$G_{\beta\gamma}$-independent constitutive association of $G_{\alpha s}$ with SHP-1 and angiotensin II receptor AT_2 is essential in AT_2-mediated ITIM-independent activation of SHP-1

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Communicated by Frederick C. Robbins, Case Western Reserve University, Cleveland, OH, July 9, 2002 (received for review February 18, 2002)

Conventional mode of activation of SH2 domain-containing phosphatase 1 (SHP-1) by a single transmembrane (TM) inhibitory receptor such as killer cell inhibitory receptor, Fcγ receptor type IIb1, and paired Ig-like receptors of inhibitory types requires tyrosine phosphorylation of immunoreceptor tyrosine-based inhibitory (ITIM) motifs in the cytoplasmic domains of the inhibitory receptors. Contrary to this paradigm, AT_2, a G protein-coupled 7TM receptor that does not undergo tyrosine phosphorylation in response to angiotensin II (Ang II) stimulation, also activates SHP-1. Here we show that SHP-1 constitutively and physically associates with AT_2 receptor in transfected COS-7 cells. On stimulation by Ang II, SHP-1 becomes activated and dissociated from AT_2 receptor, independent of pertussis toxin. Cotransfection of transducin $G_{\beta\gamma}$ inhibits SHP-1/AT_2 association and the SHP-1 activation, whereas cotransfection of C-terminal of β-adrenergic receptor kinase, which abrogates $G_{\beta\gamma}$ signaling, facilitates SHP-1 activation. Surprisingly, SHP-1/AT_2 association and the SHP-1 activation requires the presence of $G_{\alpha s}$ as shown by differential coimmunoprecipitation, dominant negative $G_{\alpha s}$, constitutively active $G_{\alpha s}$, and G_α peptides. A mutant AT_2 receptor D141A–R142L that is inactive in G_α protein activation constitutively associates with SHP-1 and activates it. Together, these results indicate that $G_{\alpha s}$ alone, rather than exclusively in the form of $G_{\alpha\beta\gamma}$ heterotrimer may facilitate signal transduction for G protein-coupled receptors, suggesting a novel mechanism distinct from the classic paradigm of heterotrimeric G proteins. The AT_2-mediated ITIM-independent activation of SHP-1 that is distinct from the conventional mode of activation, may represent a general paradigm for activation of SHP-1/2-class tyrosine phosphatases by G protein-coupled receptors.

GPCR | signal transduction

The G protein-coupled receptors (GPCR) AT_1 and AT_2 are the two major subtypes that transduce signals for angiotensin II (Ang II), a potent vasoactive octapeptide (Asp^1–Arg^2–Val^3–Tyr^4–Ile^5–His^6–Pro^7–Phe^8) and a growth factor. Activation of AT_1 and AT_2 receptors by Ang II results in opposing actions of stimulation and inhibition. The AT_1-mediated stimulatory actions as well as signal transductions are well documented, whereas the AT_2-mediated inhibitory actions and signal transductions remain poorly understood (1–4). Most recently, studies have shown that the AT_2 receptor not only couples to pertusis toxin (PTX)-sensitive $G_{i\alpha 2}$ and $G_{i\alpha 3}$ proteins (5), but also activates a SH2 domain-containing tyrosine phosphatase (SHP-1) (5–10). Deletion or alanine substitutions of residues 240–244 within the intermediate portion of the cytoloop 3 has been shown to result in a complete loss of AT_2-mediated apoptosis, inhibition of extracellular signal-regulated kinases, and SHP-1 activation (10).

SHP-1, an intracellular protein tyrosine phosphatase (PTPase) containing two SH2 domains at the N-terminal end, plays a central role in blocking cell activation signals and prevent target cell lysis by the natural killer cells. The SH2 domains of SHP-1 interact with the catalytic domain to self-constrain the SHP-1 activity in an inactive state. Removal of this self-constraint imposed by the SH2 domains thus activates the SHP-1. The inhibitory receptors of the immune system such as killer cell inhibitory receptor, Fcγ receptor type IIb1, and paired Ig-like receptors of inhibitory types that initiate an inhibitory signal of SHP-1 activation bear an immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic tail (11–13). Stimulation of the inhibitory receptors result in tyrosine phosphorylation of the ITIM and subsequently activates SHP-1 because the SH2 domain of SHP-1 selectively binds to the phosphoylated ITIM. The activated form of SHP-1 can then associate through its catalytic active site, $C(X)_5R$ motif, with diverse target molecules such as JAK2 that bear phosphorylated tyrosine sequence signature. The ITIM has become the hallmark of a growing family of receptors with inhibitory potential, which are expressed in various cell types such as monocytes, macrophages, dendritic cells, leukocytes, and mast cells. The presence of an ITIM in a cytoplasmic tail is of sufficient predictive value that it can be used as an initial criterion for identification of candidate inhibitory receptors (14–17). However, sporadic reports have shown that GPCRs bearing no ITIM also can activate SH2 domain containing PTPases such as SHP-1 and SHP-2 (18–21).

