Negative regulation of mitogenic pathways is a fundamental process that remains poorly characterized. The angiotensin II AT2 receptor is a rare example of a 7-transmembrane domain receptor that negatively cross-talks with receptor tyrosine kinases to inhibit cell growth. In the present study, we report the molecular cloning of a novel protein, ATIP1 (AT2-interacting protein), which interacts with the C-terminal tail of the AT2 receptor, but not with those of other receptors such as angiotensin AT1, bradykinin BK2, and adrenergic β2 receptor. ATIP1 defines a family of at least four members that possess the same domain of interaction with the AT2 receptor, contain a large coiled-coil region, and are able to dimerize. Ectopic expression of ATIP1 in eukaryotic cells leads to inhibition of insulin, basic fibroblast growth factor, and epidermal growth factor-induced ERK2 activation and DNA synthesis, and attenuates insulin receptor autophosphorylation, in the same way as the AT2 receptor. The inhibitory effect of ATIP1 requires expression, but not ligand activation, of the AT2 receptor and is further increased in the presence of Ang II, indicating that ATIP1 cooperates with AT2 to trans-inactivate receptor tyrosine kinases. Our findings therefore identify ATIP1 as a novel early component of growth inhibitory signaling cascade.

The potent vasoactive peptide angiotensin II (Ang II) is also an important regulator of cellular proliferation and hypertrophy. This peptide binds to two main subtypes of receptors (AT1 and AT2) that both belong to the superfamily of G protein-coupled receptors (GPCR) but display opposite biological and physiological effects. The AT1 receptor mediates most of the known cardiovascular and central actions of Ang II. This subtype has mitogenic and trophic effects in many tissues and cell types and transduces multiple intracellular signaling cascades typically associated with GPCR activation. In contrast, AT2 behaves like a "natural antagonist" of the AT1 subtype on most physiological functions, and induces anti-proliferative and pro-apoptotic effects in vivo and in vitro (for reviews, see Refs. 1–5).

The AT2 receptor activates unconventional signaling pathways that in most cases do not involve coupling to classical regulatory G proteins. A growing body of evidence indicates that anti-growth effects of the AT2 receptor are associated with activation of tyrosine phosphatases and inhibition of protein kinases, which ultimately lead to inhibition of extracellular regulated kinase (ERK2). In many cell types, AT2 is functionally coupled to the Src homology 2 domain-containing tyrosine phosphatase SHP-1 (6–8). This phosphatase has been shown to play a central role in the AT2 signaling cascades leading to inhibition of AT1-induced PYK2 and Jun kinase (9), AT1-trans-activated EGF receptor tyrosine kinase (10), and insulin-inhibited phosphatidylinositol 3-kinase and Akt activation (11).

AT2 negatively cross-talks with receptor tyrosine kinases (RTK) such as bFGF, EGF, and insulin receptors (10, 12, 13) by targeting a very early step of RTK activation, i.e. autophosphorylation of the receptor. In vascular smooth muscle cells (VSMC) from AT2-transgenic mice, EGF receptor trans-inactivation induced by AT2 stimulation was found to involve rapid activation of tyrosine phosphatase SHP-1 and its increased association with the EGF receptor (10). In Chinese hamster ovary (CHO) cells, however, AT2-mediated trans-inactivation of the insulin receptor does not involve protein dephosphorylation by orthovanadate-sensitive tyrosine phosphatases nor coupling to pertussis toxin-sensitive regulatory heterotrimeric G/i proteins (12), suggesting that another yet undefined mechanism may link AT2 receptor stimulation to growth inhibition.

A number of recent studies have revealed that GPCRs can mediate their intracellular effects through signaling pathways
ATIP, a Novel Family of AT2 Receptor-interacting Proteins

that are independent of G proteins (14, 15). Over the past few years, many groups have identified novel intracellular proteins that directly interact with C-terminal tails of GPCRs and function as scaffolds to regulate receptor trafficking or signaling (16, 17). ATRAP is one such example of a novel protein that selectively interacts with the AT1 receptor C terminus and down-regulates its activity (18, 19). Regarding the AT2 receptor, recent studies have documented a direct interaction of its C-terminal tail with ErbB3, a member of the EGF receptor family (20, 21), and with the transcription factor promyelocytic zinc finger containing protein (PLZF) abundantly expressed in the heart (22).

