VSV-G-MAS-YFP with PMA produced a high level of c-Myc–AT1R, R-CFP up-regulation without induction of VSV-G-MAS-YFP expression (Fig. 5C). The effect of PMA was also blocked by the co-addition of Ro 31-8220 and was not increased further by induction of VSV-G-MAS-YFP expression (Fig. 5C). Simple quantitation of the levels of CFP fluorescence in living cells (Fig. 5D) confirmed the immunoblot results. As anticipated, PMA treatment of cells expressing c-Myc–AT1R, R-CFP and induced to express VSV-G-I138D MAS-YFP also resulted in marked up-regulation of c-Myc–AT1R, R-CFP, which was again blocked by the co-addition of Ro 31-8220 (Fig. 6).

**FIGURE 6. PMA treatment up-regulates AT1 receptor levels in cells expressing I138D MAS.** Flp-In T-REx HEK293 cells harboring VSV-G-I138D MAS-YFP and constitutively expressing c-Myc–AT1R, R-CFP were uninduced (left panels) or treated with doxycycline (+ Dox) as indicated. Such cells were also treated with PMA alone or with Ro 31-8220 as described in the legend to Fig. 5D. Cells were then imaged. CFP fluorescence is quantitated in the graph. A.F.U., arbitrary fluorescence units. Open bar, – doxycycline; filled bars, + doxycycline.
**MAS Up-regulates the AT₁ Receptor**

**FIGURE 7.** YM-254890 is a potent and highly selective inhibitor of Gᵢₒ and Gᵢ₁₁ activation. A, HEK293 cells were transfected to transiently express either the α₁β₂-adrenoceptor-Gᵢₒ (α₁β₂) or α₁ β₁-adrenoceptor-Gᵢ₁₁ (α₁β₁) fusion protein. The stimulation of [³⁵S]GTPγS binding in Gᵢₒ/Gᵢ₁₁ immunoprecipitates produced by 100 nM phenylephrine and the effect on this of varying concentrations of YM-254890 are displayed (means ± S.E., n = 3). B, HEK293 cells were transiently transfected to express the α₁β₂-adrenoceptor-Go₁₃ or corticotropin-releasing factor-1 receptor-CRF-Go₁₃ fusion protein as indicated. The stimulation of [³⁵S]GTPγS binding by maximally effective concentrations of cognate receptor agonists and the effect of 100 nM YM-254890 on this were assessed following immunoprecipitation with an anti-G-protein subunit antisem specific for each construct.

Gᵢₒ, via a corticotropin-releasing factor-1 receptor-Goᵢ₃ fusion protein, Gᵢ₁₁, via a GPR41-Goᵢ₃ fusion protein, or Go₁₃ via an α₂₅ adrenoceptor-Go₁₃ fusion protein (Fig. 7). At 100 nM, YM-254890 also blocked the up-regulation of c-Myc-AT₁R-CFP produced following induction of VSV-G-MAS-YFP expression (Fig. 8). Because PKC is downstream of Gᵢₒ/Gᵢ₁₁, we predicted that YM-254890 would not be able to block PKC-mediated c-Myc-AT₁R-CFP up-regulation, and this was confirmed (Fig. 8).

**Induction of MAS, but Not 1138D MAS, Causes Constitutive Activation of Gᵢₒ/Gᵢ₁₁ in Flip-In T-REx Cells**—In membranes of Flip-In T-REx cells harboring VSV-G-MAS-YFP at the Flip-In locus, [³⁵S]GTPγS binding assays followed by immunoprecipitation with antiserum CQ resulted in low levels of recovered nucleotide in the absence of doxycycline treatment. This was increased substantially when VSV-G-MAS-YFP expression was induced, and this elevated level of [³⁵S]GTPγS binding was blocked by the presence of YM-254890, but not the AT₁ receptor blocker losartan. Similar levels of basal [³⁵S]GTPγS binding were present in equivalent experiments using cell membranes harboring VSV-G-1138D MAS-YFP at the Flip-In locus, but binding was not increased by induction of VSV-G-1138D MAS-YFP expression (Fig. 9).

The AT₁ receptor and the MAS proto-oncogene are often coexpressed in, for example, vascular smooth muscle cells. After the cloning of MAS cDNA (9), some early experiments suggested that it might be a receptor for angiotensin peptides (10), but it is now well appreciated that MAS does not bind Ang II. However, Ang(1–7), the product of ACE2 activity (26), has recently been described as an
endogenous agonist of MAS (12). Furthermore, because Ang-(1–7) counters many of the regulatory actions of Ang II, there has been considerable interest in the interplay between MAS and the AT1 receptor. Following expression of the AT1 receptor in CHO-K1 cells, Ang II produces a strong, concentration-dependent increase in intracellular [Ca2+] (14). However, with coexpression of MAS, the effect of a maximally effective concentration of Ang II is substantially reduced, an effect also observed when inositol phosphate accumulation is measured (14). Despite these effects on Ang II-generated signals, coexpression with MAS actually results in higher levels of [3H]Ang II binding (14). The interplay between MAS and the AT1 receptor is further underlined when measuring Ang II-mediated contraction of mouse mesenteric microvessels. Ang II produces greater contraction in vessels from MAS knock-out animals than in vessels from wild-type controls (14). It was concluded that MAS forms a complex with the AT1 receptor that is inhibitory to AT1 receptor function, as had been described previously for AT2/AT1 receptor interactions (5).