The AT_2 receptor belongs to the superfamily of GPCR. Interestingly, It exceptionally contains an ITIM-like motif ($VIY^{148}PFL$) in the cytoloop domain. Substitution of this cytoloop with corresponding region of the AT_1 receptor abolishes AT_2-mediated SHP-1 activation. Although results from our laboratory and Dzau's laboratory (10) have shown that the cytoloop 3 also plays an important role in AT_2-mediated SHP-1 activation, the underlying mechanism is not clear. This ambiguity is mainly because no direct evidence is available to show that the cytoloop 3 is involved in AT_2/SHP-1 association. Thus, it is logical to hypothesize that an inhibitory receptor-like mechanism of activation could be used by the AT_2 receptor in activating SHP-1. We show here that, contrary to our initial hypothesis, mutations of Y148F and Y148A (the tyrosine of the ITIM-like motif) for the AT_2 receptor, however, fail to inhibit the SHP-1 association and activation. We also show that $G_{\beta\gamma}$-independent constitutive association of $G_{\alpha s}$ with SHP-1 and AT_2 is essential in AT_2-mediated activation of SHP-1. Moreover, the AT_2-mediated SHP-1 activation is ITIM-independent. These data contribute to our understanding of how GPCR activates SHP-1 with a mechanism distinct from ITIM-bearing inhibitory receptors.

Methods and Materials

Materials. The monoclonal anti-c-Myc antibody (Roche Molecular Biochemicals, clone 9E10), monoclonal anti-hemagglutinin (HA) antibody (Roche Molecular Biochemicals, clone 12CA5), monoclonal anti-1D4 antibody (Cell Culture Center, Endotronics, Minneapolis), oligonucleotides (Sigma-Genosys and MWG Biotech, Ebersburg, Germany), analogues of [Sar^1]Ang II (GeneMed Syn-

Abbreviations: GPCR, G protein-coupled receptor; TM, transmembrane helix; SHP-1, SH2 domain containing phosphatase 1; ITIM, immunoreceptor tyrosine-based inhibitory motif; βARK, β-adrenergic receptor kinase; PTX, pertusis toxin; GDT, guanosine diphosphate; HA, hemagglutinin.

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thesis, San Francisco), and G_α protein peptides (Biosynthesis, Lewisville, TX, and GeneMed Synthesis) were synthesized. Ang II and [Sar^1]Ang II (Bachem), PD123319 and CGP42112A (Research Biochemicals, Natick, MA), Protein Tyrosine Phosphatase Assay kit (Upstate Biotechnology), DTT, and other chemicals (Sigma) were purchased. ^{125}I-[Sar^1,Ile^8]Ang II (2,200 Ci/mmol; 1 Ci = 37 GBq) was supplied by Robert Speth (Univ. of Washington, Pullman). Genes of $G_{\alpha s}$, dominant negative $G_{\alpha s}$, and constitutively active $G_{\alpha s}$ were purchased from American Type Culture Collection. The gene of SHP-1 was a gift from Matthew L. Thomas, Washington University (St. Louis), and genes of $G\alpha_i$, $G\beta_1\gamma_2$, and β-adrenergic receptor kinase (βARK) were gifts from Robert Lefkowitz, Duke University (Durham, NC). Rat AT_2 receptor gene and synthetic rat AT_1 receptor gene were gifts from Sadashiva Karnik, Cleveland Clinic Foundation (Cleveland).

Plasmid Construction, Mutagenesis, and Expression of the AT_2 Receptors. The rat AT_2 and AT_1 receptor genes in vector pcDNA3.1 were constructed based on the gift genes from Sadashiva Karnik (22, 23). These receptor genes were tagged with c-Myc (EQKLISEEDL) and HA (YPYDVPDYA) epitope at the N terminus after the start coden to generate c-Myc-AT_2, HA-AT_2, C-Myc-AT_2, and HA-AT_2 receptor genes. The original 1D4 epitope tag (TETSQVAPA) attached to the C terminus was removed from the receptor genes. The SHP-1 gene received as gift from Matthew L. Thomas, was subcloned into pcDNA3.1 vector for expression in mammalian cells. All these genes in pcDNA3.1 vector contain a consensus Marilyn–Kozak sequence at the N terminus to facilitate the expression in mammalian cells. Mutant AT_2 and chimera AT_2/AT_1 receptors were prepared based on HA-AT_2 receptor gene unless otherwise indicated. All plasmid construction and mutagenesis were performed with the restriction fragment-replacement method and the PCR method (22, 23). All genes, mutations, and replaced regions were sequenced by Cleveland Genomics to confirm the sequences.

To express the genes, 3–8 μg of column (Qiagen) purified plasmid DNA per 10^7 cells was used in transfection. N1E-115, Chinese hamster ovary (CHO)-K1, and COS-7 cells (American Type Culture Collection), cultured in DMEM supplemented with 10% FBS and 1% MEM nonessential amino acids (CHO-K1 only), were transfected by the GenePORTER transfection reagents (Gene Therapy Systems, San Diego). Transfected cells cultured for 48 h were used for either ligand binding assay, SHP-1 activity assay, immunoprecipitation, or cell membrane preparation. The receptor expression was assessed in each case by immunoblot analysis and by ^{125}I-[Sar^1,Ile^8]Ang II saturation binding analysis for receptors. Low-level expression was used for all signal activation related experiments. For ligand binding and other experiments, overexpression was applied. The expression levels were controlled and optimized by altering the amounts of plasmid DNA in transfections with GenePORTER transfection reagents. Efficiency of peptide transfection was examined by fluorescent microscopy (24).

