Angiotensin II stimulates cellular hypertrophy in cultured vascular smooth muscle and renal proximal tubular cells. This effect is believed to be one of earliest morphological changes of heart and renal failure. However, the precise molecular mechanism involved in angiotensin II-induced hypertrophy is poorly understood. In the present study we report the isolation of a novel angiotensin II type 1 receptor-associated protein. It encodes a 531-amino acid protein. Its mRNA is detected in all human tissues examined but highly expressed in the human kidney, pancreas, heart, and human embryonic kidney cells as well as rat vascular smooth muscle and renal proximal tubular cells. Protein synthesis and relative cell size analyzed by flow cytometry studies indicate that overexpression of the novel angiotensin II type 1 receptor-associated protein induces cellular hypertrophy in cultured rat vascular smooth muscle and renal proximal tubular cells. In contrast, the hypertrophic effects were reversed in renal proximal tubular cell lines expressing the novel gene in the antisense orientation and its dominant negative mutant, which lacks the last 101 amino acids in its carboxyl-terminal tail. The hypertrophic effects are at least in part mediated via protein kinase B activation or cyclin-dependent kinase inhibitor, p27kip1 expression level in vascular smooth muscle, and renal proximal tubular cells. Moreover, angiotensin II could not stimulate cellular hypertrophy in renal proximal tubular cells expressing the novel gene in the antisense orientation and its mutant. These findings may provide new molecular mechanisms to understand hypertrophic agents such as angiotensin II-induced cellular hypertrophy.

Angiotensin II (Ang II) elicits a wide range of physiological responses in a variety of cell types. This octapeptide hormone plays an important role in the regulation of cardiovascular and renal functions via diverse mechanisms (1), which include arteriolar vasoconstriction, stimulation of aldosterone production, and electrolyte homeostasis (2). Although its vasoactive effects were initially considered to be a unique feature for maintaining blood pressure, recent studies have demonstrated that Ang II exerts many other important actions on the cardiovascular and renal systems. For example, Ang II stimulates the growth of diverse cell types, such as vascular smooth muscle cells (VSMC), cardiac myocytes, and proximal tubular cells (3–6), and it increases the expression of enzymes that produce mediators of inflammation (7, 8). These observations suggest that Ang II may play an important role in various cardiovascular and renal diseases associated with abnormal cell growth (cellular hypertrophy or cell proliferation) and inflammation, such as hypertension, congestive heart failure, atherosclerosis, postangioplastic restenosis, and renal failure. Clinical trials and animal studies demonstrated that angiotensin-converting enzyme inhibitors and Ang II receptor blockers prevented vascular, left ventricular, and renal proximal tubular cellular hypertrophy (9–13), supporting the importance of Ang II in the pathogenesis of cardiovascular and renal diseases. In vitro, Ang II has also been shown to stimulate the growth of VSMC and renal proximal tubular cells (hypertrophic effect) as well as cardiac myocytes and fibroblasts (hyperplastic action) (14–17).

The physiological actions of Ang II are mediated by two major subtypes of G-protein-coupled receptors termed AT1 and AT2 (18–22). Most known actions of Ang II are believed to be mediated through AT1 receptors, whereas the function of AT2 receptors may be the opposite of AT1 receptors (23–26). Like most G-protein-coupled receptors, the AT1 receptor exerts its function through its carboxyl-terminal domain in addition to its intracellular cytoplasmic loops. The carboxyl-terminal domain of the AT1 receptor has been reported to directly interact with several downstream effectors (10–16). Mutagenesis of this region has shown that discrete amino acids are required for receptor desensitization and internalization (27–34). As for many G-protein-coupled receptors, the carboxyl-terminal domain of the AT1 receptor presumably interacts with G-protein-coupled receptor kinases and arrestins, causing the functional desensitization of the receptor (35–33). Two novel proteins, ATRAP and ARAP1, have recently been isolated by us and another using the yeast two-hybrid smooth muscle cell; GEF, GDP/GTP exchange factor; GLP, GEF-like protein; IRPTC, immortalized renal proximal tubular cell; GFF, green fluorescence protein; PBS, phosphate-buffered saline; DPI, diphenylenedione; sodium chloride; PKB, protein kinase B; PI, kinase; phosphatidylinositol 3-kinase; kb, kilobase(s); cdk, cyclin-dependent kinase; HEK cells, human embryonic kidney cells.
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system (35–37). ATRAP is a small protein that interacts specifically with the carboxyl-terminal domain of the AT1 receptor (35). Overexpression of ATRAP causes a marked inhibition of the AT1 receptor-mediated activation of phospholipase C. Further studies also demonstrate that ATRAP overexpression enhances AT1 receptor internalization and inhibits cell growth in VSMCs (36). On the other hand, ARAP1 is another protein that was found to interact with the carboxyl-terminal domain of the AT1 receptor (37). Characterization of ARAP1 in HEK-293 cells has revealed that ARAP1 binds and promotes the AT1 receptor to the plasma membrane, indicating its role in the receptor recycling pathway. The Ang II-induced hypertrophic effect is associated with increased expression of the proto-oncogenes c-fos, c-myc, and c-jun and autocrine growth factors PDGF-AA and bFGF and activation of extracellular signal-regulated kinase 1/2 mitogen-activated protein kinases in cultured VSMCs (38, 39). Despite the identification of these proto-oncogenes and mitogens, Ang II does not induce hyperplasia but instead causes hypertrophy in confluent quiescent VSMCs in serum-free medium. Recent studies on the molecular mechanisms of Ang II-induced VSMC and mesangial cell hypertrophy showed they are mediated at least partially at the level of the molecular machinery regulating cell-cycle progression (40), protein kinase B (PKB) (41, 42), and NADH/NADPH oxidase pathways (43). However, the precise molecular mechanisms of cellular hypertrophy remain poorly understood. In the present study we describe the identification of a novel GDP/GTP exchange factor (GEF)-like protein, GLP, and the characterization of the GLP gene on cell growth in cultured VSMCs and immortalized renal proximal tubular cells (IRPTCs). We report here that GLP induces cellular hypertrophy in both VSMC and IRPTC-overexpressing GLP genes.