To confirm MAS-induced increases in [3H]Ang II binding when coexpressed with the AT1 receptor in a separate cell system and to explore the molecular basis of this effect required a means to control MAS expression in the face of AT1 receptor expression. We thus employed Flp-In T-REx HEK293 cells. Introduction of a construct at the single defined Flp-In locus results in induction of expression only when the cells are exposed to tetracycline or the related antibiotic doxycycline (0.1 μg/ml) for 24 h (+Dox). Membranes from these were used in [35S]GTPγS binding assays with end of assay Gα11/Gα11, immunoprecipitation. Assays received no ligand (Basal) or were treated with Ang II (1 μM), losartan (Los; 10 μM), YM-254890 (100 nM), or Ang II (1 μM) and YM-254890 (100 nM).

**FIGURE 9.** Induction of MAS, but not I138D MAS, stimulates constitutive activation of Gα11/Gα11 in membranes of Flp-In T-REx HEK293 cells. Flp-In T-REx HEK293 cells constitutively expressing c-Myc-AT1R-CFP and harboring either VSV-G-MAS-YFP or VSV-G-I138D MAS-YFP at the Flp-In locus were left untreated (−Dox) or were treated with doxycycline (0.1 μg/ml) for 24 h (+Dox). Membranes from these were used in [35S]GTPγS binding assays with end of assay Gα11/Gα11, immunoprecipitation. Assays received no ligand (Basal) or were treated with Ang II (1 μM), losartan (Los; 10 μM), YM-254890 (100 nM), or Ang II (1 μM) and YM-254890 (100 nM).
compartment that may be the Golgi. It is well established that effective core glycosylation is an important quality control check prior to cell-surface delivery of many GPCRs (28, 29) and that a substantial amount of expressed protein fails this control and is routed to the proteasome for destruction. Immunoblot studies showed strong up-regulation of the AT₁ receptor by both MAS and activation of protein kinase C and also demonstrated a mixture of highly and less well glycosylated forms. Cell-surface biotinylation studies indicated that only the fully glycosylated form was delivered to the cell surface. Thus, because the imaging studies cannot discriminate between these forms, the combination of cell imaging, immunoblot, ligand binding, and cell-surface biotinylation studies offers the best means to understand the molecular diversity of the forms and cellular distribution of the AT₁ receptor construct.

Because PKC lies downstream of Gq/G₁₁, we reasoned that PKC inhibitors should also block MAS-induced up-regulation of AT₁R-CFP and that PKC activators should do so without induction of constitutively active MAS. Both these expectations were fully met. Many analyses have shown roles for agonist- and PKC-mediated phosphorylation of the C-terminal tail of the AT₁ receptor (23–25). Although key previous studies have been performed with the rat receptor, the C-terminal tail of the AT₁ receptor is well conserved between human and rat with three clear potential sites for PKC-mediated phosphorylation. Truncation to amino acid 325 removes these three sites. As such, it was satisfying that MAS-YFP was unable to cause significant up-regulation of c-Myc-Δ325AT₁R-CFP.

Although our study does not directly address the mechanistic basis for enhanced Ang II-mediated contraction in mesenteric vessels of MAS knock-out mice, it is likely that it may also reflect a loss of constitutive MAS-induced activation of PKC and phosphorylation of the AT₁ receptor. PKC-mediated phosphorylation of the AT₁ receptor is associated with decreased function, whereas truncation to eliminate PKC phosphorylation sites, as in c-Myc-Δ325AT₁R, is associated with an increased capacity of Ang II to stimulate inositol phosphate production because of reduced desensitization. Elimination of constitutive MAS-induced PKC activation and hence AT₁ receptor phosphorylation in vessels of MAS knock-out animals is therefore also consistent with the enhanced function of Ang II in producing contraction of mesenteric microvessels from MAS knock-out mice (14); and of course, heterologous desensitization of a coexpressed receptor by either constitutive or agonist-induced activation of a GPCR is a commonly employed regulatory strategy (30). It still remains to be established if the MAS-Gq/G₁₁-PKC-mediated up-regulation of the AT₁ receptor reflects enhanced stability of the protein and hence slower turnover. Future studies will assess this issue.

**FIGURE 10.** Induction of MAS does not cause up-regulation of the AT₁ receptor lacking C-terminal PKC phosphorylation sites. Flp-In T-REx HEK293 cells harboring VSV-G-MAS-YFP at the Flp-In locus (A₁ and B₁) and constitutively expressing c-Myc-Δ325AT₁R-CFP (A₂ and B₂) were left untreated (A₁ and A₂) or were treated with doxycycline (Dox; 0.1 μg/ml) for 24 h (B₁ and B₂) to induce VSV-G-MAS-YFP expression. The fluorescence corresponding to YFP (A₁ and A₂) and CFP (A₂ and B₂) was then imaged in living cells. Cell lysates of Flp-In T-REx HEK293 cells harboring VSV-G-MAS-YFP at the Flp-In locus and either c-Myc-AT₁R-CFP or c-Myc-Δ325AT₁R-CFP were treated as described above, resolved by SDS-PAGE, and immunoblotted with anti-c-Myc antibody (C).

**FIGURE 11.** Both MAS and PMA produce up-regulation of c-Myc-AT₁R-CFP in transiently transfected CHO-K1 and HEK293 cells. CHO-K1 (left panel) or HEK293 (right panel) cells were transiently transfected with c-Myc-AT₁R-CFP with (+) or without (−) VSV-G-MAS-YFP. 24 h after transfection, cells were treated for 16 h with combinations of PMA and Ro 31-8220 (both at 100 nM) or with 0.1 μg/ml doxycycline (Dox). Cell lysates were then resolved by SDS-PAGE and immunoblotted with anti-c-Myc antibody.
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