Ligand Binding Study. ^{125}I-[Sar^1,Ile^8]Ang II binding experiments were carried out under equilibrium conditions as described earlier (22, 23). For competition binding studies, membranes expressing the wild-type or mutant receptors were incubated for 1 h with 0.3 nM ^{125}I-[Sar^1,Ile^8]Ang II and various concentrations of the ligands in assay buffer. The K_d value of ^{125}I-[Sar^1,Ile^8]Ang II was determined by competition binding study in the presence of various concentrations (0.03 to 3 nM) of ^{127}I-[Sar^1,Ile^8]Ang II. For the saturation binding study, a 10-fold higher increasing concentrations of ^{125}I-[Sar^1,Ile^8]Ang II than the K_d value of the receptor was used to get >90% bound form of the receptor. Nonspecific binding (which was <5% of total binding in our experiments) of the radioligand to the membrane was measured in the presence of 50 μM ^{127}I-[Sar^1,Ile^8]Ang II. All binding experiments were carried out at 22°C for 1 h in a 250-μl volume. After equilibrium was reached, the binding reactions were stopped by filtering the binding mixture under vacuum (Brandel Type M-24R) through FP-200 GF/C glass fiber filters (Whatman), which were extensively washed further with binding buffer to wash the free radioligand. The bound ligand fraction was determined from the cpm remaining on the membrane. Equilibrium binding kinetics were determined by using the computer program LIGAND. The K_d values represent the mean ± SEM of three to five independent determinations.

Immunoprecipitation and Western Blot. At 24 h after cotransfection of AT_2 receptors and SHP-1, CHO-K1, or COS-7 cells were starved overnight and then treated with Ang II. The stimulation of cells was stopped by putting the cells on ice and rinsing briefly with ice-cold PBS. The cells were then solubilized for 30 min at 4°C in lysis buffer (50 mM Tris·HCl, pH 7.5/1.5% CHAPS/150 mM NaCl/5 mM EDTA), containing 0.5 mM sodium orthovanadate, 10 μg/ml of benzamidine, 10 μg/ml of bacitracine, 10 μg/ml of leupeptin, and 2 μg/ml of aprotinine, and 50 μg/ml PMSF. After centrifugation at 15,000 × g for 15 min, the resulting supernatant (1–1.5 mg of proteins) was incubated for 3 h at 4°C with either 10 μl of monoclonal anti-SHP-1 antibody prebound to Sepharose Protein A (Santa Cruz Biotechnology) or 10 μl of anti-HA antibody. The immunocomplexes were washed five times with washing buffer (identical to the lysis buffer). At the end of washing, the immunocomplexes were used for SDS/PAGE and Western blotting analysis. Proteins were visualized by using their specific antibodies and corresponding secondary antibodies coupled to horseradish peroxidase and enhanced chemiluminescence system (Amersham Pharmacia).

Immunoprecipitation and SHP-1 Activity Assay. After transfection and treatment, cells were solubilized for 30 min at 4°C in lysis buffer containing 0.5 mM sodium orthovanadate and protease inhibitors. After centrifugation at 15,000 × g for 15 min, the resulting supernatant (1–1.5 mg of proteins) was incubated for 3 h at 4°C with 10 μl of monoclonal anti-SHP-1 antibody prebound to Sepharose Protein A. The immunocomplexes were washed five times with washing buffer (identical to the lysis buffer but without 1.5% CHAPS and 0.5 mM sodium orthovanadate). At the end of washing, the immunocomplexes were resuspended in PTPase buffer (50 mM Tris·HCl, pH 7.0/1 mg/ml BSA/5 mM DTT). The PTPase activity was assayed by measuring release of inorganic phosphate from phosphopeptides based on a malachite green detection system (11) using PTPase assay kit (Upstate Biotechnology) following the company's protocol.

Production of Total Inositol Phosphates (IP). COS-7 Cells cultured in 60 mm Petri dishes, 24 h after transfection, were labeled for 24 h with [^{3}H]*myo*-inositol (1 μCi/ml) at 37°C in inositol-free DMEM containing 10% bovine calf serum. On the day of IP assay (i.e., 48 h after transfection), the labeled cells were washed three times with serum-free medium and incubated with the media containing 10 mM LiCl for 20 min. Then medium alone or ligands were added to the cells. After incubation for 45 min at 37°C, the medium was removed, and total soluble IP was extracted from the cells by perchloric acid extraction method as described (23).

Statistical Analysis. The results are expressed as the mean ± SEM of 2–5 independent determinations. The significance of measured values was evaluated with an unpaired Student's t test.

Results and Discussion

Inactive SHP-1 Constitutively and Physically Associates with AT_2 Receptor, Whereas Active SHP-1 Dissociates from AT_2 Receptor. Consistent to the previously reported observation (8), stimulation of AT_2 receptor by 0.1 μM of Ang II in COS-7 cells cotransfected with HA-AT_2 and SHP-1 induced rapid SHP-1 activation as assayed in immunocomplexes prepared with anti-SHP-1 antibodies (Fig. 1*A*). The activation reached the maximum of 180% at about 5 min and returned to the basal level at 30 min. Similar

