Constitutively Active Homo-oligomeric Angiotensin II Type 2 Receptor Induces Cell Signaling Independent of Receptor Conformation and Ligand Stimulation

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Members of the G-protein-coupled receptor superfamily (GPCRs) undergo homo- and/or hetero-oligomerization to induce cell signaling. Although some of these show constitutive activation, it is not clear how such GPCRs undergo homo-oligomerization with transmembrane helix movement. We previously reported that angiotensin II (Ang II) type 2 (AT2) receptor, a GPCR, showed constitutive activation and induced apoptosis independent of its ligand, Ang II. In the present study, we analyzed the translocation and oligomerization of the AT2 receptor with transmembrane movement when the receptor induces cell signaling. Constitutively active homo-oligomerization, which was due to disulfide bonding between Cys38 in one AT2 receptor and Cys399 in another AT2 receptor, was localized in the cell membrane without Ang II stimulation and induced apoptosis without changes in receptor conformation. These results provide the direct evidence that the constitutively active homo-oligomeric GPCRs by intermolecular interaction in two extracellular loops is translocated to the cell membrane and induces cell signaling independent of receptor conformation and ligand stimulation.

Recently, there have been many new insights regarding G-protein-coupled receptor superfamily (GPCRs), such as on homo- and hetero-oligomerization (1, 2), constitutive activation (3, 4), and receptor-associated protein. GPCR oligomers may be “contact” oligomers (5), where two binding pockets are formed from regions donated by both monomers instead of one binding site formed by one “subunit” receptor. Moreover, the dissociation of receptor homo-oligomers by reducing agents has been observed for several rhodopsin-like GPCRs (6–9). δ-Opioid receptor is unable to form oligomers when the terminal 15 amino acids are truncated (6), indicating that the C terminus might be a site of intermolecular interaction. Although these observations indicate that a disulfide linkage is important in the formation of receptor oligomers, it is not known whether the bond is inter- or intramolecular. Here we demonstrate that constitutively active homo-oligomeric (by intermolecular contact) angiotensin II type 2 receptor induces cell signaling independent of receptor conformation and ligand stimulation.

EXPERIMENTAL PROCEDURES

Materials—The following antibodies and reagents were generously provided as indicated or purchased: AT2 receptor-selective non-peptide antagonist PD123319 (Research Biochemical International) and the p38 MAP kinase inhibitor SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-(pyridyl)1H-imidazole (Upstate Biotechnology); the caspase-3 inhibitor Ac-DEVD-chloro methyl ketone (Calbiochem); anti-AT2 receptor antibody catalog number sc-7420 (Santa Cruz Biotechnology). 125I-[Sar1,Ile8]Ang II was purchased from Amersham Biosciences. The highly reactive, sulfhydryl-specific alkylating reagent CH3SO2CH2CH2NH3+ (MTSEA+), adduct size of about 4.726 Å, was purchased from Toronto Research Chemicals, Inc., North York, Ontario, Canada.

Cell Cultures and Treatments—Mouse fibroblast R1T3 cells and Chinese hamster ovary (CHO-K1 cells (ATCC CCL-61) were maintained in 10% fetal bovine serum with penicillin- and streptomycin-supplemented Dulbecco’s modified Eagle’s medium (Invitrogen) in 5% CO2 at 37 °C. Apoptosis was measured in cells maintained for 48 h in Dulbecco’s modified Eagle’s medium without serum. Cell viability was >95% by trypan blue exclusion analysis in control experiments. The level of AT2 receptor stimulation was measured in the presence of 0.1 μM [Sar1]Ang II (Kd = 0.3 nM), and inhibition was measured in the presence of 10 μM AT2 selective non-peptide antagonist PD123319 (Kd = 5 nM). [Sar1]Ang II was added every 12 h to compensate for potential degradation. Inhibition experiments were performed in the presence of the p38 mitogen-activated protein kinase (p38 MAPK)-specific inhibitor SB203580 (10 μM) and the caspase-3 inhibitor (1 μM) for 48 h after serum starvation.