In the present study, we have used the C-terminal part of the AT2 receptor as bait in a two-hybrid system to identify new interacting partners of the receptor. We describe here the molecular cloning and functional characterization of ATIP1, a novel coiled-coil domain containing protein that selectively interacts with the AT2 receptor and mediates inhibition of growth factor-induced ERK2 activation and cell proliferation.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid and cDNA Cloning**—The 52 C-terminal residues of the human AT2 receptor were PCR amplified and subcloned into pGBT9 vector in-frame with the Gal4-DNA binding domain. Independent transformants (3 × 10⁴) from a mouse fetal cDNA library containing inserts of 300 to 700 bp in VP16 vector (a kind gift of Dr. A. Vojtek) were screened by the two-hybrid system cloning method in the His7 strain as described (23) using the 52 C-terminal residues of the AT2 receptor as a bait. A 354-bp cDNA clone designated ATIP was repeatedly isolated from two independent screenings of the library. Specificity of the interaction was verified with pGBT9 vectors containing lamin or RAS (kind gifts of Drs. J. Camonis) as unrelated baits. cDNA fragments corresponding to the 65 C-terminal residues of the human bradykinin B2 receptor (kind gift of Dr. W. Müller-Esterl), the last 86 residues of the human β-adrenergic receptor (kindly provided by Dr. R. Fouche, Institut Cochin, Paris) and human cell lines. Proteins (20 μg) were separated on a 10% SDS-PAGE and immunoblotted using rabbit anti-ATIP antibodies was affinity purified by passage through glutathione-agarose beads coupled to the glutathione S-transferase-ATIP fusion protein (obtained by subcloning the 354-bp ATIP-ID cDNA fragment into the pGEX-AT1 (Amersham Biosciences) and purification of glutathione S-transferase-ATIP (as described by the manufacturer). Specificity of anti-ATIP antibodies was confirmed by immunoblotting cell lysates transfected with each ATIP cDNA. A single polypeptide migrating at the expected molecular weight (18,000, 55,000, 55,000, and 50,000 for ATIP-ID, mATIP1, hATIP1, and hATIP2, respectively) was detected in each case.

**Measurement of ERK2 Phosphorylation**—Stably transfected CHO-hAT2 cells (clones L11, L14, V11, V13, C11, and C12) were seeded at a density of 2 × 10⁵ cells/well in 6-well dishes and treated as described (12). Total cell lysates were analyzed by immunoblotting with polyclonal anti-phospho-ERK antibodies (Cell Signaling). Blots were reprobed with monoclonal anti-ERK2 antibodies (UBI) as an internal control, and further incubated with monoclonal anti-phosphostereospecific antibodies (Cell Signaling). Alternatively, total cell lysates were submitted to 10% SDS-PAGE and immunoblotted under conditions (6) that allow to visualize the activated, slower migrating form of endogenous ERK2.

**Transient Expression and Phosphorylation of Tagged ERK2**—For measurement of ERK2 phosphorylation in transient transfections, COS-wt or COS-hAT2 cells were seeded at a density of 3 × 10⁴ cells/well in 6-well dishes and transfected with mATIP1 cDNA or empty vector (1 μg) in 5 μl of FuGENE (Roche) as indicated by the manufacturer. To avoid high background because of stimulation of endogenous ERK2 in non-transfected cells, co-transfections were performed with 0.5 μg of a construct (“ERK2-Myc”) encoding ERK2 fused to six Myc epitopes (a kind gift of Dr. Sabine Traver, Paris). The resulting ERK2-Myc polypeptide was visualized by immunoblotting cell lysates (60 μg) with anti-phospho-ERK antibodies (Cell Signaling) and anti-Myc polyclonal antibodies (4G10, UBI). Alternatively, total cell lysates were submitted to 10% SDS-PAGE and immunoblotted with anti-Myc antibodies (Santa Cruz) to assess expression levels of ERK2-Myc in each lane.

**Measurement of Thymidine Incorporation**—DNA synthesis was assayed by measuring [3H]thymidine incorporation as described (25). Stably transfected CHO-hAT2 cells (clones V11, V13, L11, and L14) were seeded at a density of 2 × 10⁵ cells/well in 24-well dishes (90% density) in Dulbecco’s modified Eagle’s medium for 18 h before appropriate treatment with EGF as indicated (12), then lysed in 60 μl of Laemmli’s sample buffer and analyzed by immunoblotting (20 μl) with anti-phospho-ERK antibodies (Cell Signaling) and anti-Myc polyclonal antibodies (sc-9040, Santa Cruz). Membranes were stripped and reprobed with goat anti-Myc antibodies (sc-7421, Santa Cruz) for internal control.

