The carboxyl-terminal cytoplasmic domain of the angiotensin II type 1 (AT₁) receptor has recently been shown to interact with several classes of cytoplasmic proteins that regulate different aspects of AT₁ receptor physiology. Employing yeast two-hybrid screening of a mouse kidney cDNA library with the carboxyl-terminal cytoplasmic domain of the murine AT₁a receptor as a bait, we have isolated a novel protein with a predicted molecular mass of 18 kDa, which we have named ATRAP (for AT₁ receptor-associated protein). ATRAP interacts specifically with the carboxyl-terminal domain of the AT₁a receptor but not with those of angiotensin II type 2 (AT₂), m₃ muscarinic acetylcholine, bradykinin B₂, endothelin B, and β₂-adrenergic receptors. The mRNA of ATRAP was abundantly expressed in kidney, heart, and testis but was poorly expressed in lung, liver, spleen, and brain. The ATRAP-AT₁a receptor association was confirmed by affinity chromatography, showing co-localization of these proteins in intact cells. Overexpression of ATRAP in COS-7 cells caused a marked inhibition of AT₁a receptor-mediated activation of phospholipase C without affecting m₃ receptor-mediated activation. In conclusion, we have isolated a novel protein that interacts specifically with the carboxyl-terminal cytoplasmic domain of the AT₁a receptor and affects AT₁a receptor signaling.

G protein-coupled receptors (GPCRs) interact with different classes of intracellular proteins, including heterotrimeric G proteins, kinases, and arrestins (1–3). Although the intracellular third loop of a number of GPCRs is a key structural determinant of coupling of the receptor to heterotrimeric G proteins (4–9), recent studies have highlighted the functional importance of the carboxyl-terminal cytoplasmic domain in receptor signaling and desensitization (10–16). Angiotensin II (AngII) is a key regulator of the cardiovascular system. AngII exerts its biological effects through two major subtypes of high affinity GPCRs designated AT₁ and AT₂ receptors. Recently, the carboxyl-terminal cytoplasmic domain of the AT₁ receptor has been reported to directly associate with several downstream effectors (12–15). By means of mutational analysis, this domain has also been shown to contain discrete amino acid sequences that are required for receptor desensitization (17, 18) and internalization (17, 19, 20). As for many GPCRs, the carboxyl-terminal cytoplasmic domain of the AT₁ receptor presumably interacts with G protein-coupled receptor kinases and arrestins, causing functional desensitization of the receptor (18, 21).

These observations raise the possibility that the carboxyl-terminal cytoplasmic domain of the AT₁ receptor interacts with additional cellular proteins that may play an important role in the efficacy and/or specificity of receptor-G protein coupling. We have investigated this possibility by searching for novel protein interactions with the carboxyl-terminal cytoplasmic domain of the murine AT₁a receptor. Using interaction cloning as well as biochemical and immunocytochemical techniques, we report here the identification of a novel protein that specifically interacts with the AT₁ receptor tail. Functional studies suggest that this protein interaction may play a role in the regulation of receptor-mediated signaling.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The carboxyl-terminal cytoplasmic domain of the murine AT₁a receptor (AT₁a C-ter; amino acids 297 to 359) was polymerase chain reaction-amplified and fused to the Gal4 binding domain in the yeast shuttle vector pBD-Gal4 (Stratagene). In a similar manner, 3 carboxyl-terminal deletions of the AT₁a receptor tail (AT₁a C-ter Δ349, Δ339, and Δ329) were generated by polymerase chain reaction with use of reverse primers containing stop codons at the desired locations, and the deleted cDNAs were subcloned into pBD-Gal4. The carboxyl-terminal cytoplasmic domains of human AT₁ (amino acids 314 to 363), human m₃ muscarinic acetylcholine (amino acids 548 to 590), human bradykinin B₂ (amino acids 299 to 364), endothelin B (amino acids 390 to 442), and β₂-adrenergic (amino acids 328 to 413) receptors were polymerase chain reaction-amplified and subcloned into pBD-Gal4. All the constructs were verified by DNA sequencing using the Sanger dideoxynucleotide termination method adapted to the Applied Biosystems model 373S Automated DNA Sequencer.