CHO Cell Transfection and Establishment of CHO Cell Lines—The rat AT2 receptor gene described earlier was used (10). The wild-type (WT), N127G, inactive mutant AT2 receptor (4), and C35A, C290A, and C35A/C290A genes (10) cloned in the pEGFP (enhanced green fluorescent protein), pEYFP (enhanced yellow fluorescent protein), pECFP (enhanced cyan fluorescent protein), and pDsRed (discosoma red fluorescent protein) vectors that contain the neomycin (G418) resistance gene were transfected into CHO cells using the Lipofectamine 2000 liposomal reagent according to the manufacturer’s instructions (Roche Applied Science). Clonal cell lines that permanently expressed AT2 receptor were selected by 800 μg/ml G418 and evaluated by fluorescence-activated cell sorter.

PAGE, Western Blotting, and Immunoprecipitation—Cell membrane and cytosolic fraction were prepared as described previously (11, 12). For Western blotting, equal amounts of samples on a protein basis as determined using the Bradford reagent (Bio-Rad) were resolved on non-reducing 12% SDS-PAGE. Cell membrane was incubated with or without 1 mM dithiothreitol (DTT) at 30 °C for 1 h and with loading

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buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40) at room temperature for 30 min. Western blotting was performed with primary antibodies as specified in each case. Horseradish peroxidase-conjugated secondary antibody and the enhanced chemiluminescent substrate system (Amersham Biosciences) were used as a detection system. The signal was independently quantified by a digital image analysis system.

**Analysis of Apoptosis**—Nuclear fragmentation and condensation analysis with 4′, 6-diamidino-2-phenylindole (DAPI) staining was used to identify apoptotic cells at the cellular level with a DMRB digital fluorescent microscope (Leica) and a LSM410 laser scanning confocal microscope (Carl Zeiss Co. Ltd.). For imaging, the cells were plated on lysis-coated microscope slides and then treated for up to 48 h. The cells were washed and fixed with 1% paraformaldehyde at 4 °C for 15 min and then stained with DAPI. Five images of separate locations were taken for each sample, and the percentage of apoptotic cells was quantified. The percentage of apoptotic cells was also determined by the in situ cell death detection kit, TMR red™, according to the manufacturer’s protocol (Roche Diagnostics). Briefly, cells were fixed in 2% paraformaldehyde, and terminal deoxynucleotidyl transferase-mediated deoxyuridine 5'-triphosphate nicked end labeling (TUNEL) reaction mix was added. Cells were analyzed by flow cytometry.

**Caspase-3-like Activity Assay**—Caspase-3-like protease activity was determined by fluorometric assay using the CaspACE™ fluorometric system (Promega) according to the manufacturer’s instructions. Transfected and control cells (10⁶) were lysed in lysis buffer by freezing and thawing followed by centrifugation (15,000 × g for 20 min at 4 °C). Caspase-3-like activity was measured in supernatant following proteolytic cleavage of the colorimetric substrate Ac-DEVD-pNA. DEVD-pNA was used as a standard in the assay buffer (405 nm).

**Radioligand Binding Studies**—Kₐ and Bₘₐₓ of the receptor were estimated by ¹²⁵I-[Sar¹,Ile⁸]Ang II binding experiments carried out under equilibrium binding and Scatchard plot analysis, as described previously (11, 12). Membranes expressing the WT or the mutant receptor were incubated with 300 pM ¹²⁵I-[Sar¹,Ile⁸]Ang II for 1 h at 22 °C in a 125-μl volume. The binding experiments were stopped by filtering the binding reaction mixture through Whatman GF/C glass fiber filters, which were extensively washed further with binding buffer. The bound ligand fraction was determined from the cpm remaining on the membrane. Binding kinetics values were determined using the computer program Ligand.