**Antibody production**—The 354-bp ATIP-ID cDNA fragment was subcloned into the pRSETA vector (Invitrogen) and the resulting polypeptide His6-ATIP fused to six histidine residues, was purified from bacterial lysates by passage through a nickel column as described by the manufacturer. Purified His6-ATIP (100 μg) was injected three times intradermally into rabbits at 2-week intervals for production of polyclonal antisera. Anti-ATIP polyclonal antibodies were affinity purified by passage through glutathione-agarose beads coupled to the glutathione S-transferase-ATIP fusion protein (obtained by subcloning the 354-bp ATIP-ID into pGEX-AT1) and purification of glutathione S-transferase-ATIP (as described by the manufacturer). Specificity of anti-ATIP antibodies was confirmed by immunoblotting cell lysates transfected with each ATIP cDNA. A single polypeptide migrating at the expected molecular weight (18,000, 55,000, 55,000, and 50,000 for ATIP-ID, mATIP1, hATIP1, and hATIP2, respectively) was detected in each case.

**Production of Rabbit Anti-ATIP Polyclonal Antibodies**—The 354-bp ATIP-ID cDNA fragment was subcloned into the pRSETA vector (Invitrogen) and the resulting polypeptide His6-ATIP fused to six histidine residues, was purified from bacterial lysates by passage through a nickel column as described by the manufacturer. Purified His6-ATIP (100 μg) was injected three times intradermally into rabbits at 2-week intervals for production of polyclonal antisera. Anti-ATIP polyclonal antibodies were affinity purified by passage through glutathione-agarose beads coupled to the glutathione S-transferase-ATIP fusion protein (obtained by subcloning the 354-bp ATIP-ID into pGEX-AT1) and purification of glutathione S-transferase-ATIP (as described by the manufacturer). Specificity of anti-ATIP antibodies was confirmed by immunoblotting cell lysates transfected with each ATIP cDNA. The resulting cDNA fragment was subcloned into the pGBT9 vector and expressed in yeast two-hybrid system. Specificity of interaction was verified with pGBT9 vectors containing lamin or RAS (kind gifts of Drs. J. Camonis) as unrelated baits. cDNA fragments corresponding to the 65 C-terminal residues of the human bradykinin B2 receptor (kind gift of Dr. W. Müller-Esterl), the last 86 residues of the human β-adrenergic receptor (kindly provided by Dr. R. Fouche, Institut Cochin, Paris) and human cell lines. Proteins (20 μg) were separated on a 10% SDS-PAGE and immunoblotted using rabbit anti-ATIP antibodies was affinity purified by passage through glutathione-agarose beads coupled to the glutathione S-transferase-ATIP fusion protein (obtained by subcloning the 354-bp ATIP-ID into pGEX-AT1) and purification of glutathione S-transferase-ATIP (as described by the manufacturer). Specificity of anti-ATIP antibodies was confirmed by immunoblotting cell lysates transfected with each ATIP cDNA. A single polypeptide migrating at the expected molecular weight (18,000, 55,000, 55,000, and 50,000 for ATIP-ID, mATIP1, hATIP1, and hATIP2, respectively) was detected in each case.
cDNA or empty vector (0.4 μg/well). Forty-eight hours after transfection, cells (80–90% confluency) were cultured for 48 h in serum-free medium to induce quiescence. For measurement of DNA synthesis, quiescent cells were treated with the indicated growth factor for 40 h and pulsed with 1 μCi/ml [3H]thymidine (PerkinElmer Life Sciences) for an additional 24 h. Cells were washed with ice-cold phosphate-buffered saline and treated as described previously (25) to measure radioactivity of the cell lysate.