**Two-hybrid Screen**—A cDNA library from mouse kidney poly(A)⁺ RNA was constructed in fusion with the Gal4 activation domain in pAD-Gal4 (Stratagene) with a cDNA synthesis kit from Stratagene using XhoI/(dT)₁₈ primer and EcoRI adapters. The yeast reporter strain YRG-2 (Stratagene) containing 2 Gal4-inducible reporter genes (HIS3 and LacZ) was sequentially co-transformed with the AT₁a C-ter hybrid constructs. All the yeast transformants were plated on yeast drop-out media and screened by X-gal staining for interactions with the GAL4 expression library.
media lacking tryptophan, leucine, and histidine. The transformants were grown for 3 days, and His⁺ colonies were then picked on selective media, replica-plated on nitrocellulose filters, and tested for β-galactosidase activity (23). Positive clones were rescued and tested for specificity by retransformation into strain YRG-2 with either AT₁a C-ter or the extraneous targets human lamin C and murine p53 (Stratagene). The cDNA inserts from positive clones were then sequenced.

Northern Blot Analysis—The Northern blot was purchased from CLONTECH and hybridized with α-32P-ATRAP and β-actin cDNAs according to the manufacturer’s recommendations.

Maltose Binding Protein (MBP) Fusion Protein Affinity Chromatography—The cytoplasmic AT₁a and AT₂ receptor tails were fused to maltose polymerase chain reaction and cloned into a pMal-c2 prokaryotic expression vector (New England Biolabs). MBP fusion polypeptides were expressed in Escherichia coli DH5α and purified according to the manufacturer’s instructions. The MBP fusion protein load of individual amylase residues was normalized by densitometric scanning of SDS-PAGE gels stained with Coomassie Blue. For affinity chromatography, a 50-μl volume of the MBP fusion protein-loaded resin (50% (w/v) suspensions) was preblocked in binding buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1 μg of aprotinin/ml) with bovine serum albumin (10 mg/ml) for 1 h at 4 °C. The MBP fusion protein resin was then incubated with a lysate (100 μg of protein) of COS-7 cells transiently transfected with an HA epitope-tagged version of ATRAP (HA-ATRAP) in binding buffer for 16 h at 4 °C. Resins were washed four times with binding buffer and eluted with SDS-PAGE sample buffer. Samples were then subjected to SDS-PAGE, transferred to nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech), and probed with anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim). Epitope-tagged ATRAP was detected with peroxidase-conjugated sheep anti-mouse secondary antibody 12CA5 (Boehringer Mannheim). AT₁a receptor was detected with the monoclonal mouse anti-receptor 522 (CLONTECH) and hybridized with [32P]-ATRAP and [3H]inositol phosphate. For affinity chromatography, membranes were incubated in serum-free medium (Ham’s F12; Life Technologies) for 16 h. Quiescent cells were then treated with 100 nM AngII for 3.5 h, washed with phosphate-buffered saline, and lysed for 10 min with 250 μl of lysis buffer (luciferase assay system; Promega) at 4 °C. 10 μl of cell extract was mixed with 100 μl of luciferase reagent, and the light produced was measured for 10 s using a LUMAT LB 9507 luminometer (EG & G Berthold). Results were normalized to the β-gal activity using a β-galactosidase assay system (Promega).

Coom-Immunoprecipitation—The NH₂-terminal HA epitope-tagged ATRAP in pCDNA3 was transiently co-transfected with a FLAG-tagged murine AT₁a receptor (24) in COS-7 cells according to the LipofectAMINE protocol (Life Technologies, Inc.). The ratio of AT₁a and ATRAP DNA was 1:3. Cells were harvested 48 h after transfection, and membrane fractions prepared from the transfected cells (25) were solubilized in 50 mM Tris-HCl (pH 7.5), 140 mM NaCl, 1 mM CaCl₂, 1 mM phenylmethylsulfonyl fluoride, and 1 μg of aprotinin/ml (buffer A) in the presence of 1% CHAPS. The mixture was gently agitated for 30 min at 4 °C and thereafter centrifuged at 13,000 × g for 20 min. Cleared supernatants (100 μg of protein) were diluted 1:10 in buffer A and incubated for 16 h at 4 °C with M1 monoclonal antibody recognizing the FLAG epitope (Eastman Kodak Co.) and protein A/G PLUS-agarose beads (Santa Cruz Biotechnology). The beads were then washed in buffer A, and the samples were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim). Epitope-tagged ATRAP was detected with peroxidase-conjugated sheep anti-mouse secondary antibody (Amersham Pharmacia Biotech) and enzyme-linked chemiluminescence (ECL, Amersham Pharmacia Biotech).

Immunocytochemistry—COS-7 cells were seeded in glass coverslips and co-transfected with NH₂-terminal HA-tagged ATRAP and FLAG epitope-tagged AT₁a, using the method described above. The cells were then fixed and permeabilized with ice-cold methanol for 5 min. HA-ATRAP was detected with either the monoclonal mouse antibody 12CA5 and a fluorescein isothiocyanate-conjugated rat anti-mouse IgG₁ monoclonal antibody (PharMingen) or an HA-specific rabbit antibod (Bdbo) and CY3 goat-anti-rabbit antibody (Zymed Laborato ries Inc.). AT₁α receptor was detected with the monoclonal mouse antibody M1 and a fluorescein isothiocyanate-conjugated rat anti-mouse IgG₁ monoclonal antibody.