**Results with MTS Reagents by Reporter Cysteine Accessibility Mapping**—Aliquots of cell membranes (20 μl) were incubated with or without MTS reagents at the stated concentrations (0.1–12.5 mM) at 22 °C for the indicated times (2–10 min) in 20 mM HEPES buffer (pH 7.4). The reaction mix was then diluted 75-fold with cold buffer to stop the reaction, centrifuged for 10 min at 16,000 × g at 4 °C, and resuspended in 200 μl of buffer. A 150-μl aliquot was used for ¹²⁵I-[Sar¹,Ile⁸]Ang II binding analysis. The percent inhibition of ¹²⁵I-[Sar¹,Ile⁸]Ang II binding was calculated as [1−(specific binding after MTS reagents binding without reagent)] × 100%. The experiment was performed 4–10 times.

**Statistical Analysis**—The results are expressed as the mean ± S.E. of three or more independent determinations. Significant differences in measured values were evaluated with an analysis of variance using Bonferroni’s test and the unpaired Student’s t test. Pearson product moment correlation coefficients were calculated. Statistical significance was set at <0.05.

**RESULTS AND DISCUSSION**

The PC12W pheochromocytoma cell line has been used as a model for study because apoptosis in these cells (13) resembles the in vivo situation described in ovarian granulosa cells and other remodeling tissues (14–16). Serum starvation leads to a ~5-fold increase in AT₂ receptor expression (Fig. 1a). Immunoblotting showed that the AT₂ receptor underwent homo-oligomerization in the cell membrane (Fig. 1a). AT₂ receptor-expressing native cell lines are not a suitable model for concluding the localization and homo-oligomerization of constitutively active AT₂ receptor for apoptosis and are even less appropriate for concluding a role for the induced overexpression of AT₂ receptor protein. Since CHO cells are an established epithelial lineage of non-transformed cells that are capable of growing under serum starvation with appropriate supplements and since we previously reported that exogenous overexpression of the AT₂ receptor induced apoptosis in CHO cells (4), we used these cells as surrogate models to link the de novo expression of the AT₂ receptor to apoptosis.

Using molecular imaging techniques, which show the translocation of the AT₂ receptor to the cell membrane in living cells, we analyzed the translocation of AT₂ WT-EGFP receptor that had been permanently transfectantly in CHO cells (Fig. 1b). Although the expression level of the AT₂ receptor in normal adult tissues is ~10–20 fmol/mg of protein (Bₘₐₓ), it is re-expressed ~5–25-fold over basal levels during the remodeling of tissues, where the AT₂ receptor is thought to play a role in the apoptosis of smooth muscle cells, fibroblasts, and endothelial cells (14, 15). Therefore, a CHO cell line, which showed an expression level of 341 ± 13 fmol/mg of protein in the cell membrane (Table I), was used for pharmacological examinations.

As shown in Fig. 1b, AT₂ WT-EGFP receptor was localized in the cell membrane after 24 h under serum-free conditions, whereas serum starvation by itself did not induce EGFP translocation. The expression level of AT₂ WT-EGFP receptor in the cell membrane after 24 h under serum-free conditions was about 2.4-fold higher than the expression under serum conditions (Table I). In addition, AT₂ WT-EGFP receptor was localized in the cell membrane after 24 h under serum-free conditions (Fig. 1b), suggesting that AT₂ WT-EGFP receptor translocation in the cell membrane may induce cell signaling.

