Angiotensin II (Ang II) binds to specific G protein-coupled receptors and is mitogenic in Chinese hamster ovary (CHO) cells stably expressing a rat vascular angiotensin II type 1A receptor (CHO-AT1A). Cyclin D1 protein expression is regulated by mitogens, and its assembly with the cyclin-dependent kinases induces phosphorylation of the retinoblastoma protein pRb, a critical step in G1 to S phase cell cycle progression contributing to the proliferative responses. In the present study, we found that in CHO-AT1A cells, Ang II induced a rapid and reversible tyrosine phosphorylation of various intracellular proteins including the protein-tyrosine phosphatase SHP-2. Ang II also induced cyclin D1 protein expression in a phosphatidylinositol 3-kinase and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK)-dependent manner. Using a pharmacological and a co-transfection approach, we found that p21Vm, Raf-1, phosphatidylinositol 3-kinase and also the catalytic activity of SHP-2 and its Src homology 2 domain are required for cyclin D1 promoter/reporter gene activation by Ang II through the regulation of MAPK/ERK activity. Our findings suggest for the first time that SHP-2 could play an important role in the regulation of a gene involved in the control of cell cycle progression resulting from stimulation of a G protein-coupled receptor independently of epidermal growth factor receptor transactivation.

Received for publication, February 28, 2000, and in revised form, May 16, 2000 Published, JBC Papers in Press, June 7, 2000, DOI 10.1074/jbc.M001614200

Laurent Guillemot‡, Arlette Levy, Zhizhuang Joe Zhaoš, Gilbert Béréziat, and Bernard Rothrut‡
From the Laboratoire de Signalisation Cellulaire, Médiateurs Lipidiques et Contrôle de l’Expression des Gènes, CNRS UPRES-A 7079, Université Pierre et Marie Curie, 7 Quai St-Bernard, Bâtiment A, 5ème étage, 75005 Paris, France and the §Division of Hematology/Oncology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

The octapeptide angiotensin II (Ang II) binds to specific high affinity receptors present in the adrenal cortex, liver epithelial cells, and vascular smooth muscle cells (VSMC), where it elicits a vast array of biological effects (1). In VSMC, Ang II has been shown to stimulate proliferative and hypertrophic growth via binding to type 1 receptor (AT1-R), a seven-transmembrane-spanning receptor coupled to the Gq/11 subtype of heterotrimeric G proteins (2). Ang II initiates early biochemical events including rapid production of diacylglycerol (an activator of protein kinase C) and inositol 1,4,5-trisphosphate (that induces release of Ca2+ from the sarcoplasmic reticulum) by phospholipase C-mediated hydrolysis of inositol phospholipids (3–8) and activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) family members ERK1 and ERK2 (9, 10) and the c-Jun N-terminal kinase (11), which are known to influence c-Jun and c-Fos transcription. Indeed, some of the intracellular signals mediated by the AT1 receptor are similar to those activated by mitogens such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), leading to induction of transcription of several immediately early growth response genes such as c-fos, c-jun, and c-myc (12–15).

Phosphorylation and dephosphorylation of proteins on tyrosine residues are now well recognized as important mechanisms for transmitting extracellular stimuli in cellular events such as cell attachment, proliferation, differentiation, and migration (16). The phosphorylation level of the molecules within signaling pathways are regulated by the activity of protein-tyrosine kinases and protein-tyrosine phosphatases (PTPs).

An increase in protein tyrosine phosphorylation has been attributed in part to the activation of Src (17, 18), the Jak family proteins Jak2 and Tyk2 (10, 19), Tyk-2/CADTK (20), and focal adhesion kinase pp125FAK (21). A decrease in the level of tyrosine phosphorylation of cellular proteins is finely controlled by the activity of PTPs, some containing Src homology-2 (SH2) domains (22). Two of the SH2 domain-containing PTPs that have been studied are SHP-1 (also known as PTP1C, SHPTP1, SHPTP2, and SHPTP3, by guest on July 27, 2018http://www.jbc.org/Downloaded from