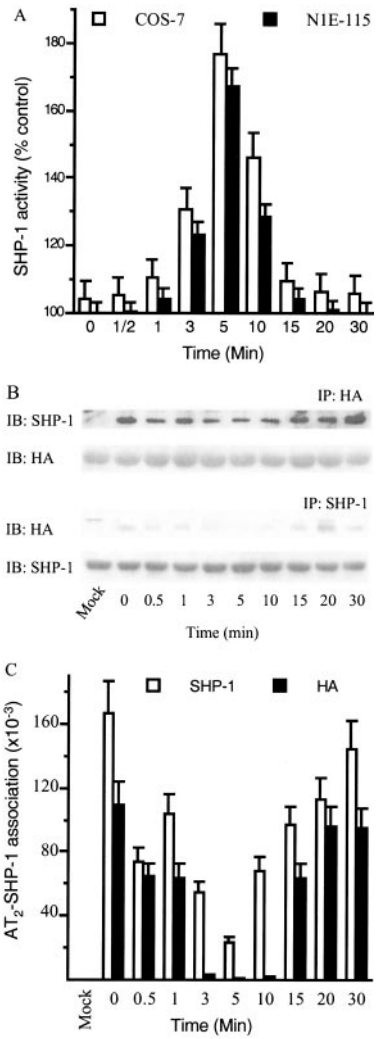

Fig. 1. AT_2 receptor mediates SHP-1 activation. (*A*) N1E-115, and COS-7 cells were incubated with 0.1 μM Ang II for various periods of time before immunoprecipitation with polyclonal anti-SHP-1 antibodies. The phosphatase activities measured as described in the experimental section were expressed as percentage of basal activity of mock-transfected COS-7 cells. Results are means ± SEM of at least three independent experiments performed in duplicate. (*B*) Immunodetection of physical interaction between AT_2 and SHP-1. Cell lysates (500 μg of protein) or immunocomplexes using anti-SHP-1 and anti-HA antibodies were prepared from COS-7 cells cotransfected with HA–AT_2 and SHP-1. After separation on 10% SDS/PAGE, immunoblotting were performed with antibodies as indicated in the figure. (*C*) Quantitation of the relative extent of SHP-1/AT_2 association. The amount of SHP-1 immunoprecipitated by anti-HA antibodies and the amount of AT_2 immunoprecipitated by anti-SHP-1 antibodies, as assessed by densitometric scanning (arbitrary units) were normalized to the similarly quantitated amount of AT_2 and SHP-1, respectively, recovered in each immunoprecipitate. The mock background were substracted.

activation profile was observed with N1E-115 cells expressing endogenous AT_2 and SHP-1. With respect to the basal phosphatase activity in the absence of Ang II, COS-7 cells cotransfected with genes of HA-AT_2 receptor and SHP-1 displayed no significant difference from COS-7 cells transfected with SHP-1 alone. However, mock-transfected or AT_2-transfected COS-7 cells show significantly less basal phosphatase activity (Fig. 1*A*). This suggests that the AT_2 receptor is not constitutively active in SHP-1 activation or the assay itself is not sensitive enough to demonstrate the constitutive activity of AT_2 receptor as reported in an AT_2-mediated apoptosis study (25).

To determine whether SHP-1 physically associates with AT_2 receptor, we carried out differential immunoprecipiation and immunoblotting experiments. Genes of HA-AT_2 and SHP-1 were cotransfected in COS-7 cells and the immunocomplexes of the cell lysates precipitated with anti-HA or anti-SHP-1 antibodies were detected by immunoblot with anti-SHP-1 or anti-HA antibodies. Fig. 1*B* shows that SHP-1 constitutively and physically associates with AT_2 receptor in the absence of Ang II. On stimulation by 0.1 μM of Ang II, the association decreases. The decrease in association reached the maximum at about 5 min and returned to the basal level at 30 min. This pattern is very similar to the pattern of increase in SHP-1 activity as shown in Fig. 1*A*. It indicates that the activated SHP-1 dissociates from activated AT_2 receptors. To further confirm this conclusion, phosphatase activity was assayed in immunocomplexes precipitated with anti-HA rather than anti-SHP-1 antibodies. As expected, the phosphatase activity of immunocomplexes precipitated with anti-HA antibodies remains unchanged regardless of the time course.

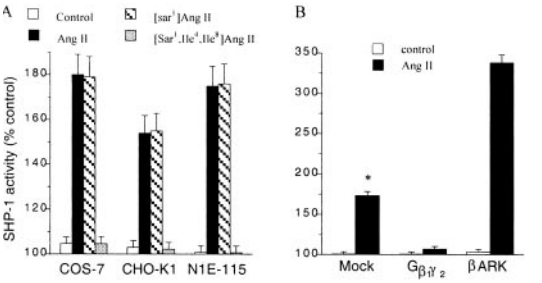

Fig. 2. (*A*) Ang II analogs induce SHP-1 activity. COS-7 cells transfected with HA-AT_2 and SHP-1, CHO-K1 cells transfected with HA-AT_2, and N1E-115 cells were incubated with Ang II (0.1 μM) and [Sar^1]Ang II (0.1 μM), and [Sar^1,Ile^4,Ile^8]Ang II (50 μM) for 5 min before immunoprecipitation with anti-SHP-1 antibodies. (*B*) Ang II (0.1 μM) induces SHP-1 activity in N1E-115 cells transfected with or without $G_{\beta 1\gamma 2}$ and βARK. Each data point represents the mean ± SE of two duplicate determinations. *, $P < 0.01$ with comparison to $G_{\beta 1\gamma 2}$ and βARK.

In N1E-115 cells and transfected COS-7 cells, 0.1 μM of Ang II induces maximal SHP-1 activity. No significant difference in SHP-1 activity was seen between 0.1 μM and 50 μM of Ang II concentrations.

The maximal decrease in physical association (about 3-fold) is much greater than the maximal increase in SHP-1 activity (180%) at 5 min after Ang II stimulation. This difference possibly reflects the relatively low sensitivity of phosphatase assay.

AT_2 Receptor Is Not Constitutively Active in SHP-1 Activation. Three lines of evidence suggest that wild type AT_2 receptor is constitutively active. First, in an AT_2/AT_1 chimera receptor with the cytoloop 3 domain of AT_2 replaced by the corresponding region of AT_1 receptor, Ca^{2+} movement was observed in the absence of Ang II stimulation (26). Second, Ang II binding profile has shown that in agreement with constitutively active AT_1 mutant N111G (27–29), AT_2 is insensitive to modifications of Ang II side chains at any position (22, 30), suggesting that AT_2 receptor is already in a relaxed active state (R*). Third, Karnik's group has shown that AT_2 induces apoptosis in the absence of Ang II (25). However, stimulation by Ang II failed to facilitate the AT_2-mediated constitutive apoptosis (25).