Morphological features associated with apoptotic cells, such as nuclear DNA condensation and fragmentation, began ~24 h (the time at which growth arrest was established) after shifting to serum starvation and was complete after 48 h in a CHO cell line expressing AT₂ WT-EGFP receptor (Fig. 1c). The TUNEL method was used to analyze the percentage of apoptotic cells under various treatments (Fig. 1d); the CHO cell line expressing AT₂ WT-EGFP receptor showed 64 ± 5% apoptosis. These cells also exhibited membrane blebbing, cytoplasmic shrinkage, and inhibition of protein synthesis (data not shown). Although we established five different AT₂ WT-EGFP receptor-expressing CHO cell lines, all of them showed similar apoptotic effects under serum-free conditions. In contrast, DAPI staining of cells that expressed AT₂ N127G-EGFP receptor, which is an inactive AT₂ receptor (4), revealed cell nuclei with sharp round edges and diffuse chromatin (Fig. 1c). In addition, the expression level of AT₂ N127G receptor in the cell membrane after 24 h under serum-free conditions was about 2.5-fold higher than the expression under serum conditions, and this increase in the expression level of AT₂ N127G receptor is comparable with that of AT₂ WT receptor (Table I). The translocation of the AT₂ receptor to the cell membrane may be necessary but not sufficient to induce apoptosis. Since we previously reported that AT₂ receptor-induced apoptosis in AT5 cells was blocked by the pharmacological inhibition of p38 MAPK and caspase-3 (4), the effects of p38 MAPK inhibitor and caspase-3 inhibitor were analyzed in this cell system. The p38 MAPK-specific inhibitor SB203580 blocked AT₂ receptor-induced apoptosis by about 60%. The caspase-3 inhibitor Ac-DEVD-cmk (1 mM) inhibited AT₂ receptor-induced apoptosis by about 40% (Fig. 1d). Combined treatment with SB203580 and Ac-DEVD-cmk almost completely blocked AT₂ WT-receptor-induced apoptosis.

To determine whether the homo-oligomerization of the AT₂ receptor in the cell membrane induces apoptotic signaling, immunoblotting of the AT₂ WT and AT₂ N127G receptors was performed. AT₂ WT receptor homo-oligomerized in the cell membrane under serum-free conditions for 24 h (Fig. 1e). Although AT₂ N127G receptor mainly homo-oligomerized in the cell membrane, a monomer band was also observed. After being pretreated with DTT under non-reducing conditions, AT₂ WT
FIG. 1.  
(a) AT₂ receptors form homo-oligomerization and up-regulate after serum-free conditions in PC12W cells. Cell membrane was prepared under serum or serum-free conditions, subjected to SDS-gel electrophoresis under non-reducing conditions, and immunoblotted with anti-AT₂ receptor antibody. 30 μg of protein was used in each lane. Bmax on the AT₂ receptors was also analyzed by Scatchard plot analysis.  
(b) AT₂-WT-EGFP receptor localized in the cell membrane after 24 h under serum-free conditions. EGFP- and AT₂-WT-EGFP receptor-expressing CHO cell lines were grown under serum or serum-free conditions and stained with DAPI to visualize nuclear morphology as assessed by a laser scanning confocal microscope. Arrows indicate AT₂-WT-EGFP receptor translocation in the cell membrane.  
(c) AT₂-WT-EGFP receptor induced apoptosis after 48 h under serum-free conditions. AT₂-WT-EGFP receptor- and AT₂-N127G-EGFP receptor-expressing CHO cell lines were grown under serum conditions or for up to 48 h under serum-free conditions, stained with DAPI, and imaged by a digital fluorescent microscope.  
(d) Pharmacological intervention in apoptosis mediated by AT₂-WT-EGFP receptor and AT₂-N127G-EGFP receptor was analyzed by treating cells under serum conditions and with or without 10 μM p38 MAPK inhibitor (SB203580) and 1 μM caspase-3 inhibitor (DEVD-cmk) for 48 h under serum-free conditions. Data are shown as the percentage of apoptotic cells in three independent experiments as assessed by TUNEL as described under “Experimental Procedures.” Histograms show that AT₂-WT-EGFP receptor transfected CHO cells induced apoptosis under serum (A) or after 48 h serum-free conditions (B). 65% of AT₂-WT-EGFP receptor transfected CHO cells showed apoptosis after 48 h of serum-free conditions. *p < 0.05 versus serum conditions.  
(e) The AT₂ receptor formed a homo-oligomer in the cell membrane. Cell membranes were prepared in serum-free conditions for 24 h, subjected to SDS-gel electrophoresis after being pretreated with (−) or without (−) DTT under non-reducing conditions, and immunoblotted with anti-EGFP antibody. 20 μg of protein was used in each lane. Arrows indicate the monomer and dimer species of the receptor.  
(f) AT₂-WT-EGFP receptor formed only homo-oligomer, whereas AT₂-N127G-EGFP receptor formed both homo-oligomer and monomer in the cell membrane. Cell membranes were prepared in serum (+) or serum-free (−) conditions for up to 48 h, subjected to SDS-gel electrophoresis under non-reducing conditions, and immunoblotted with anti-EGFP antibody. 40 μg of protein was used in each lane. Arrows indicate the monomer and dimer species of the receptor.  
Representative immunoblots (a, d, and f) and pictures (b and c) are shown. Three independent determinations were performed, and similar results were observed (a–d and f).
The presence of DsRed-fused AT2 receptors. In addition, AT2 receptor with anti-EGFP antibody and immunoblotting of the pre-
brane, whereas treatment with [Sar1]Ang II or PD123319 did not induce the delocalization of AT2-WT-EGFP from the cell membrane to the cytoplasm (Fig. 2d). Immunoblotting indicated that the homo-oligomerization of AT2-WT-EGFP receptors was not affected by [Sar1]Ang II or PD123319 for up to 24 h under serum-free conditions (Fig. 2e). In addition, the expression level of AT2-WT-EGFP receptor in the cell membrane also did not show any changes in the presence of [Sar1]Ang II or PD123319 after 24 h under serum-free conditions. AT1 receptor is stabilized in the R state (inactive state), and agonist binding causes transition to the R* state (fully active state). In the AT1 receptor, this transition may proceed through a relaxed inter-
mediate activated R’ state (partially active state) (20). In the case of the AT2 receptor, constitutively active AT2 receptor may be in the R’ state but not in the R state. Since PD123319 is a neutral antagonist but not an inverse agonist for constitutively active AT2 receptor-induced apoptosis (4), the antagonist may not affect AT2 receptor translocation, and the AT2 receptor may remain in the R’ state in the cell membrane. Taken together, ligand-independent AT2 receptor-induced signaling may cause apoptosis associated with AT2 receptor translocation and homo-oligomerization.