EXPERIMENTAL PROCEDURES

Materials—Phospho-specific (Ser-473) antibody against Akt2 and antibody against Akt2 were purchased from Cell Signaling Technology Inc., Beverly, MA. p27kip1 antibody was from Transduction Laboratories Inc., Mississauga, Ontario, Canada. Antibody against β-actin, Na3VO4, aprotinin, leupeptin, phenylmethylsulfonyl fluoride, pepstatin, and diphenylethionon (DPI) were purchased from Sigma. 9E10 monoclonal antibody against Myc epitope tag and fetal bovine serum were from Invitrogen. LY294002 was obtained from Calbiochem. Yeast Two-hybrid Screening—The yeast two-hybrid system was carried out essentially as described previously (44). Briefly, the bait plasmid was pB7M116, expressing the carboxyl-terminal 64-amino acid residues (295–359) of rat AT1a receptor in-frame with the Lex A DNA binding domain (pB7M116-AT1a). The L40 yeast strain was transformed with pB7M116-AT1a and subsequently with a YEp16 10.5-day mouse embryo cDNA library.

Plasmids—To overexpress the GLP gene in VSMCs and IRPTCs, human GLP cDNA was subcloned into a retroviral vector, pLNCX (45, 46), and pcDNA3, respectively. To facilitate detection by Western blot analysis, GLP was tagged with an Myc epitope tag (MGREEQKQL4-SEEDLL) followed by the GLP sequence (pcDNA3-Myc-GLP). A dominant negative mutant of GLP, lacking the last 101 amino acids, was created with an addition of a stop codon at position 429 (pcDNA3-Myc-GLP-M1). GLP in antisense orientation was also produced (pcDNA3-GLP-AS) to reverse the effects observed in GLP overexpression in IRPTCs. For cellular localization experiments, GLP was transiently transfected into the medium. Cells were washed twice with PBS and fixed with 37 °C under 5% CO2. To establish a VSMC line—

Flow Cytometry Analysis—For measurement of forward-angle light scatter, cells were harvested by trypsinization, fixed with 75% methanol, washed, and incubated with 100 μg/ml RNase and 10 μg/ml propidium iodide in PBS at 1 h at 37 °C. Samples were analyzed using standard methods on a FACScan flow cytometer (BD Biosciences). Data was computer analyzed with Cell Quest Pro software (BD Biosciences).

Northern Blot Analysis—Total RNA was extracted from different human tissues and cells by a modified version of the guanidinium thiocyanate procedure. Poly(A)+ mRNAs were then extracted with an oligotex mRNA kit (Qiagen). Two μg of poly(A)+ mRNA were resolved by electrophoresis on a 1.2% agarose gel containing 1.8% formaldehyde, transferred to a Hybond-N membrane, fixed, and hybridized with a 32P-labeled probe. Full-length human GLP cDNA was used as a probe. After exposition, the membrane was stripped and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase probe. Microscopy Analysis—HEK-293 cells expressing pcDNA3-GFP-GLP were grown on glass coverslips and fixed in 4% paraformaldehyde. The cells were visualized and photographed under a Zeiss microscope with Eastman Kodak Co. film.

5HThymidine Incorporation—To determine cell proliferation, DNA synthesis was measured as described previously (49). Briefly, 50–80% confluent cells were washed with PBS and incubated in serum-free medium for 48 h for VSMCs or 24 h for IRPTCs, respectively. Four hours before harvesting the cells, 0.25 μCi/ml 5Hthymidine was added into the medium. Cells were washed twice with PBS and fixed with ethanol/acetic acid (3:1, v/v), and the acid-insoluble material was precipitated with ice-cold perchloric acid. Then the DNA was extracted in 1 ml of 0.5 N perchloric acid at 80 °C. Supernatants were transferred to plastic tubes and counted in a scintillation counter. The experiments were performed in triplicate and repeated at least three times.