β-Galactosidase Assay—The yeast reporter strain SPY526 was co-transformed with the deleted versions of the AT₁a C-ter and the ATRAP hybrid expression plasmid. The amounts of β-galactosidase from three independent transformants grown in liquid selective media were measured in a chlorophenol red-β-D-galactopyranoside-based assay (26).

Inositol Phosphate Determination—COS-7 cells were transiently co-transfected with NH₂-terminal HA epitope-tagged ATRAP and FLAG epitope-tagged AT₁a receptor or human m3 muscarinic acetylcholine receptor subunit in LipofectAMINE reagent. The ratio of AT₁a and ATRAP DNA was 1:3. Transfected cells plated at 1 × 10⁶ cells/well were labeled overnight with [myo-³H]inositol (5 μCi/ml; NEN Life Sciences Products) in serum-free Dulbecco’s modified Eagle’s medium. After 1 h of stimulation with increasing concentrations of AngII or carbachol in the presence of 10 μM LiCl, inositol phosphate was extracted and separated on Dowex AG1-X8 columns (Bio-Rad). Total inositol phosphate was eluted with 2 M ammonium formate, 0.1 M formic acid.

fos-Luciferase Assay—Chinese hamster ovary (CHO) K1 cells were co-transfected with the FLAG-tagged murine AT₁a receptor expression vector (24) and pSV × Neo with the LipofectAMINE reagent (using a 30:1 DNA ratio). Stably transfected cells were selected in G418 (800 μg/ml) for 3 weeks, and the cells expressing high levels of AT₁a receptors were sorted by fluorescence-activated cell sorting after immunolabeling with the anti-FLAG M1 monoclonal antibody. The immunoselected, stably transfected CHO AT₁a cells were maintained in G418 and used for up to four passages. 3.5 × 10⁶ CHO AT₁a cells were seeded in 6-well plates and transiently co-transfected with pCMVβ-galactosidase reporter gene (pCMVβ; CLONTECH) by lipofection using the LipofectAMINE reagent. The fos-luciferase reporter gene consists of two copies of the -fos 5′-regulated enhancer element (-357 to -276), the herpes simplex virus thymidine kinase gene promoter (-200 to +70), and luciferase gene (4). The ratio of HA-ATRAP, fos-luciferase, and β-gal DNA was 3:1:1. Forty-eight h after transfection, transfected cells were incubated in serum-free medium (Ham’s F12; Life Technologies) for 16 h. Quiescent cells were then treated with 100 nM AngII for 3.5 h, washed with phosphate-buffered saline, and lysed for 10 min with 250 μl of lysis buffer (luciferase assay system; Promega) at 4 °C. 10 μl of cell extract was mixed with 100 μl of luciferase reagent, and the light produced was measured for 10 s using a LUMAT LB 9507 luminometer (EG & G Berthold). Results were normalized to the β-gal activity using a β-galactosidase assay system (Promega).

Radioligand Binding Assay—For AT₁a receptor-transfected COS-7 and CHO K1 cells, ligand binding assays were performed using membrane preparations as described elsewhere (25). For m3 receptor-transfected COS-7 cells, binding assays using N'-[3H]methylylcopolamine (NEN Life Science Products) were carried out with membrane homogenates as described previously (28). Non-specific binding was measured in the presence of 1 μM atropine.

RESULTS AND DISCUSSION

The yeast two-hybrid system was used to identify candidate proteins that interact with the carboxyl-terminal cytoplasmic tail of the mouse AT₁a receptor. Screening of 1.5 × 10⁶ transformants from a mouse kidney primary cDNA library resulted in the isolation of three independent clones that interacted specifically with the AT₁a carboxyl-terminal tail. No interaction with another major subtype of Ang II receptor (AT₂) was observed. Sequence analysis revealed that the three library plasmids contained different lengths of the same cDNA. All inserts contained an open reading frame, and inspection of the sequence of the longest cDNA revealed a potential initiator ATG that matched well the consensus sequence for translational initiation (29). This clone spans an open reading frame of 483 base pairs encoding a predicted protein of 17.8 kDa (Fig. 1). We named this protein ATRAP for AT₁a receptor-associated protein. The failure of 5′-rapid amplification of cDNA ends to lead to the isolation of longer cDNA suggested that the clone isolated from the two-hybrid screen represents the full-length gene. Moreover, the mobility of an in vitro translation product was in agreement with the molecular mass predicted for ATRAP by sequence analysis (data not shown). The ATRAP-predicted amino acid sequence was used to search available data bases by means of the BLAST program network server. ATRAP does not show homology with known proteins. However, it is similar to at least three mouse EST clones (accession numbers AA718794, AA840135, and W577121), all of which homology to a number of rat and human EST clones, ATRAP has a potential N-glycosylation site, one potential phospholipidation site for protein kinase C, and one potential phosphorylation site for casein kinase II (Fig. 1). This protein also contained several extensive hydrophobic domains in its NH₂-terminal portion.