RESULTS

Molecular Cloning of ATIP1 from Mouse and Man—The yeast two-hybrid system cloning method was developed to identify novel interacting partners of the AT2 receptor. The last 52 amino acids of the human AT2 receptor were used as a bait to screen 3 × 10⁶ clones of a mouse fetal cDNA library constructed in the pVP16 cloning vector (23). One positive clone containing an insert of 354 bp was repeatedly isolated from two independent screenings of the library. This insert encoded an open reading frame of 118 amino acids that we have designated AT2 receptor-interacting protein-(interacting domain). As shown in Fig. 1A, ATIP-ID interacts with the C-terminal intracellular tail of the AT2 receptor but not with that of the AT1 receptor subtype, nor with C-terminal domains of other GPCR such as β2-adrenergic or bradykinin B2 receptors. The interaction between ATIP-ID and the AT2 C terminus was further confirmed by in vitro binding assays using purified domains of each protein (data not shown).

The 354-bp fragment of ATIP-ID was used as a probe to screen a mouse fetal cDNA library constructed in the pcDNA1 expression vector. A full-length cDNA clone of 1803 nucleotides was isolated (GenBank™ accession number AF173380). This clone contains an open reading frame of 1323 bases that we designated mouse ATIP1 (mATIP1) (Fig. 1B). Several in-frame stop codons were found upstream of the initiating methionine. The human homolog of mATIP1 was further isolated by screening a human adult lung cDNA library, using as a probe a 755-bp cDNA fragment corresponding to the 3′ end of the mATIP1 coding sequence. Full-length human ATIP1 (hATIP1) cDNA encompassed 1977 nucleotides (GenBank™ accession number AF293357) and encodes a 436-amino acid polypeptide that shares 86% amino acid sequence identity with mATIP1. While this manuscript was in revision, a nucleotide sequence (MTSG1) identical to hATIP1 was published in the data banks (GenBank™ accession number AF121259).

Mice and human ATIP1 proteins are mainly hydrophilic and contain no transmembrane domain. The major part of ATIP1 is composed of a large coiled-coil domain (residues 106 to 375 of mATIP1) including two leucine zippers (Fig. 1B). ATIP1 is also characterized by a high proportion of basic residues (16%) and a stretch of 30 C-terminal residues rich in proline, serine/threonine, and arginine (“PSR” region). A BLAST search for homologous proteins in the data banks indicated that ATIP1 is a novel protein that shares 25% amino acid sequence identity with myosins in the coiled-coil region.

Immunoprecipitation experiments were carried out to investigate whether ATIP1 was also able to interact with the AT2 receptor in a cellular context. Full-length mATIP1 cDNA was fused to the Myc epitope and transfected into CHO cells expressing the AT2 receptor. Co-immunoprecipitation experiments (Fig. 1C) revealed constitutive interaction between ATIP1 and AT2, which is not significantly modified upon treatment with Ang II.

A Family of Homologous ATIP Proteins—We then analyzed the tissue distribution of ATIP1 mRNA. The 354-bp fragment of ATIP-ID was used as a probe to hybridize a Northern blot of mRNA from various human tissues (Fig. 2A). Expression of a 1.9-kb transcript likely corresponding to hATIP1 mRNA was detected in all tissues examined. Additional hybridizing transcripts were detected at 4.2 and 6.9 kb in spleen, prostate, ovary, small intestine, colon (Fig. 2A), as well as in heart, placenta, skeletal muscle, pancreas, and lung (data not shown), suggesting the existence of additional mRNAs homologous to ATIP1.

Western blot analysis of total lysates from human tissues and cell lines using a polyclonal antibody directed against the ATIP-ID domain (Fig. 2A, right panel) further confirmed endogenous expression of hATIP1-like proteins. Four major polypeptides of apparent molecular weights 30,000, 60,000, 120,000, and 180,000, respectively, were expressed at variable levels in different tissues. Further work will be required to unambiguously identify each of these polypeptides, and determine the relationship between the various mRNA transcripts and polypeptides detected with the ATIP-ID probe and specific antibodies, respectively.

Nucleotide sequence comparisons with GenBank™ data bases allowed the identification of human cDNA sequences related to hATIP1 in uterus (accession number AL096842), brain (partial sequence, accession number AB033114), and fetal brain (accession number AK125188). All three sequences contained the 354-bp sequence of ATIP-ID and were therefore designated hATIP2 (uterus), hATIP3 (brain), and hATIP4 (fetal brain). Close examination of genomic sequences in the data bases revealed that all four hATIP mRNAs are derived from a single gene by alternative promoter utilization and exon/intron splicing. ²