5HLeucine Incorporation—To determine cellular hypertrophy, 5Hleucine incorporation was measured. Cells were grown in 24-well plates made quiescent in serum-free medium for 48 h for VSMCs or 24 h for IRPTCs. Cells were pulses with 0.5 μCi/ml 5Hleucine for 24 h, then three times with PBS. Cellular proteins were collected at 10 min at 4 °C with trichloroacetic acid, and lipid fractions were solubilized by washing twice with ice-cold ethanol. Precipitated proteins were resuspended in 0.25 N NaOH, transferred to plastic tubes, and counted in a scintillation counter. The experiments were performed in triplicate and repeated at least three times.

Acetylation of Histone H3—Acetylation of histone H3 was determined using the antibody H3K9Ac (Cell Signaling Technology). Immunoprecipitation was performed with the antibody and protein A agarose beads as described previously (49). 

To determine positive IRPTC lines expressing pcDNA-Myc-GLP and its derivatives, ARAP1, and ARAP2 were purchased from Cell Signaling Technology. 

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FIG. 1. A, sequence of human GLP cDNA and its predicted amino acid sequence. B, comparison of seven tandem repeats motif with their amino acid sequences. C, structure comparison of human GLP and RCC1 genes. GLP contains a seven tandem repeats motif similar to that of RCC1 protein. RCC1 is a GEF for small G-protein Ran, which belongs to the Ras small GTPase superfamily. The number of amino acids in each tandem repeat is indicated. The putative AT1 receptor binding site is also shown in the carboxyl-terminal domain of GLP gene.

### A. Sequence of Human GLP cDNA and Predicted Amino Acid Sequence

| Position | Amino Acid | Appearance |
|----------|------------|------------|
| 51       | Gly         |             |
| 53       | Gly         |             |
| 53       | Phe         |             |
| 53       | Phe         |             |
| 53       | Asp         |             |
| 53       | Asp         |             |
| 164      | Ser         |             |

### B. Comparison of Seven Tandem Repeats Motif with Amino Acid Sequences

#### Consensus Sequence

- GQLG...N Q...P.RV...L.K.V...A CG...H....T GEYW..W...N

#### Seven Tandem Repeats

| Clone | Amino Acid |
|-------|------------|
| 371   | Gly         |
| 351   | Ser         |
| 347   | Thr         |
| 343   | Thr         |
| 341   | Thr         |
| 332   | Ser         |
| 324   | Ser         |
| 321   | Ser         |
| 316   | Asp         |
| 314   | Asp         |
| 312   | Asp         |
| 307   | Asp         |
| 304   | Asp         |
| 303   | Asp         |
| 300   | Asp         |

### C. Structure Comparison of Human GLP and RCC1 Genes

- GLP contains a seven tandem repeats motif similar to that of RCC1 protein. RCC1 is a GEF for small G-protein Ran, which belongs to the Ras small GTPase superfamily.

#### Table

| Clone | Amino Acid |
|-------|------------|
| 1     | Ser         |
| 51    | Ser         |
| 53    | Ser         |
| 53    | Ser         |
| 53    | Ser         |
| 53    | Ser         |
| 157   | Ser         |
| 210   | Ser         |
| 262   | Ser         |
| 262   | Ser         |
| 262   | Ser         |
| 531   | Ser         |

#### Diagram

- The putative AT1 receptor binding site is also shown in the carboxyl-terminal domain of GLP gene.
against glutathione S-transferase-GLP fusion protein, which consists of 101 amino acid residues in the carboxyl-terminal tail of GLP, were produced. Proteins were visualized by enhanced chemiluminescence detection system (Amersham Biosciences). The relative densities of Akt2/PKB, β27-kDa, and β-actin bands were determined with a computerized laser densitometer. We analyzed the data quantitatively by applying a commercially available system (Kodak 1D image analysis software).

Cellular Protein Content—To assess the cellular protein content, the cells were rendered quiescent for 48 h and then harvested with 0.05% EDTA. The number of cells per well was counted and lysed in 100 μl of 2 M NaOH, and cellular protein content was determined by protein assay kit (Bio-Rad). Bovine serum albumin was used as a standard. Cellular protein content is an indicator of cellular hypertrophy, as shown by Mackovic-Basic et al. (50).