Northern blot analysis of messenger RNA from various mouse tissues, with full-length ATRAP cDNA as a probe, re-
revealed two transcripts of 1.2 and 0.8 kilobases; this result further suggested that the cDNA clone represents the full-length gene. ATRAP was expressed at a relatively high level in kidney, testis, and heart but at lower levels in lung, liver, spleen, and brain (Fig. 2). Using reverse transcription-polymerase chain reaction, we also detected ATRAP transcripts in mouse aortic tissue and vascular smooth muscle cells (data not shown).

To biochemically confirm the association between ATRAP and AT1a C-ter, in vitro interactions were examined in studies using AT1a and AT2 receptor cytoplasmic tails fused to MBP. When added to detergent-solubilized extracts of ATRAP-transformed COS-7 cells, ATRAP was recovered with the recombinant MBP-AT1a C-ter but not with MBP-AT2 C-ter or the MBP alone (Fig. 3A). These results further suggested that ATRAP associates specifically with the AT1 receptor.

The binding of ATRAP to full-length AT1a receptors in vivo was confirmed by co-immunoprecipitation from transiently transfected COS-7 cells. The AT1a receptor was tagged at the amino-terminal extracellular domain with a FLAG epitope to facilitate specific immunoprecipitation of receptors (24). For the immunodetection of ATRAP, the protein was HA-tagged at the amino terminus, and a polypeptide of the expected size was observed by immunoblotting in transfected cells (Fig. 3B, 1st lane). ATRAP was co-immunoprecipitated specifically from cell membrane lysates in association with the AT1a receptor (Fig. 3B, 4th lane). ATRAP was not detected in control immunoprecipitates, including those prepared from cells expressing ATRAP without FLAG-tagged receptors (Fig. 3B, 2nd lane); this result confirmed the specificity of this protein association in vivo. We did not observe a significant difference in the amount of ATRAP co-immunoprecipitated with the AT1 receptor before or after AngII stimulation (data not shown).

The subcellular localization of epitope-tagged ATRAP was examined in transfected COS-7 cells by fluorescence microscopy. Using optical sectioning of antibody-labeled cells by confocal microscopy, ATRAP was visualized in a diffuse cytoplasmic distribution, with a more intensive staining near the cell membrane.
periphery (Fig. 4A). Immunoblotting of extensively washed membrane fractions prepared from transfected cells confirmed that a significant fraction of ATRAP was membrane-associated (data not shown). The association of ATRAP with AT1a receptors was further examined by immunofluorescence co-localization experiments. COS-7 cells were co-transfected with HA-tagged ATRAP protein and FLAG-tagged AT1a receptor. Immunostaining and fluorescence microscopy were carried out as described under “Experimental Procedures.” A, in addition to its cytoplasmic distribution, ATRAP localized to the plasma membrane. B–D, co-transfected and immunostained cells were imaged by dual-color confocal microscopy: AT1a receptor (green channel, B) and ATRAP (red channel, C) co-localized to the plasma membrane and in intracellular compartments as shown in yellow in the two-color merged image (D).

**TABLE I**

Yeast two-hybrid interaction of ATRAP to deletion mutants of the AT1a receptor tail

| Gal4 binding domain hybrid | Gal4 activation domain hybrid | β-Gal units | % Binding |
|---------------------------|-----------------------------|------------|----------|
| AT1a, C-ter wild type     | ATRAP                       | 160        | 100      |
| AT1a, C-ter Δ349          | ATRAP                       | 60         | 37.5     |
| AT1a, C-ter Δ339          | ATRAP                       | 0          | 0        |
| AT1a, C-ter Δ329          | ATRAP                       | 0          | 0        |

**FIG. 4.** Immunocytochemical co-localization of ATRAP and AT1a receptor. COS-7 cells were transiently co-transfected with HA-tagged ATRAP protein and FLAG-tagged AT1a receptor. Immunostaining and fluorescence microscopy were carried out as described under “Experimental Procedures.” A, in addition to its cytoplasmic distribution, ATRAP localized to the plasma membrane. B–D, co-transfected and immunostained cells were imaged by dual-color confocal microscopy: AT1a receptor (green channel, B) and ATRAP (red channel, C) co-localized to the plasma membrane and in intracellular compartments as shown in yellow in the two-color merged image (D).