[Sar^1,Ile^4,Ile^8]Ang II that is completely incapable of activating wild-type AT_1 receptor has been shown to fully activate constitutively active AT_1 receptor mutant N111G (27–29). [Sar^1,Ile^4,Ile^8]Ang II binds wild type AT_2 receptor with >100-fold greater affinity with comparison to the wild-type AT_1 receptor. However, 5 or 50 μM of [Sar^1,Ile^4,Ile^8]Ang II fails to activate AT_2-mediated SHP-1 activity (Fig. 2*A*). Consistently, this inactive Ang II analog also fails to induce any significant change in AT_2/SHP-1 association (data not shown). These observations are

consistent to the findings regarding basal phosphotase activities as described above.

Taken together, these findings indicate that the wild type AT_2 receptor is not constitutively active in activation of SHP-1. However, these findings do not exclude the possibility that AT_2 receptor may be constitutively active in activation of other signal pathways as exemplified by Perez *et al.* (31) with α1-adrenergic receptor.

AT_2-Mediated SHP-1 Activation Is Independent of G Protein Activation. It is known that AT_2 receptor couples to the $G_{\alpha i}$ proteins only (5). However, the AT_2-mediated SHP-1 activation is PTX-insensitive (8). These contradictory findings do not support an involvement of $G_{\alpha i}$ proteins in AT_2-mediated SHP-1 activation. To examine whether AT_2-mediated SHP-1 activation could occur independently of activation of any Gα protein, we need an AT_2 mutant receptor that does not activate any Gα protein but is still capable of activating SHP-1. Initially, we constructed a mutant AT_2 receptor (D141A–R142L) in which the highly conserved Asp-141–Arg-142 residues were substituted with $Ala^{141}Leu^{142}$. In structurally related GPCRs, this sequence is the switch for release of GDP from the receptor-coupled G_α protein. Mutations of the sequence destabilize receptor–G protein complex and prevent the mutant receptor from inducing GDP–GTP exchange (32). The D141A–R142L mutant receptor expressed in COS-7 cells binds Ang II and AT_2 specific antagonist PD123319 with similar affinities to wild-type AT_2 receptor. Interestingly, this mutant receptor fully activates SHP-1 (Fig. 3*A*) and physically associates with SHP-1 (Fig. 3*B*). Consistently, the AT_2-mediated SHP-1 activation was PTX-insensitive. These findings indicate that AT_2-mediated SHP-1 activation can occur in the absence of Gα protein activation.

Significantly less SHP-1 activity of D141A–R142L mutant may reflect reduced binding affinity between mutant receptor and SHP-1 (Fig. 3*A*). This speculation is consistent to the immunoblot result as shown in Fig. 3*B*.

Angonist-induced conformational change of a receptor from inactive state (R) to active state (R*) is an absolute prerequisite for homologous activation of an effector pathway. AT_2 activation of SHP-1 must involve similar conformational change of the receptor triggered by the binding of agonist Ang II. Interruption of the conformational change should paralyze the AT_2 receptor in SHP-1 activation but probably not in SHP-1 association. To determine whether AT_2-mediated SHP-1 activation requires an active conformation (R*) of the AT_2 receptor, we constructed a mutant AT_2 receptor (D90A) in which the highly conserved Asp^{90} residue in the TM3 domain was replaced with Ala^{90}. In structurally highly conserved GPCRs, mutation of the Asp residue has been shown in many receptors, including AT_1 receptor, to disable the receptors in signal activation (2, 33). As expected, the D90A mutant receptor recognizes Ang II and AT_2-specific antagonist PD123319 with similar affinities of the wild type. Interestingly, the D90A mutant associates with SHP-1 like the wild type but fails to activate SHP-1 (Fig. 3).

These findings indicate that activation of SHP-1 by AT_2 receptor requires conformational transition of the receptor from inactive state to active state, in a way similar to Gα protein activation but distinct from classical SHP-1 activation by immunoreceptors (11–17). In these non-GPCR receptors, phosphorylation of a tyrosine located in the ITIM is required for SHP-1 binding and activation (11–17).

Synergism of Intracellular Domains Rather than the ITIM-Like Motif (VIYPFL) of the AT_2 Receptor Mediates SHP-1 Activation. An ITIM-like motif (VIYPFL) located in the cytoloop 2 of AT_2 receptor was identified originally by sequence analysis using PHI-BLAST program (34). However, substitutions of the tyrosine residue at this motif (Y148F and Y148A) with Phe and Ala failed to block SHP-1 association and activation in the presence of Ang II (data not shown). This result should be conclusive because Tyr^{148} is the only