Translated amino acid sequences of hATIP2, hATIP3, and hATIP4 comprise 415, 1270, and 517 residues, respectively, and are 100% identical to hATIP1 in their last 395 amino acids (Fig. 2B). It is of note that the C-terminal sequence shared by all four hATIP members includes the large coiled-coil domain and leucine zippers as well as the stretch of 118 amino acids (ATIP-ID) that interact with AT2. In the N terminus, hATIP proteins differ both in length and sequence, and exhibit specific motifs that suggest differential intracellular localizations and/or association with distinct cytosolic partners (Fig. 2B). hATIP2 thus exhibits a very short (20 amino acids) N-terminal region that contains a bipartite nuclear localization signal, and three polyproline-rich region (PPXXP) known to play an important role in protein-protein interactions with WW or Src homology 3 domains (28). hATIP3 has a long N-terminal region (874 residues) containing one nuclear localization signal and four polyproline-rich motifs (Fig. 2B). The N-terminal part of hATIP4 (122 amino acids) contains a stretch of 24 hydrophobic amino acids flanked on each side by a charged residue, which is a typical feature of membrane spanning regions. hATIP4 thus likely consists of a transmembrane protein with a short (36 residues) N-terminal extracellular domain and two polyproline-rich motifs at the inner face and close vicinity of the membrane (Fig. 2B).

Dimerization of ATIP Proteins—The presence of a large coiled-coil domain with two leucine zippers in the C-terminal part of all ATIP proteins suggested that these proteins may be able to dimerize. Co-immunoprecipitation experiments were undertaken to investigate this possibility. The cDNAs encoding hATIP1 and hATIP2 were fused either to the Myc or HA epitopes, and co-transfected into COS cells prior to immunoprecipitation with anti-Myc antibodies. As seen in Fig. 3 (left panel), HA-hATIP2 was revealed in anti-Myc immunoprecipitates from cells co-transfected with Myc-hATIP2 and HA-hATIP2 (lane 3) but not from cells transfected either with Myc-hATIP2 or HA-hATIP2 alone (first and fourth lanes).

² M. Di Benedetto, manuscript in preparation.
therefore indicating that hATIP2 homodimerizes inside the cell. Similarly, HA-hATIP1 was detected in anti-Myc immunoprecipitates from COS cells co-transfected with Myc-hATIP1 and HA-hATIP1 (Fig. 3, right panel, fourth lane), but not from cells transfected with either Myc-hATIP1 or HA-hATIP1 alone (first and third lanes), therefore indicating homodimerization of hATIP1. Finally, the ability of hATIP1 and hATIP2 to heterodimerize was demonstrated by co-immunoprecipitation of HA-hATIP1 and Myc-hATIP2 using anti-Myc antibodies (Fig. 3, right panel, second lane).

Inhibitory Effect of mATIP1 on Growth Factor-induced ERK2 Activation—We then sought to examine the functional role of ATIP1, and we first analyzed whether ectopic expression of ATIP1 in eukaryotic cells was able either to block, or mimic, AT2 receptor activation. Our previous studies had demonstrated negative cross-talk between AT2 and growth factor
receptors, leading to an inhibitory effect of AT2 receptor on insulin-induced ERK2 activation and receptor autophosphorylation in CHO-hAT2 cells (12). In the present study, we therefore analyzed the consequence of ectopic ATIP1 expression on growth factor-induced ERK2 activity. CHO-hAT2 cells stably transfected with full-length mATIP1 cDNA and two independent clones (L11 and L14) expressing detectable amounts of mATIP1 migrating at 55 kDa (Fig. 4A) were selected for further studies. Binding studies performed on whole cells using radiodinated ligand [Sar1,Ile8]Ang II revealed that mATIP1 expression in L11 and L14 only slightly modifies the number and affinity of AT2 binding sites as compared with empty vector-transfected clones V11 and V13 (Kd = 0.5 ± 0.16, 0.21 ± 0.03, and 0.25 ± 0.01 nM (n = 3), for V13, L11, and L14, respectively, and Bmax = 2.33 ± 0.58, 1.14 ± 0.15, and 1.04 ± 0.21 fmol/10^6 cells (n = 3), for V13, L11, and L14, respectively).