GLP mRNA Expression—To determine GLP mRNA expression, a specific RT-PCR was performed using the primer sets 5'-AGTCATGG-TGGATGTCCGAAA-3' (sense) and 5'-ATGAGCTCCACAGCCTTCCTC-3' (antisense), corresponding to the nucleotide sequences of -4 to +17 and +393 to +414 of human GLP cDNA, respectively. Total RNAs were extracted with TRIzol reagent, (Invitrogen) according to the protocol of the supplier and quantified by absorbance at 260 nm, and were used in RT-PCR to quantify the amount of GLP mRNA expressed in IRPTCs. PCR reactions were performed for 5 min at 95 °C initial denaturing followed by 32 cycles of 95 °C for 30 s, 54 °C for 45 s, 72 °C for 30 s, and 72 °C for 5 min in the thermal cycler (MJ Research Inc.). The GLP mRNA expression was normalized with β-actin mRNA expression with the primer sets of 5'-ATGAGCACTCCTGCTGACCTGGC-3' (sense) and 5'-AGCATTTGCGTGCACGATGGAGG-3' (antisense), corresponding to the nucleotide sequences of +155 to 139 of exon 3 and nucleotide sequences of +155 to +139 of exon 5 of the rat β-actin gene, respectively (51).

Statistical Analysis—Three to thirteen separate experiments were performed, and each treatment group was assayed in triplicate unless indicated. The data were analyzed with the Student’s t test or analysis variance. A probability level of p < 0.05 was considered as statistically significant.

RESULTS

Identification of GLP Gene—To identify proteins that interact with the carboxyl-terminal domain of the AT1A receptor, a yeast two-hybrid screen was performed in a system that included the AT1A carboxyl terminus domain (residues 295–359) fused to the VP16 transactivation domain. One positive clone, A8, which contained 162-amino acid residues of mouse GLP (residues 371–533), was isolated. Clone A8 was subsequently retested in the yeast two-hybrid assay with the truncated AT1A receptor (residues 295–318). Although the entire AT1A carboxyl terminus domain (residues 295–359) interacted with mouse clone A8, the truncated fragment AT1A-T318, consisting of residues 295–318, failed to do so. The result indicates that the region between residues 319 and 359 of AT1A is required for interaction with the GLP gene in the yeast two-hybrid system.

A total of 23 clones were subsequently isolated from a λgt11 human VSMC primary cDNA library employing mouse clone A8 as a probe. A 2.6-kb clone has a putative ATG start codon at position 228 and contains an open reading frame of 1593 bp encoding a hydrophilic protein of 531 amino acid residues with a calculated molecular mass of 58,564 daltons (Fig. 1A). The mobility of the in vitro translation product was in agreement with the molecular mass predicted for the 2.6-kb clone (data not shown). An amino acid sequence database search found a similar human cDNA from GenBank™ (accession numbers AF334406, AJ319660, AK096654, AL838231, and NM_018191). There are three amino acid differences between the 2.6-kb clone and the published amino acid sequence from GenBank™; valine 239 is changed to phenylalanine, tyrosine 242 to asparagine, and threonine 245 to asparagine. Analysis of the 2.6-kb clone amino acid sequence revealed that it contains a motif of seven tandem repeats similar to that of RCC1 protein. RCC1 has been identified as a GEF for Ran small G-protein (52–57). Fig. 1B shows the amino acid comparison between the seven tandem repeats of GLP protein.

We believe that the 2.6-kb clone is a novel GEF-like protein and putatively named the 2.6-kb clone as GLP. Comparison of GLP and RCC1 protein revealed that the GLP protein has an additional carboxyl tail (amino acid residues 368–531) in addition to the seven tandem repeat motif (Fig. 1C). We found that the amino acid residues between 371 and 531 of the GLP protein are important for the interaction with the carboxyl-terminal domain of the AT1 receptor. This may explain why GLP protein has an additional carboxyl tail, whereas RCC1 protein does not.

Tissue and Cell Distribution of GLP Gene—Northern blot analysis showed that GLP mRNA is expressed in the human heart, brain, lung, liver, kidney, pancreas, skeletal muscle, and placenta but is particularly abundant in the kidney, pancreases, and heart (Fig. 2A). GLP mRNA was detected in cultured rat VSMCs and IRPTCs and human HEK-293 cells (Fig. 2B). Only a single GLP transcript of about 4.0 kb was found in the human tissues and cell lines. The 4.0-kb size is in agreement with the
human cDNA published in GenBank™. This may explain why the 2.6-kb clone lacks both a polyadenylation signal AAUAAA sequence as well as a poly(A) tail in the 3’-untranslated terminus. GLP protein expression was also detected in rat VSMCs and IRPTCs and human HEK-293 cells analyzed by Western blotting with polyclonal antibody against the last 101 amino acids of GLP (Fig. 2C). The size of bands detected by Western blot analysis is about 58 kDa, which is similar to the molecular mass predicted from its protein sequence.