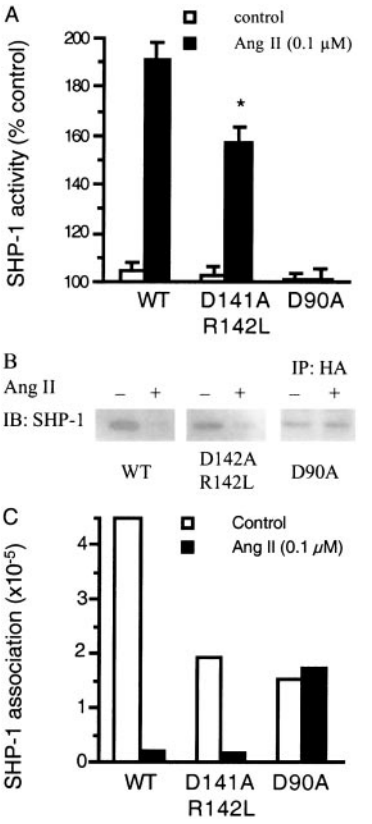

Fig. 3. AT_2 receptor mutants mediate SHP-1 activation. (*A*) COS-7 cells cotransfected with SHP-1 and HA-tagged AT_2 wild type (WT), or D141A–R142L or D90A mutants were incubated with Ang II (0.1 μM) for 5 min before immunoprecipitation with anti-SHP-1 antibodies. The SHP-1 activities were measured in duplicate. *, $P < 0.01$ between wild type and D141A–R142L in the presence of Ang II. (*B*) Immunodetection of receptor-SHP-1 association in the presence or absence of 0.1 μM of Ang II. Each data point represents the mean ± SE of at least two duplicate determinations. (*C*) Quantitation of the relative extent of SHP-1/AT_2 association. The amount of SHP-1 immunoprecipitated by anti-HA antibodies as assessed by densitometric scanning (arbitrary units) were normalized to the similarly quantitated amount of AT_2 receptors recovered in each immunoprecipitate. The mock background were substracted.

tyrosine residue available in the cytoloop 2 of the AT_2 receptor (Tyr^{143} is not included because Tyr^{143} is part of the DRY motif that is highly conserved in most GPCRs, including AT_1 receptor). In addition, AT_2 receptor activation by Ang II resulted in dissociation from SHP-1, rather than association with the SHP-1. This dissociation is accompanied with increase in SHP-1 activity (Fig. 1). This finding does not support any significant role of this ITIM-like motif in AT_2-medaited SHP-1 activation.

To identify structural determinants of AT_2 receptor for SHP-1 association and activation, we took an AT_2/AT_1 chimera receptor approach. Because intracellular domains of GPCR often serve as the interface for coupling to signaling molecules, four chimera receptors that contain only minimal exchange of intracellular segments of AT_1 and AT_2 receptor in various combinations were used (Table 1). These receptors bind Ang II with high affinity (0.46–1.56 nM). They also bind subtype-specific antagonist losartan or PD123319 with affinity similar to their structurally dominant wild types, respectively. Among these chimera receptors, CR14, which contains the cytoloop 3 of AT_1 receptor, showed no SHP-1 association and activation (Table 1). This result indicates that the cytoloop 3 of AT_2 receptor is involved in AT_2-mediated SHP-1 activation. This finding is in agreement with Lehtonen's report (10) in which the key residues of Lys^{240}, Asp^{242}, and Ser^{243} located in the

Table 1. Functions of Ang II receptors

Receptor	K_d (nM)			IP	SHP-1	SHP-1
	Ang II	PD	losartan	(%)	coupling	activity
AT_2	0.57	5.6	ND	2.5	++++	100
AT_1	1.56	ND	9.8	100	-	0.0
CR13	0.68	5.9	ND	2.4	+++	82.5
CR14	0.46	5.3	ND	2.5	-	0.2
CR25	0.52	5.8	ND	3.2	+++	78.3
CR26	0.55	5.4	ND	2.1	-	0.3

SHP-1 activity and coupling to the receptor were determined at 5 min of incubation with 0.1 μM Ang II. The maximal SHP-1 activity of wild-type AT_2 was referred as 100%. The maximal inositol phosphate (IP) production of wild type AT_1 in the presence of 0.1 μM Ang II was referred as 100%. CR13, the C-terminal region (309–363) of AT_2 replaced by the corresponding region (293–359) of AT_1; CR14, the cytoloop 3 (230–254) of AT2 replaced by the corresponding region (214–239) of AT_1; CR25, a combination of CR13 and CR14 replacements; CR26, the cytoloop 2 (134–162) of AT_2 replaced by the corresponding region (118–146) of AT_1. ND, not detectable.

third cytoloop of AT_2 receptor have been found important for AT_2-induced apoptosis, extracellular signal-regulated kinase inhibition and SHP-1 activation.

However, CR26 that has the cytoloop 2 replaced also show no SHP-1 association and activation, suggesting an important role for the second cytoloop. Interestingly, CR25 that contains both cytoloop 3 and C terminus of AT_1 receptor associates with SHP-1 and activates SHP-1 (Table 1). CR13 with replacement of the C-terminal alone preserved the capability of wild-type AT2 receptor to associate with and activates SHP-1 (Table 1).

Replacement of the second or third cytoloop alone results in loss of SHP-1 activation, whereas replacement of C-terminal in CR25 rescued the CR14 in SHP-1 activation. These findings suggest that all three intracellular domains are important in AT_2-mediated SHP-1 activation. It is their collective actions that dictate the SHP-1 activation. This is reminiscent to what we learned from receptor–G protein interactions. Interestingly, somatostatin receptor sst2-mediated SHP-1 activation also requires participation of $G_{\alpha i}$ protein (35).

Cotransfection of $G_{\beta\gamma}$ Inhibits AT_2-Mediated SHP-1 Activation, Whereas Cotransfection of βARK Potentiates the Function. Because AT_2-mediated SHP-1 activation involves multiple intracellular domains, it becomes logical to hypothesize that ancillary molecules are required for AT_2-SHP-1 interaction. To test whether $G_{\beta\gamma}$ proteins contribute to the interactions, N1E-115 cells were cotransfected with $G_{\beta 1}$ and $G_{\gamma 2}$ proteins. It has been shown that overexpression of G_{b1g2} proteins in rabbit tubule epithelial cells augments Ang II-induced arachidonic acid release, whereas overexpression of the C terminus of βARK abrogates Ang II-induced arachidonic acid release under conditions with and without overexpression of $G\beta_{1\gamma 2}$ proteins (36). Surprisingly, overexpression of $G_{\beta 1\gamma 2}$ proteins inhibited AT_2-mediated SHP-1 activation by 92%, whereas overexpression of βARK potentiated the action by 95% (Fig. 2*B*). This result supports a role of G_α rather than $G_{\beta 1\gamma 2}$ in AT_2-mediated SHP-1 activation.