Fluorescence resonance energy transfer (21) and biolumines-
cence resonance energy transfer (22) are valuable approaches for analyzing the oligomerization of receptor after ligand stim-
ulation. Although fluorescence resonance energy transfer analysis was performed using CHO cells, which co-expressed both
the AT2-EYFP and the AT2-EGFP receptors (data not shown), there was no energy transfer after Ang II stimulation because the AT2 receptor undergoes constitutive homo-oligomerization independent of Ang II. Since fluorescence resonance energy transfer and bioluminescence resonance energy transfer were not useful in this study, several AT2 mutant receptors were used to analyze the specificity of the constitutive homo-
oligomerization of the AT2 receptor. Four Cys residues, Cys117, Cys195 and Cys290, in the AT2 receptor are located in the extracellular domain, where the redox environment may facil-
itate two disulfide bonds. A disulfide bond linking transmem-
brane (TM) 3 and extracellular loop 2 occurs in >91% of GPCRs (23). In the case of the AT2 receptor, a disulfide bond between
Cys117 and Cys195 in the extracellular domain is critical for the polypeptide to gain a ligand binding conformation, and in its absence, the polypeptide is irreversibly misfolded (15). Cys290 in the N terminus and Cys290 in extracellular loop 3 in the AT2 receptor may play a central role in homo-oligomerization of the receptor through intermolecular or intramolecular interaction by disulfide linkage. AT2 receptor translocation was analyzed using C35A-EGFP, C290A-EGFP, and C35A/C290A-EGFP receptors (Fig. 3c). C35A-EGFP and C290A-EGFP receptors but not C35A/C290A-EGFP receptor were homo-oligomerized by immuno-
blotting (Fig. 3b) and induced apoptosis after 48 h under serum-free conditions (Fig. 3c), suggesting that the interaction of Cys290 in the homo-oligomerization of the AT2 receptor may be critical for inducing apoptosis.