**Cellular Localization of GLP Gene**—To determine the intracellular localization of GLP gene, HEK-293 cells stably expressing pcDNA3-GFP-GLP were used. As shown in Fig. 3, GFP-GLP protein was mainly localized in small vacuoles in the cytoplasm and also clearly on the plasma membrane but not in the nucleus.

**Hypertrophic Effects of GLP Gene in VSMCs**—Although several groups have reported the GLP sequence to the GenBank™, little is known about the function of this novel gene. To determine the biological roles of the GLP gene, VSMCs stably expressing pLNCX-GLP (GLP) and empty vector, pLNCX (control), were established. The morphology of VSMCs expressing pLNCX or pLNCX-GLP after viral infection was not changed, but VSMCs expressing pLNCX-GLP (Fig. 3C) seem to be larger than control cells expressing pLNCX alone (Fig. 3B). Confluent, quiescent VSMCs were used to examine whether GLP regulates cell growth. Fig. 4A shows that [³H]thymidine incorporation, an indicator of new DNA synthesis, decreased by 32% in VSMCs overexpressing the GLP gene compared with the control cells. However, [³H]leucine incorporation, a measure of protein synthesis and also an indicator of cellular hypertrophy, significantly increased by 238% in VSMCs overexpressing the GLP gene when compared with the control cells (Fig. 4B). The hypertrophic effects of the GLP gene in VSMCs were further demonstrated by a rightward shift of the forward-angle light scatter, a relative measure of cell size on flow cytometry (data not shown). The relative cell size, as arbitrary units, increased by 178% in VSMCs overexpressing the GLP gene compared with the control cells (Fig. 4C). In parallel experiments, Ang II increased in relative cell size by only 112% in VSMCs stimulated with 100 nM Ang II for 48 h (data not shown). As a third hypertrophic marker, total protein content was measured. Fig. 4D shows that total protein content was 2.2-fold higher in VSMCs overexpressing GLP than in control cells. These results demonstrate that overexpression of GLP gene induces VSMC hypertrophy.

**Effect of the GLP Gene on Phosphorylation of Akt2 in VSMCs**—Activation of Akt2/PKB has been shown to play an important role in cellular hypertrophy in VSMCs and mesangial cells. To explore the signaling pathways involved in GLP-induced VSMC hypertrophy, phosphorylation of Akt2/PKB was determined by Western blot analysis. As shown in Fig. 5A, Akt2/PKB was significantly more activated in VSMCs overexpressing the GLP gene than in the control cells. Activation of Akt2/PKB increased by 2.2-fold in VSMCs overexpressing the
GLP gene compared with the control cells (Fig. 5B). Because the levels of cellular p27kip1, an inhibitor for cyclin-dependent kinase (cdk), have been demonstrated to be indicators of cellular hypertrophy in VSMCs and murine and rat proximal tubular cells, cellular p27kip1 levels were also determined by Western blot analysis (Fig. 5C). Cellular p27kip1 protein expression decreased by 12% in VSMCs overexpressing the GLP gene compared with the control cells (Fig. 5D). These data suggest that the activation of Akt2/PKB is involved in VMSC hypertrophy induced by GLP gene overexpression but that cellular p27kip1 is not.

Effects of a PI3 Kinase Inhibitor, LY294002, and NADH/NADPH Oxidase Inhibitor, DPI, in VSMCs Overexpressing the GLP Gene—Akt2/PKB is stimulated by a number of receptor-tyrosine kinases, Ang II, and insulin by activation of PI3 kinase. Interestingly, Akt2/PKB is also activated through a PI3 kinase-independent but reactive oxygen species-dependent pathway. To further explore upstream signaling events of ac-
tivation of Akt2/PKB, VSMCs overexpressing the GLP gene were treated with LY294002, a PI3 kinase inhibitor, and DPI, an NADH/NADPH oxidase inhibitor, then activation of Akt2/PKB and relative cell size were determined. As shown in Fig. 6A, phosphorylated Akt2/PKB was significantly reduced in the cells treated with both inhibitors compared with the untreated cells, as examined by Western blot analysis. Activation of Akt2/PKB was decreased by 72% in the cells treated with LY294002 and 50% in the cells treated with DPI, respectively, compared with the untreated cells (Fig. 6C).

**Hypertrophic Effects of the GLP Gene in IRPTCs**—IRPTCs have been used as in vitro model to examine the molecular mechanism on Ang II-induced cellular hypertrophy. To confirm the cellular hypertrophic effects of the GLP gene observed in VSMCs, four IRPTC lines stably expressing pcDNA3 (Control), pcDNA3-Myc-GLP (GLP), GLP deletion mutant M1 pcDNA3-Myc-GLP-M1 (GLP-M1), GLP in antisense orientation (GLP-AS), and pcDNA3 (Control). IRPTC lines expressing pcDNA3-Myc-GLP (GLP) and its mutant M1 (GLP-M1) were determined with Western blot analysis using 9E10 monoclonal antibody against Myc epitope tag sequences (A). IRPTC lines expressing GLP in the antisense orientation (GLP-AS) were determined by RT-PCR as described under “Experimental Procedures”; GLP mRNA expression in control and GLP cells are also shown (B).