As seen in Fig. 4B, ERK2 phosphorylation induced by a 5-min treatment with increasing doses of insulin was markedly reduced in clones L11 and L14 when compared with V13. Autophosphorylation of the insulin receptor \( \beta \) chain migrating at 97 kDa was also significantly reduced in L11 and L14 as compared with V13 cells (Fig. 4B). These results therefore indicate that, in the same way as previously reported for the AT2 receptor (12), mATIP1 interferes at the initial step of the insulin receptor intracellular cascade (i.e. insulin receptor autophosphorylation), thereby blocking its downstream signaling.

The inhibitory effect of ATIP1 is not solely restricted to the insulin receptor, as ERK2 activation induced by bFGF was also decreased in clones L11 and L14 as compared with V13 (Fig. 4C). To examine the involvement of the AT2-interacting domain of mATIP1 in this effect, the 118-amino acid fragment corresponding to ATIP-ID was transfected into CHO-hAT2 cells and two independent clones (C11 and C12) were isolated and analyzed. Both clones C11 (Fig. 4D and C12 (not shown) behaved like L11 and L14, showing reduced sensitivity to bFGF and insulin as compared with V13. Taken together, these data indicate that mATIP1 mimics the inhibitory effect of AT2 on growth factor-induced ERK activation and insulin receptor autophosphorylation, and that expression of the sole AT2-interacting domain of ATIP is sufficient to inhibit growth factor receptor-coupled signaling.

The Inhibitory Effect of mATIP1 Requires Expression of the AT2 Receptor—Transient transfections of COS cells were undertaken to further analyze the intracellular effects of mATIP1 expression and avoid any possible bias because of clonal selection of stable transfectants. To eliminate background because of high activation of endogenous ERK2 in response to growth factors, cells were co-transfected with a tagged ERK2-Myc cDNA construct as described under “Experimental Procedures.” Experiments were conducted in parallel in wild-type COS cells (COS-wt) and in COS-hAT2 cells that permanently express the human AT2 receptor (26). As shown in Fig. 5 (left panel), in COS-wt transfected with empty vector, EGF (50 ng/ml) induced early and transient activation of ERK2-Myc being maximal at 10 min. The same time course of ERK2 activation was observed in COS-hAT2 cells transfected with pcDNA3 (Fig. 5, right panel). Transient transfection of mATIP1 into COS-hAT2 cells led to a 50% reduction of EGF-induced ERK2 phosphorylation at 10 min (Fig. 5, right panel), in agreement with our findings on CHO-hAT2 cells (Fig. 4). Transfec-
**Fig. 4.** **mATIP1 inhibits insulin- and bFGF-induced ERK2 activation in CHO-hAT2 cells.**

**A.** Immunoblotting of cell lysates from CHO-hAT2 cells transfected with mATIP1 (L11, L14) or pcDNA3 vector alone (V13) were revealed using polyclonal anti-ATIP antibodies raised against the ATIP-ID domain. Arrow (p55) on the right indicates the migration of mATIP1 in addition to endogenous ATIP from CHO cells. Transfected CHO-hAT2 cells were treated at 37°C for 5 min with the indicated dose of insulin, and whole cell lysates were submitted to 10% SDS-PAGE and analyzed by immunoblotting using anti-phospho-ERK2 antibodies (Cell Signaling). The same blots were stripped and reprobed with anti-ERK2 (UBI) as an internal control of the amount of protein loaded onto each lane. Densitometry scanning was performed using the NIH Image software. Values shown below each lane are expressed as phosphorylation of ERK2 (anti-phospho-ERK2) relative to the amount of endogenous ERK2 in each lane (anti-ERK2).

Membranes were further immunoblotted with anti-phosphotyrosine monoclonal antibodies (4G10, UBI) and the arrow (p97) on the left shows migration of auto-phosphorylated insulin receptor β-chain at 97 kDa.

**B.** Transfected CHO-hAT2 cells were treated for 5 min with the indicated dose of bFGF, and whole cell lysates were immunoblotted using anti-ERK2 antibodies in conditions (6) that allow visualization of the phosphorylated, slower migrating form of ERK2 (indicated by a star). Values shown below are the results of densitometry scanning of the upper band (activated ERK2) relative to that of the lower band (inactive enzyme).