$G_{\alpha s}$ Constitutively and Physically Associates with SHP-1 and AT_2 and Is Essential in AT_2-Mediated SHP-1 Activation. It is well established that both α and $\beta\gamma$ subunits of signal-transducing G proteins are maintained in inactive states by their mutual association in a heterotrimeric complex, which is coupled to 7TM GPCRs. As a result, neither GDP-bound α nor $\beta\gamma$ can interact with or activate downstream effectors. Conformational change of the receptor induced by specific agonist binding can result in conformational changes in GDP-bound α to favor GTP binding. The GTP-bound α dissociates from the receptor and releases $\beta\gamma$. These two activated signal generating molecules, the GTP-bound α and the free $\beta\gamma$, will activate their own effectors, initiating signal transductions (37). However, whether α protein or $\beta\gamma$ dimer alone can serve as an independent adapter protein or ancillary protein for other signal pathways is unknown. The fact that AT_2-mediated SHP-1 activation can be inhibited by overexpression of $G_{\beta 1\gamma 2}$ and potentiated by overexpression of βARK strongly suggests that a G_α protein is involved.

To test this possibility, the immunocomplexes precipitated with anti-HA antibody in cell lysates of COS-7 cells expressing HA-AT_2 and SHP-1 were immunoblotted with various anti-G_α antibodies. We identified a band with anti-$G_{\alpha s}$ antibodies (Fig. 4). The AT_2-$G_{\alpha s}$ association decreases in the presence of Ang II. Similar bands were also demonstrated from immunocomplexes precipitated with anti-SHP-1 antibodies in cell lysates of COS-7 cells expressing HA-AT_2

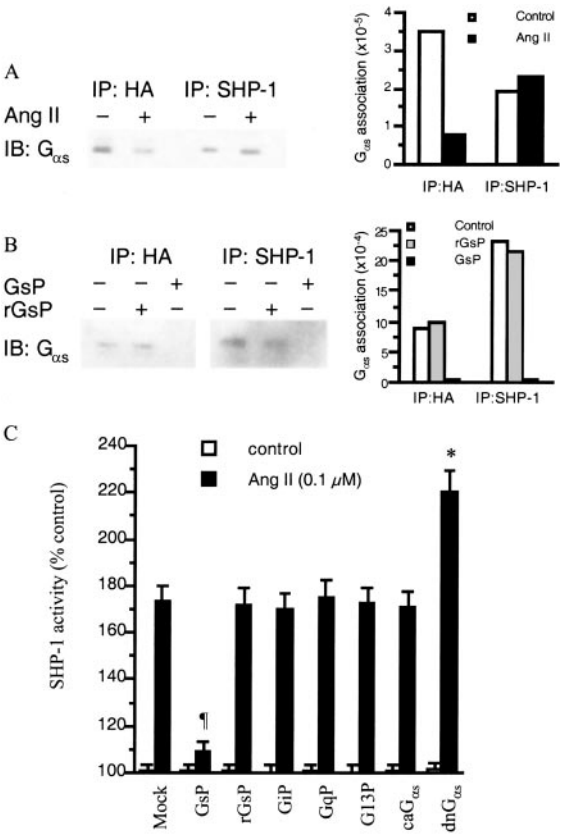

Fig. 4. Role of G_α proteins in AT_2-mediated SHP-1 activity. (*A*) Immunodetection of physical association of Gas with AT_2 or SHP-1 in COS-7 cells cotransfected with HA-AT_2, SHP-1 and $G_{\alpha s}$ genes. (*B*) Immunodetection of AT_2-SHP-1 and $G_{\alpha s}$-SHP-1 associations in the presence or absence of $G_{\alpha s}$ peptides. (*C*) SHP-1 activities induced by 0.1 μM Ang II in N1E-115 cells in the presence of $G_{\alpha s}$ peptides and $G_{\alpha s}$ mutants. $G_{\alpha s}$ peptides are: GiP, IKNNLKDCGLF; rGiP, NGIKCLFNDKL (Randomized peptide of Gi); GqP, LQLNLKEYNAV; rGqP: LAYQVNKLNLE (Randomized peptide of Gq); GsP, QRMHLRQYELL; rGsP: QQRLEMYLRHL (Randomized peptide of Gs); G13P, LHDNLKQLMLQ; rG13P, MQLDNKLQLHL (Randomized peptide of G13). Data for rGiP, rGqP, and rG13P peptides are not included in *C*. Here caG$_{\alpha s}$ and dnG$_{\alpha s}$ represent constitutively active $G_{\alpha s}$ mutant R201C and dominant negative mutant A366S-G226A-E268A, respectively. Each data point represents the mean ± SE of at least two duplicate determinations. *, $P < 0.01$ compared with mock-transfected N1E-115 cells; ¶, $P < 0.01$ compared with mock-transfected N1E-115 cells.

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and SHP-1. However, the $G_{\alpha s}$-SHP-1 association shows no significant decrease in the presence of Ang II. This observation suggests that $G_{\alpha s}$ protein remains associated with activated SHP-1 molecule and the sustained association may serve as a mechanism to maintain the activated SHP-1 in its active conformation until it binds to its target molecules of dephosphorylation.