**TABLE II**

Binding parameters of 125I-sarcosine AngII in transiently co-transfected COS-7 cells

| Parameter | AT1a + pcDNA3 | AT1a + ATRAP |
|-----------|---------------|--------------|
| Kd (nM)   | 0.25 ± 0.05   | 0.21 ± 0.02  |
| Bmax (pmol/mg of membrane protein) | 5.84 ± 2.05 | 5.5 ± 1.4 |

**FIG. 5.** Effect of ATRAP overexpression on agonist-dependent activation of phospholipase C in transiently transfected COS-7 cells. Total inositol phosphate (IP) production was measured in COS-7 cells co-transfected either with AT1a receptor and the control vector ( ■) or with AT1a receptor and ATRAP ( ■) in the presence of increasing amounts of Ang II. Results are expressed as the ratio of stimulated to unstimulated cells normalized to maximal binding capacity (Bmax). Values are means ± S.E. of 4 independent experiments performed in duplicate. *p < 0.05.
and the control plasmid. Radioligand binding assays performed with the same populations of transfecteds used in the PLC assay indicated that ATRAP overexpression did not significantly affect the affinity or the number of AT_{1a} receptors (Table II). The magnitude of the inhibitory effect of ATRAP overexpression was influenced by the expression of endogenous ATRAP in COS-7 cells, as detected by Northern blot analysis (data not shown). Furthermore, the effect of ATRAP on receptor signaling may depend on its interaction with other cellular partners that may be present in limiting amounts relative to overexpressed ATRAP. To assess whether ATRAP associates with other GPCRs, we examined its interaction with the carboxyl-terminal cytoplasmic domains of several G_{i}-coupled receptors in the yeast two-hybrid system. ATRAP did not interact with the carboxyl-terminal cytoplasmic tails of the m_{3} muscarinic, bradykinin B_{2}, or endothelin B receptors, nor did it associate with the G_{i}-coupled β_{2}-adrenergic receptor (data not shown). Accordingly, no effect of ATRAP overexpression was observed on m_{3} receptor-mediated PLC activity over a wide range of agonist concentrations (10^{-9} to 10^{-5} M carbachol) or on basal PLC activity. Moreover, ATRAP did not affect PLC (β and γ isoforms) expression level as determined by immunoblot analysis (data not shown). Taken together, these observations are consistent with the hypothesis that ATRAP specifically inhibits signaling by interacting directly with AT_{1a} receptors rather than by affecting receptor expression or downstream signaling components. Moreover, the specificity of this inhibition is consistent with the specificity of ATRAP association with the receptor tail in vitro. The observation that AngII binding did not affect the ATRAP-AT_{1} receptor interaction would suggest that ATRAP function may be regulated by other means such as post-transcriptional modifications (phosphorylation/dephosphorylation) or alternatively by its association with other cellular proteins in response to AT_{1} receptor stimulation.

To assess whether ATRAP may influence a more downstream AT_{1a} receptor-dependent signaling event, we examined the effect of ATRAP overexpression on AngII-induced c-fos gene expression. CHO AT_{1a} cells (K_{D} = 0.3 ± 0.05 nM; B_{max} = 4.2 ± 1.2 pmol/mg of membrane protein) were transiently co-transfected with a fos-luciferase reporter gene together with ATRAP or a control vector. The fos-luciferase reporter construct contains the serum response element of the c-fos promoter (4), which has been shown to be sufficient for AngII-induced activation of the c-fos promoter (27). AngII-induced c-fos expression was determined by measuring the increase in fos-luciferase activity in lysates of co-transfected cells after AngII treatment. ATRAP overexpression did not significantly affect AngII-dependent increase in c-fos expression when compared with cells transfected with the control plasmid (data not shown). AngII-induced activation of the serum response element of the c-fos promoter has been proposed to involve protein kinase C and ERK1/2 (extracellular signal-regulated kinase) stimulation (30). In contrast to the proximal effector PLC, induction of c-fos expression is a downstream signaling event that requires the activation of a cascade of effectors. Therefore, it is conceivable that the attenuated PLC response observed in cells overexpressing ATRAP is insufficient to affect the downstream effectors involved in the induction of the c-fos reporter gene expression.

In conclusion, we have identified a novel, membrane-localized protein that interacts specifically with the carboxyl-termi-