![Fig. 6](image6.png)

**Fig. 6.** Effect of LY294002 and DPI on phosphorylation of Akt2 and relative cell size in VSMCs overexpressing GLP gene. To determine upstream signaling pathways of activation of Akt2/PKB, a PI3 kinase inhibitor, LY294002, and an NADH/NADPH oxidase inhibitor, DPI, were used in VSMCs overexpressing the GLP gene. A shows a representative Western blotting result of phosphorylation of Akt2/PKB. B shows a histogram of percentage decrease by LY294002 and DPI on phosphorylation of Akt2 in VSMCs overexpressing the GLP gene, LY294002 (n = 3; *, p < 0.001), and DPI (n = 3; **, p < 0.001), respectively. C shows the effect of LY294002 and DPI on the relative cell size measured by flow cytometry, LY294002 (n = 3; *, p < 0.05) and DPI (n = 3; **, p < 0.001), respectively. All experiments were performed in duplicate.

![Fig. 7](image7.png)

**Fig. 7.** Establishment of IRPTC lines stably expressing pcDNA3-Myc-GLP (GLP), GLP deletion mutant M1 pcDNA3-Myc-GLP-M1 (GLP-M1), GLP in antisense orientation (GLP-AS), and pcDNA3 (Control). IRPTC lines expressing pcDNA3-Myc-GLP (GLP) and its mutant M1 (GLP-M1) were determined with Western blotting with 9E10 monoclonal antibody against Myc epitope tag sequences (A). IRPTC lines expressing GLP in the antisense orientation (GLP-AS) were determined by RT-PCR as described under “Experimental Procedures”; GLP mRNA expression in control and GLP cells are also shown (B).
IRPTCs expressing GLP were 117% larger than control IRPTCs, but no changes were observed in IRPTCs expressing GLP in the antisense orientation (GLP-AS), and GLP mutant M, pcDNA3-Myc-GLP-M1 (GLP-M1) were serum-starved for 24 h, then incubated with [3H]thymidine or [3H]leucine for 4 or 24 h before harvesting the cells. [3H]Thymidine (A) and [3H]leucine (B) incorporation experiments were performed (n = 5; *, p < 0.01). Relative cell sizes were determined with flow cytometry (C) (n = 4; *, p < 0.05). Total protein content experiments were shown in D (n = 4; *, p < 0.01).

Effect of Ang II in Different IRPTC Lines—Ang II has been implicated to induce cellular hypertrophy in cultured RPTCs (15, 58, 59). To explore whether GLP plays an important role in GLP overexpression induces p27kip1 protein expression but not activation of Akt2/PKB in different IRPTC lines. A, a representative Western blot of phosphorylated Akt2/PKB in different IRPTC lines, pcDNA3 (Control), pcDNA3-Myc-GLP (GLP), GLP in the antisense orientation (GLP-AS), and GLP mutant M1 (GLP-M1); all blots were quantified by laser densitometry (B). C, a representative Western blot of p27kip1 protein expression, and all blots were quantified by laser densitometry (D). All experiments were performed four times; *, p < 0.01.
Ang II-induced hypertrophy in IRPTCs, three IRPTC lines expressing pcDNA3 (Control), GLP in the antisense orientation (GLP-AS), and GLP mutant M1 (GLP-M1) were used, and [3H]leucine incorporated, relative cell size, and total protein content were studied. As shown in Fig. 10A, Ang II increased [3H]leucine incorporation by 36% in control cells compared with unstimulated cells but failed to increase [3H]leucine incorporation in the cells expressing GLP in the antisense orientation (GLP-AS) and GLP mutant M1 (GLP-M1). Ang II also increased relative cell size by 8.9%, analyzed by flow cytometry, as well as total protein content by 46% in control cells but failed to do so in IRPTCs expressing GLP in the antisense orientation (GLP-AS) and GLP mutant M1 (GLP-M1). These data suggest that the carboxyl-terminal tail of GLP is required for Ang II-induced cellular hypertrophy in IRPTCs, and GLP expression level is essential for Ang II-induced cellular hypertrophic effect in IRPTCs.