To test whether specific blockade of AT_2–$G_{\alpha s}$ interaction would inhibit AT_2-mediated SHP-1 activation, 0.5 μM of peptides (Fig. 4) derived from the last 11 residues of $G_{\alpha q}$, $G_{\alpha s}$, G_{13}, and $G_{\alpha i}$ proteins are introduced into N1E-115 cells by transfection with Lipofectamine reagent (Life Science). These peptides are able to block G protein coupling to GPCR (37). Fig. 4 shows that only Gs peptide (GsP) inhibits the AT_2-mediated SHP-1 activation by about 88%. The rGsP, the control peptide of scrambled sequence of Gs peptide and other G_{α} peptides failed to inhibit the SHP-1 activation. In COS-7 cells cotransfected with HA-AT_2 and SHP-1, Gs peptide also blocked AT_2-SHP-1 association.

Because AT_2-mediated SHP-1 activation requires only conformational change of AT_2 receptor to its active state but not G protein activation as described earlier with D141A–R142L and D90A mutants, we speculated that constitutively active $G_{\alpha s}$ would not constitutively activate SHP-1, whereas dominant negative $G_{\alpha s}$ would facilitate the SHP-1 activation. As expected, overexpression of constitutively active $G_{\alpha s}$ (R201C mutant) failed to show any effect on SHP-1 activation, whereas overexpression of dominant negative $G_{\alpha s}$ (A366S-G226A-E268A mutant) augmented SHP-1 activation up to 78%. Constitutively active $G_{\alpha s}$ is monomer in GTP-bound form and no longer binds to the receptor. However, the dominant negative $G_{\alpha s}$ forms heterotrimer with $G_{\beta\gamma}$ subunits with reduced binding affinity for GDP and GTP. It consumes endogenous $G_{\beta\gamma}$ to facilitate AT_2–SHP-1 interaction. The 78% increase in SHP-1 activation reflects a mixed effect of sequestration of $G_{\beta\gamma}$ and modified binding to the receptor (38).

SHP-1 Activation by $G_{\alpha s}$-Coupled AT_2 Receptor: ITIM vs. Non-ITIM. SHP-1 binding to tyrosine-phosphorylated ITIM (I/VxYxxL) of inhibitory receptors represents a general paradigm for SHP-1 activation (11–17). This finding is further supported by evidence that has identified sequence YxxL as a previously uncharacterized ITIM in the death domain of death receptor of the tumor necrosis factor and nerve growth factor superfamily (39). The resultant free catalytic domain becomes active and ready to interact with its substrate. However, similar ITIMs are not present in the GPCRs that activate SHP-1 (18–21, 35). Moreover, no similar ITIMs are present in any of G protein subunits as indicated by our sequence analysis. The ITIM-like motif identified in the AT_2 receptor is not required for the SHP-1 activation. Thus, it is conceivable that GPCR-mediated SHP-1 activation is ITIM-independent and certainly distinct from the general paradigm of ITIM-mediated SHP-1 activation.

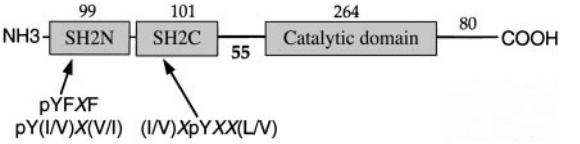

Fig. 5. Functional domains of SHP-1 and the ITIMs.

The fact that $G_{\alpha s}$ involves in AT_2-mediated SHP-1 activation has illustrated a previously undescribed mode of SHP-1 activation by GPCRs. Besides physical interaction with AT_2 receptor, SHP-1 may interact with $G_{\alpha s}$ protein at the interface to which $G_{\beta\gamma}$ proteins bind. Alternatively, the SHP-1 binding site may overlap with the $G_{\beta\gamma}$ binding site in $G_{\alpha s}$ protein. AT_2, $G_{\alpha s}$, and SHP-1 are capable of forming complexes at basal state.

SHP-1 (11, 14, 15) consists of (*i*) two SH2 domains, designated SH2N and SH2C, (*ii*) a catalytic domain, (*iii*) a 55-residue fragment connecting the SH2C and CA, and (*iv*) an 80-residue C-terminal tail (Fig. 5). It is well known that SH2 domains are functional protein motifs that bind tyrosine-phosphorylated targets by recognizing phosphotyrosine and specific adjacent residues. It is thought that the free cytosolic SHP-1 maintains a self-constrained inactive state by interaction of the catalytic domain with the two SH2 domains. Preferential interaction of the SH2 domain (mainly SH2C) with a tyrosine phosphorylated ITIM automatically removes the constraints imposed on the catalytic domain, leading to SHP-1 activation (14, 40, 41). This activation mechanism is based on evidence that removal of the SH2 domains from SHP-2, belonging to SHP-1 PTPase family, results in a 12- to 45-fold increase in phosphatase activity and exogenous SH2 domains reverse this effect (14, 40, 41). Because the functions of SH2N, the 55 fragment and C tail of SHP-1 are not yet defined, it is possible that binding of SHP-1 to AT_2 and $G_{\alpha s}$ through these functional domains may also lead to SHP-1 activation.

We thank Drs. Sadashiva Karnik, Matthew L. Thomas, and Robert Lefkowitz for providing plasmid genes. This work was supported in part by American Heart Association Scientist Development Grant 0030019N (to Y.H.F.) and National Heart, Lung, and Blood Institute Grant HL41618 (to J.G.D.).

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