### Table I

| Receptor          | $B_{max}$ (pmol/mg of protein) | Fold increase (serum(−)/serum(+) | $p$-value |
|-------------------|-------------------------------|---------------------------------|----------|
|                  | (serum(−)) | (serum(+)) |                       |
| WT                | 341 ± 13    | 821 ± 32$^a$ (No treatment) | 2.4      |
|                  | 2.3         | 788 ± 41* (+ [Sar1]Ang II)   |          |
|                  | 2.4         | 815 ± 35* (+ PD123319)       |          |
| N127G             | 389 ± 23    | 989 ± 22*                    | 2.5      |
| C35A              | 364 ± 33    | 756 ± 29*                    | 2.1      |
| C290A             | 660 ± 27*   | 708 ± 19                     | 1.1      |
| C35A/C290A$^a$    | 309 ± 20    | 289 ± 11                     | 0.9      |

$^a$ Expression levels in cytoplasm are shown.

receptor showed only a monomer band, suggesting that disul-
fide bonding may be important for homo-oligomerization (Fig.
1e). In addition, whereas both receptors mainly homo-oligomer-
ized in the cell membrane under serum-free conditions for 48 h
(Fig. 1f), only homo-oligomerized AT2-WT receptor induced apoptosis. Although the AT2 receptor contains consensus sites
for phosphorylation, it is not phosphorylated in response to Ang
II and does not internalize (17). These results suggest that a
constitutively active homo-oligomerized AT2-WT receptor but not an inactive homo-oligomerized AT2 receptor (N127G)-in-
duced apoptosis.

Next, we co-transfected CHO cells with AT2-WT-EGFP and
AT2-WT-DsRed receptors to confirm the homo-oligomerization of
AT2-WT receptor in the cell membrane. AT2-WT-EGFP and
AT2-WT-DsRed receptors were co-localized in the cell mem-
brane after 24 h under serum-free conditions (Fig. 2a). Many
studies on GPCR oligomerization have used the co-immunopre-
cipitation of differentially epitope tagged forms of GPCRs. Co-
expression of both the EGFP- and the DsRed-fusion forms of
the AT2 receptors in this study followed by immunoprecipita-
tion with anti-EGFP antibody and immunoblotting of the pre-
cipitated samples with an anti-DsRed antibody detected the
presence of DsRed-fused AT2 receptors. In addition, AT2 recep-
tors appear to be localized in a perinuclear compartment, and
this may be an artifact of oligomerization. These results indi-
cate that AT2-WT-EGFP and AT2-WT-DsRed receptors homo-
oligomerized (Fig. 2b). As shown in Fig. 2c, CHO cells, which were co-transfected with AT2-WT-EGFP and AT2-WT-DsRed receptors, also showed morphological features of apoptotic
cells, such as irregular nuclei and membrane blebbing under serum-free conditions. Although early studies using radio-
ligand binding and cross-linking had predicted homo-oligo-
meric receptors, the significance of these findings was not clear
at that time. Although the functional importance of homo-
oligomerization is much better defined for other receptors, such
as tyrosine-kinase and steroid-hormone receptors (18), in light
of several reports on GPCR oligomers, it is not yet clear whether or not these receptors exist functionally as homo-
oligomers. Although constitutive activity has been observed for
more than 60 wild-type GPCRs and different species including
humans (19), this is the first report in which a constitutively
active GPCR, the AT2 receptor, has been shown to be function-
ally homo-oligomerized in the cell membrane.