**C.** Transfected CHO-hAT2 cells transfected with empty vector (V13) or ATIP-ID domain (C11) were treated for 5 min with the indicated doses of bFGF (left panel) or insulin (right panel). Total cell lysates were immunoblotted and densitometry values analyzed as described in C. Insulin-treated cell lysates were further immunoblotted with anti-phosphotyrosine antibodies 4G10, as described in B. Results shown in panels B–D are from one representative of three to five independent experiments.
Inhibit insulin-induced ERK2 phosphorylation (Fig. 7). mATIP1-expressing clones L14 and C11, Ang II was still able to activate ERK2 phosphorylation, suggesting that ATIP1 and AT2 work in concert to negatively regulate the insulin receptor pathway.

The effect of mATIP1 on the AT2 receptor response was also investigated in transiently transfected COS-wt and COS-hAT2 cells.
ATIP, a Novel Family of AT2 Receptor-interacting Proteins

DISCUSSION

It is now well established that the Ang II AT2 receptor negatively regulates cell proliferation via atypical signaling pathways that mainly involve activation of tyrosine phosphatases and inhibition of protein kinases (1–4). However, the initial steps of the AT2 signaling cascade leading to growth inhibition still remain to be defined. The present study reports the molecular cloning of a novel AT2 receptor-interacting protein (ATIP1) that selectively interacts with the C-terminal tail of the receptor and inhibits insulin-, bFGF-, and EGF-induced intracellular signaling cascades, in the same way as previously reported for the AT2 receptor (12). Results shown here indicate that ATIP1 constitutively interacts with the AT2 receptor and reveal that expression, but not ligand-induced activation, of the receptor is required for functionality of ATIP1. Furthermore, ATIP1 and AT2 cooperate to inhibit growth factor-induced ERK2 activation and cell proliferation. Altogether these results support a role for ATIP1 as an early mediator of the AT2 receptor inhibitory pathway.

Northern blot experiments and examination of EST sequences in the data banks indicated that ATIP1 is expressed in essentially all tissues examined. This was surprising with regard to the restricted pattern of expression of the AT2 receptor, which is abundantly expressed in fetal tissues but found only at specific sites (adrenal, uterus, brain, and vasculature) in the adult (26, 30). The large tissue distribution of ATIP1 suggests that in addition to its role in the AT2 anti-proliferative pathway, ATIP1 may play other important roles inside the cell, which may be broader than those fulfilled by AT2 receptors.

Molecular studies revealed that ATIP1 is the leader member of a family of at least four proteins (ATIP1 to ATIP4) that all exhibit the same C-terminal domain able to interact with the AT2 receptor. N-terminal parts of different ATIP members diverge both in length and sequence, and carry specific motifs for localization to the nucleus (ATIP2 and ATIP3) or spanning the plasma membrane (ATIP4). It will be interesting to determine whether ATIP2, ATIP3, and ATIP4 also participate in AT2 signaling pathways, and whether their functional roles also depend on the presence of AT2 receptors.

What is the mechanism for ATIP1-mediated inhibition of RTK signaling? We first investigated the role of tyrosine phosphatase SHP-1, a major component of the AT2 signaling pathway involved in inhibition of ERK (6–8), EGF receptor phosphorylation (10), Pyk2 and c-Jun kinase (9), phosphatidylinositol 3-kinase and Akt (11), and JAK2 activity (31). Treatment with 0.1 mM sodium orthovanadate, a potent inhibitor of protein-tyrosine phosphatase activity, had no significant effect on mATIP1-

**Fig. 7.** mATIP1 cooperates with AT2 receptor to inhibit ERK2 and cell proliferation. A, transfected CHO-hAT2 cells were treated for 5 min at 37 °C with (+) or without (−) insulin (1 μg/ml) and Ang II (100 nM). Total cell lysates were analyzed by immunoblotting using anti-phospho-ERK antibodies and anti-ERK2 antibodies as described in the legend to Fig. 4. Values shown below are the results of densitometry scanning of ERK2 phosphorylation (anti-phospho-ERK) relative to the amount of endogenous ERK2 in each lane (anti-ERK2). Shown is one representative of three independent experiments. B, COS-wt (left panel) or COS-hAT2 (right panel) were co-transfected with ERK2-Myc and either empty vector or mATIP1 cDNA, as indicated. Treatments were for 5 min at 37 °C with EGF (50 ng/ml) in the presence (+) or absence (−) of Ang II (100 nM). Western blot analysis of total cell lysates and densitometry scanning were performed as described in the legend to Fig. 5. C, transfected CHO-hAT2 cells were treated for 48 h at 37 °C with (+) or without (−) insulin (1 μg/ml) and Ang II (100 nM), and [3H]thymidine incorporation was measured as described in the legend to Fig. 6. Results are expressed as mean ± S.E. of three independent experiments performed in triplicate.
induced inhibition of ERK activity or cell proliferation in transfected CHO cells (data not shown), therefore ruling out a major effect of a phosphatase activity, including SHP-1, in the inhibitory effect of ATIP1. We further explored the possibility that ATIP1 may function as a scaffold protein to bring SHP-1 at the vicinity of the AT2 receptor, as previously shown for the 5G3 protein (32). However, all attempts to visualize an interaction between ATIP1 and SHP-1, both at basal conditions and after stimulation with Ang II or growth factors, remained unsuccessful (data not shown).