Ang II Stimulates GLP mRNA Expression in IRPTCs—We further explored whether Ang II regulates GLP gene expression in IRPTCs. IRPTCs were treated with 100 nM Ang II for the indicated time periods, GLP mRNA expression was determined by a specific RT-PCR, and its expression was normalized with β-actin mRNA expression. As shown in Fig. 11, A and B, Ang II stimulated GLP mRNA expression in a time-dependent manner. The maximal expression of GLP mRNA was seen about 16 h after Ang II stimulation. The GLP mRNA expression stimulated by Ang II is also dose-dependent as shown in Fig. 11, C and D. These data demonstrate that Ang II-induced cellular hypertrophy in IRPTCs may be due to increase GLP gene expression. To determine which Ang II receptor is involved in Ang II-stimulated GLP mRNA expression in IRPTCs, specific receptor antagonists losartan and PD123319 (10−6 m) were added into medium with Ang II for 8 h, and GLP mRNA expression was examined. As shown in Fig. 11, E and F, losartan, but not PD123319, reversed Ang II-induced GLP mRNA expression, indicating that the AT1 receptor is specifically involved in the regulation of GLP mRNA expression in IRPTCs.

Discussion

In this study we identified a novel GEF-like protein, GLP gene, by a yeast two-hybrid screening with the carboxyl-terminal domain of the AT1 receptor as “bait.” We demonstrate that overexpression of GLP gene induces cellular hypertrophy in two different rat cell lines, VSMCs and IRPTCs, by five parameters as follows. 1) Protein content of VSMCs and IRPTCs expressing the GLP gene was significantly higher than the control cells, 2) Relative cell size measured by flow cytometry was significantly bigger in VSMCs and IRPTCs overexpressing the GLP gene than in the control cells, 3) The ratio of [3H]leucine/[3H]thymidine incorporation, a cellular hypertrophy indicator, was significantly higher in VSMCs and IRPTCs overexpressing the GLP gene than in the control cells, 4) A greater activation of Akt2/PKB was seen in VSMCs overexpressing the GLP gene than in the control cells, and 5) Cellular p27kip1 protein level significantly increased in IRPTCs overexpressing the GLP gene compared with IRPTCs transfected with pcDNA3 alone. We also demonstrate that the carboxyl-terminal tail of GLP gene (430–531 amino acids) not only plays an important role in GLP-induced IRPTC hypertrophy but is also required for Ang II-induced IRPTC hypertrophy.

An amino acid sequence analysis revealed that GLP protein contains a motif similar to that of the RCC1 gene, which is a GEF for small G protein Ran (52–57). In addition to a sev-ent tandem-repeat motif, GLP has an extra carboxyl-terminal tail that the RCC1 gene does not possess (Fig. 1C). The carboxyl-terminal region of GLP gene (amino acids 371–531) was identified to interact with the carboxyl-terminal domain of the AT1 receptor in the yeast two-hybrid system. The GFP-GLP fusion protein is localized in part in the plasma membrane, and the AT1 receptor has been shown to localize in the plasma membrane. These data suggest that the GLP protein may co-localize with the AT1 receptor and regulate receptor functions.

Because the carboxyl-terminal domain of the AT1 receptor has been reported to play an important role in receptor internalization, desensitization, and phosphorylation (30–35), we examined whether GLP regulates the receptor function in cultured HEK-293 cells and VSMCs. Our studies revealed that overexpression of GLP gene failed to change the receptor- ligand binding activity in both cell lines (data not shown). It also did not affect the AT1 receptor internalization and desensitization in VSMCs overexpressing the GLP gene and in HEK-293 cells co-transfected with the AT1 receptor and GLP gene (data not shown), suggesting that it may not regulate the receptor endocytosis process, but it may regulate downstream signaling pathways after the activation of the receptor.

We then investigated whether the GLP gene regulates cell growth in VSMCs. It is well known that the activation of the AT1 receptor stimulates several key signaling molecules involved in cell growth, such as mitogen-activated protein kinase, extracellular signal-regulated kinase 1/2, Akt2/PKB, and the cell cycle machinery (38–43). In the present study the activa-
tion of extracellular signal-regulated kinase 1/2 was not changed in VSMCs and IRPTCs overexpressing the GLP gene compared with the control cells (data not shown). However, Akt2/PKB was significantly activated in VSMCs overexpressing the GLP gene (Fig. 5A). Akt2/PKB is a serine/threonine protein kinase that contains a region homologous to a pleckstrin domain that is part of a slightly larger portion in the NH2 terminus, termed the Akt homology domain. The PI 3 kinase product phosphatidylinositol 3,4-bisphosphate binds in vitro directly to the pleckstrin domain and activates enzyme activity (60). Akt/PKB has been shown to be activated by factors that stimulate PI3 kinase, including insulin-like growth factor, nerve growth factor, platelet-derived growth factor, and vascular endothelial growth factor (a number of receptor tyrosine kinases), Ang II (G-protein-coupled receptors), and insulin (60–62). Interestingly, Ang II has recently been reported to activate Akt2/PKB in a PI3 kinase-independent but arachidonic acid-dependent and redox-sensitive signaling pathway (41, 63–66). Taken together, these data suggest that activation of Akt2/PKB is not only dependent on PI3 kinase activation but also on reactive oxygen species signaling pathways. In the present study we found that both LY294002, a PI3 kinase inhibitor, and DPI, an inhibitor of NADH/NADPH oxidase, significantly decreased the relative cell size measured by flow cytometry and Akt2/PKB phosphorylation in VSMCs overexpressing the GLP gene (Fig. 6), supporting that the activation of Akt2/PKB is not only dependent on PI3 kinase activation but also on the NADH/NADPH oxidase-signaling pathway. Akt2/PKB has diverse regulatory functions. It promotes glucose transport through translocation of GLUT1 and GLUT4 to the plasma membrane and controls glycogen synthesis by an insulin-dependent phosphorylation. These data demonstrate that Akt2/PKB promotes cell growth by acting at multiple stages of the cell cycle. Overexpression of Akt2/PKB in VSMCs has been demonstrated to induce hypertrophy and polyploidization (42).