The agonist [Sar1]Ang II and the antagonist PD123319 do not modulate apoptosis in AT2 receptor-transfected A7r5 cells
(4). Serum starvation by itself induced the translocation of
AT2-WT-EGFP receptor from the cytoplasm to the cell mem-
brane, whereas treatment with [Sar1]Ang II or PD123319 did not induce the delocalization of AT2-WT-EGFP from the cell membrane to the cytoplasm (Fig. 2d). Immunoblotting indicated that the homo-oligomerization of AT2-WT-EGFP receptors was not affected by [Sar1]Ang II or PD123319 for up to 24 h under serum-free conditions (Fig. 2e).
Since AT<sub>2</sub> receptor expression-linked apoptosis has been shown to activate caspase-3 (4), we examined caspase-3-like activity as an AT<sub>2</sub> receptor-induced signal. Basal caspase-3-like activity in cell lines that expressed WT-EGFP, C35A-EGFP, and C290A-EGFP receptors was much higher than that in cell lines that expressed EGFP as a control and N127G receptor, which is an inactive receptor, under serum-free conditions (Fig. 4a). Basal caspase-3-like activity in C35A/C290A-EGFP was comparable with that in cell lines that expressed EGFP as a control and N127G receptor, which is an inactive receptor, under serum-free conditions (Fig. 4a). However, [Sar<sup>1</sup>]Ang II caused a significant increase in caspase-3-like activity in cell lines that expressed EGFP as a control and N127G receptor, which is an inactive receptor, under serum-free conditions (Fig. 4a). Basal caspase-3-like activity in C35A/C290A-EGFP was comparable with that in cell lines that expressed EGFP as a control and N127G receptor. The increased activity was not suppressed when cells were pre-treated with PD123319. As shown in Table I, the expression level of C290A receptor between serum and non-serum conditions was 2.1-fold higher than that in serum conditions, which is comparable with the results in WT, whereas there was no significant difference in the expression level of C290A receptor between serum and non-serum conditions because C290A receptor is already located in the cell membrane under serum conditions. Homooligomeric AT<sub>2</sub> receptor in the cell membrane is not sufficient but may be necessary for caspase-3-like activation. The activation of caspase-3 is thought to be important for specific cleavage of cell cycle regulatory proteins, such as p21<sup>waf1</sup>/cip1 and p27<sup>kip1</sup>, in apoptotic cells (24).