The possibility was also investigated that ATIP1 may directly interact with intracellular parts of RTKs and thus provide a link between AT2 and RTKs. This was suggested by the observation that ATIP1 and AT2 attenuate RTK autophosphorylation, and by recent studies showing that ErbB3, a member of the EGF receptor family, interacts with the AT2 receptor C-terminal portion (20, 21). Experiments performed on transfected CHO-hAT2 and COS-AT2 cells failed, however, to detect direct interaction between ATIP1 and the EGF receptor or the insulin receptor β-chain, either at basal levels or after stimulation with Ang II, EGF, or insulin (data not shown).

Results presented here show that the inhibitory effect of ATIP1 depends on the presence, but not extracellular activation, of AT2 and that ERR2 inhibition is further increased upon Ang II stimulation in ATIP1-expressing cells. One simple explanation would be that ATIP1 directly targets AT2 receptor expression or activation. Radioligand binding studies performed on transfected CHO-AT2 cells revealed that ATIP1 only slightly modifies AT2 receptor binding parameters $K_D$ and $B_{max}$, suggesting that ATIP1 does not function mainly by increasing AT2 receptor expression, accessibility to the membrane, or affinity to the ligand. The possibility remains that by interacting with the C terminus of AT2, ATIP1 may induce “intracellular activation” of the receptor in a ligand-independent manner. Indeed, ligand-independent effects of the AT2 receptor on apoptosis have been demonstrated in various cell types (33). One main feature common to all ATIP members is the presence of a large C-terminal coiled-coil domain that allows homo- and hetero-dimerization of these proteins. This may suggest a role for ATIP1 in AT2 receptor dimerization, a process of major importance for GPCR regulation and function (34, 35). AT2 receptors have indeed been shown to heterodimerize with the AT1 subtype, and thereby inhibit the AT1 intracellular response by a novel mechanism that is independent of ligand/AT2 receptor interaction (36).

Other studies using the two-hybrid system have led to the identification of signaling molecules that interact with the C-terminal tails of other GPCRs including AT1 and AT2 (18–22), and regulate their functions (14, 15, 17). Of interest, although not structurally related, Homer proteins display striking similarities with the ATIP family. Homer is a family of non-transmembrane domain proteins that directly interact with the C-terminal tail of some, but not all, metabotropic glutamate receptors and provide a link with inositol 1,4,5-trisphosphate receptors and calcium signaling (37). Furthermore, members of the Homer family exhibit in their C-terminal portion a large coiled-coil domain allowing their dimerization and have been shown to intracellularly activate metabotropic receptors in an agonist-independent manner (38).

Intracellular cross-talk between GPCRs and RTKs has been extensively studied over the past few years and has been shown in most cases to involve EGF receptor trans-activation (39–41). However, little is known about negative regulation of RTK activity. We report here the cloning of ATIP1, a novel AT2 receptor-interacting protein that functions as an early media-
Trans-inactivation of Receptor Tyrosine Kinases by Novel Angiotensin II AT2 Receptor-interacting Protein, ATIP

Sandrine Nouet, Nathalie Amzallag, Jian-Mei Li, Simon Louis, Isabell Seitz, Tai-Xing Cui, Anne-Marie Alleaume, Mélanie Di Benedetto, Christine Boden, Maryline Masson, A. Donny Strosberg, Masatsugu Horiuchi, Pierre-Olivier Couraud and Clara Nahmias

J. Biol. Chem. 2004, 279:28989-28997.
doi: 10.1074/jbc.M403880200 originally published online April 29, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M403880200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 41 references, 9 of which can be accessed free at http://www.jbc.org/content/279/28/28989.full.html#ref-list-1