On the other hand, the cell cycle molecular machinery has also been implicated to play an important role in cellular hypertrophy in VSMCs and IRPTCs (40, 67–69). Cell cycle entry and progression, the final common pathway of cell-growth response, depends on the carefully regulated expression and ac-
activation of certain proteins, termed cyclin-dependent kinases (cdk), and their regulatory subunits, the cyclins (40, 70). For the G1 phase, cyclin D, cyclin E, and cyclin A play an important role (71). Cyclin D complexes with cdk4/cdk6 and regulates G1-phase progression, cyclin E/cdk2 or cyclin A/cdk2 is essential for the G1/S transition, and cyclin A/cdk1 or cyclin B/cdk1 or 2 initiates mitosis (72). Cdk5 are activated through phosphorylation and dephosphorylation at specific sites, and this activation is controlled by cdk inhibitors that bind to and inhibit the activation of the cdk-cyclin complex. In cellular hypertrophy, cell cycle entry takes place, but the progression through the cell cycle toward DNA synthesis and mitosis is blocked. Among cdk inhibitors, p27kip1 has been shown to play an important role in cell cycle regulation. High levels of p27kip1 present in quiescent (G0) cells have been shown to decline upon mitogen induction (73). This decrease in p27kip1 appears to be critical in enabling the cells to enter the cell cycle. Cellular p27kip1 levels have been shown as an indicator of cellular hypertrophy in VSMCs (40) and murine and rat proximal tubular cells (63–65). Furthermore, p27kip1 regulates growth arrest in response to transforming growth factor, rapamycin, and contact inhibition. Thus, studies of p27kip1 expression combined with assessment of relative cell size by flow cytometry and [3H]leucine incorporation will give a better assessment of the hypertrophic effect induced by GLP gene. Interestingly, cellular p27kip1 level was not changed between VSMCs overexpressing the GLP gene and the control cells, suggesting that the hypertrophic effect of GLP gene is independent of p27kip1 expression in VSMCs. However, cellular p27kip1 expression significantly increased in the IRPTC-overexpressing GLP gene, indicating that, unlike in VSMCs, the hypertrophic effect of GLP gene is dependent on cell cycle regulation in IRPTCs. IRPTCs were chosen as a second in vitro model to investigate the hypertrophic effect of the GLP gene in addition to VSMCs. The experiments are designed to eliminate the possibility of side effects due to retroviral proteins, which were used to infect VSMCs in order to deliver the plasmid DNA into the cells. Although the relative cell size of the control cells (pLNCX) measured by flow cytometry was the same as that of uninfected VSMCs (data not shown), the hypertrophy observed could not be completely ruled out as a rival protein's side effect. The results obtained from RPTCs overexpressing the GLP gene with increased [3H]leucine incorporation combined with a higher p27kip1 expression clearly demonstrated that the cellular hypertrophic effect was dependent on GLP gene overexpression. Because the mRNA components of the renin-angiotensin system including angiotensinogen, rennin, angiotensin-converting enzyme, and AT1 receptor are all expressed in murine (rat and mouse) proximal tubular cell lines, IRPTCs can be used to further investigate the molecular mechanisms of Ang II- or high glucose-induced hypertrophy. In the present study, we demonstrated that Ang II, as a hypertrophic agent, indeed significantly stimulates GLP mRNA expression in rat IRPTCs. Similar results were observed in rat VSMCs and kidney mesangial cells. These data indicate that GLP gene expression may be important in Ang II-induced cellular hypertrophy in cultured cells.

In summary, we have identified a novel GEF-like protein, GLP gene, that induces cellular hypertrophy in cultured VSMCs and IRPTCs. The signaling pathways involved in the cellular hypertrophic effect of GLP gene are different in these two cell types. In VSMCs, the activation of Akt/PKB plays an important role, whereas in IRPTCs, cellular p27kip1 expression plays an important role. The activation of Akt/PKB in VSMCs seems to be P1i kinase- and reactive oxygen species-dependent. Furthermore, the carboxyl-terminal tail of GLP plays an important role in GLP- as well as Ang II-induced cellular hypertrophy in IRPTCs.
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