Since we previously reported that constitutive activation of AT<sub>2</sub> receptor induces movement of the TM2–TM7 helix by reporter cysteine accessibility mapping using sulphydryl-specific reaction with MTSEA (11, 12), we also analyzed AT<sub>2</sub> receptor TM movement by reporter cysteine accessibility mapping (Fig. 4, b and c). Exposure of native Cys residues to a water-accessible ligand pocket was measured. The percentage of inhibition of [Sar<sup>1</sup>,Ile<sup>8</sup>]-Ang II-specific binding treated with 2.5 mM MTSEA for 2–10 min in the inactive N127G receptor was significantly lower than that in the constitutively active WT receptor (Fig. 4b). Exposure of the constitutively active WT receptor to MTSEA for 2 min reduced specific binding by nearly 47%, whereas exposure of the inactive N127G receptor reduced specific binding by 18% (Fig. 4c). In the case of N127G receptor, the Cys residues are moved to an area inaccessible by water, and this may reduce the specific binding of the peptide antagonist [Sar<sup>1</sup>,Ile<sup>8</sup>]-Ang II. There are three Cys residues in the upper site of the plasma membrane that face the extracellular space: Cys<sup>169</sup> and Cys<sup>271</sup> in TM IV and Cys<sup>271</sup> in TM VI. Since one or more of these Cys residues may expose the ligand pocket in WT receptor, we replaced these Cys residues by Ala (C169A, C172A, and C269A). Cys accessibility for C169A was only 10% of that in the active N127G receptor, which is comparable with the results in WT, whereas there was no significant difference in the expression level of C290A receptor between serum and non-serum conditions because C290A receptor is already located in the cell membrane under serum conditions. Homooligomeric AT<sub>2</sub> receptor in the cell membrane is not sufficient but may be necessary for caspase-3-like activation. The activation of caspase-3 is thought to be important for specific cleavage of cell cycle regulatory proteins, such as p21<sup>waf1</sup>/cip1 and p27<sup>kip1</sup>, in apoptotic cells (24).
WT receptor, indicating that these mutations did not induce conformational changes in the AT$_2$ receptor. In addition, Cys accessibilities for C35A/N127G, N127G/C290A and C35A/N127G/C290A receptors are similar to that for N127G receptor. C35A/C290A receptor but not N127G receptor did not express and form a homo-oligomer in the cell membrane, suggesting that both Cys35 and Cys290 are important for making a disulfide bond and translocating the receptor to the cell membrane but not for creating a constitutively active form (Fig. 3b). EGFP and DsRed in C35A/C290A receptors did not mainly overlap in the cytoplasm (Fig. 4d). Most C35A/C290A receptors did not form a homo-oligomer in the cytoplasm and mainly formed a monomer by immunoblotting (Fig. 4e). Several recent studies have identified novel intercellular proteins that interact with the C-terminal tails of GPCRs (25). AT$_2$ receptor-interacting protein has a novel coiled-coil domain that contains protein that interacts with the AT$_2$ receptor (26). This kind of protein may still form a homo-oligomer even if interaction between Cys$_{35}$ and Cys$_{290}$ is disrupted.

To reconcile the observations presented here with those reported earlier, we propose a model of constitutively active homo-oligomeric AT$_2$ receptor in the cell membrane (Fig. 4f). Inactive N127G receptor forms a homo-oligomer in the cell membrane but does not induce apoptosis. If Cys$_{35}$ in the AT$_2$ receptor binds to Cys$_{290}$ in another AT$_2$ receptor, C35A/C290A receptors do not form a homo-oligomer. Although the conformation of C35A/C290A receptor is similar to that of WT receptor, C35A/C290A mutant did not form a homo-oligomer in cytoplasm. Therefore, disulfide bonding in the extracellular loop in the WT receptor was disrupted by DTT treatment, and Cys$_{35}$ in the AT$_2$ receptor may establish some intermolecular contact such as a disulfide bond with Cys$_{290}$ in another AT$_2$ receptor. Other regions of intermolecular contact that have been proposed in other GPCRs include a region or regions within TM domains 1–3 from one receptor interacting with TM domains 1–3 from another for the V2 vasopressin receptor (27) and docking of helix 8 with the loop between helices 5 and 6 for rhodopsin (28). The relative arrangement of receptors and the
orientation of helix 8 are also based on the crystalline array of squid rhodopsin and on the hypothesis that there is both a symmetrical dimer interface that involves TM domain 4 and an interaction that involves helix 8 with the third intracellular loop. Site-directed mutagenesis studies have indicated that two conserved extracellular Cys residues (Cys140 and Cys220), which are present in almost all GPCRs of the rhodopsin family, play key roles in the formation of disulfide-linked m3 receptor dimmers (8). Since the two conserved Cys residues are likely to form an intramolecular disulfide bond, their findings indicate that the two conserved extracellular Cys residues can also participate in the formation of intermolecular disulfide bonds. In the case of constitutively active AT2 receptor, two conserved Cys residues form an intramolecular disulfide bond. Two other Cys residues (Cys35 and Cys290) may form intermolecular disulfide bonds independent of ligand stimulation and receptor conformation changes, and the homo-oligomer induces cell signaling. Although oligomerization affects GPCR signaling, other
roles still need to be identified. This discovery should help us to elucidate what is likely to be a common underlying mechanism for oligomerization of constitutively active GPCRs and to obtain a better understanding of the structure and function of these receptors in cells.

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