*This work was supported by Association pour la Recherche sur le Cancer Contract 9662, by CNRS, and by National Institutes of Health Grants HL-57393 and CA75218 (to Z. J. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Supported by a doctoral fellowship from the French Ministère de l’Education Nationale de l’Enseignement Supérieur et de la Recherche.
¶ To whom correspondence should be addressed. Tel.: 33-1-44-27-35-07; Fax: 33-1-44-27-51-40; E-mail: rothhut@ccr.jussieu.fr.
† The abbreviations used are: Ang II, angiotensin II; AP-1, activator protein 1; AT1-R, angiotensin II type 1A receptor; CDK, cyclin-dependent kinase; CHO, Chinese hamster ovary fibroblast cell line; CS, cytostatic to serine mutant; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; HA, influenza hemagglutinin protein epitope; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MBP, myelin basic protein; PAGE, polyacrylamide gel electrophoresis; PI3K, phosphatidylinositol 3-kinase; pRb, retinoblastoma protein; PTP, protein-tyrosine phosphatase; SH2, Src homology 2 domain; signal transducers and activators of transcription; VSMC, vascular smooth muscle cells; PDGF, platelet-derived growth factor; MBP, myelin basic protein.
Role of SHP-2 in Cyclin D1 Promoter Activation by Ang II

Both p70 S6 kinase and the p21 protein kinase have been shown to inhibit S phase entry in a variety of cell types. In CHO fibroblasts (56), these kinases are required for Ang II-induced S phase cell cycle progression. PI3K (LY294002 and wortmannin) have been shown to inhibit cell cycle progression in response to a variety of growth factors. PI3K is indispensable for G1 to S phase cell cycle machinery, phosphatidylinositol 3-kinase (PI3K), a member of the tyrosine phosphatase family SH2, 3p110 (SHP-2), has been shown to be involved in the control of cytoskeletal organization, cell spreading, and migration (47, 48).

The D-type cyclins (cyclin D1, D2, and D3) are thought to be key regulatory components for progression through G1 phase and for the commitment of mammalian cells to enter S phase and to replicate their DNA. Cyclin D1 and its catalytic protein partners, the cyclin-dependent kinases (CDKs), are induced as part of the delayed early response to mitogenic stimulation (49–51). Cyclin D1 preferentially associates with either CDK4 or CDK6, an association that is prevented by the interaction of CDKs with their inhibitors (52). Once activated, cyclin D1-CDK4 and/or cyclin D1-CDK6 complexes phosphorylate the 110-kDa retinoblastoma tumor suppressor gene product (pRb) (53), resulting in the release of E2F transcription factor and allowing gene transcription required for the progression of the cell cycle into S phase and DNA synthesis (54).

Early studies demonstrated that p21ras (Ras) induces DNA synthesis in the nucleus of quiescent cells and that overexpression of activated Ras is associated with the increased expression of cyclin D1 in NIH 3T3 cells (55) and with the positive regulation of the cyclin D1 promoter in human trophoblasts (JEG-3), in the mink lung epithelial cell line (Mv1Lu), and in Chinese hamster ovary fibroblasts (56). These results suggest that the Ras signaling pathway is directly linked to the G/S phase transition of the cell cycle. Indeed, p21ras activates c-Jun, and members of the c-Jun/AP-1 family are involved in promoting cellular proliferation (57) and cyclin D1 promoter activity (58), although other transcription factors such as the members of the ETS family proteins participate in this regulation (56).

Cyclin D1 activation by Ras has been attributed to the sequential activation of Raf kinase, MEK, and MAPK/ERK (59, 60), but recent findings also reveal that other effectors might contribute to G1/S phase cell cycle progression (61).

The various Ras effectors that interact with the cell cycle machinery, phosphatidylinositol 3-kinase (PI3K), a hetero-erodic protein composed of 85- and 110-kDa subunits that catalyzes the synthesis of 3-phosphorylated phosphoinositides, appears to be a key intermediate in receptor-stimulated mitogenesis (62, 63). PI3K is indispensable for G1 to S phase cell cycle progression in response to a variety of growth factors. Inhibitors of PI3K (LY294002 and wortmannin) have been shown to inhibit S phase entry in a variety of cell types. In CHO cells, selective activation of PI3K to physiological relevant levels was sufficient to stimulate DNA synthesis and required both p70 S6 kinase and the p21ras/MEK pathway (64). Inhibitors of PI3K have been shown to block MAPK/ERK activation by some stimuli, such as insulin or insulin-like growth factor-1 (65) and lysophosphatidic acid (66) but not others such as EGF (66) or PDGF (67). Recent studies demonstrate that PI3K is activated in VSMC after treatment with Ang II and that its activity is crucial for cell growth (68).

In this study, we investigated the signaling pathway linking the Ang II receptor to cyclin D1 protein expression and promoter activity in CHO-AT1A cells. We found that both PI3K and MAPK/ERK are important components of the mitogenic signal, since they are required for Ang II-induced S phase cell cycle progression, cyclin D1 protein expression, and promoter activity. In addition, we show that the catalytic activity as well as the SH2 domains of SHP-2 and class I PI3K are required for Ang II to stimulate cyclin D1 promoter activity, through a pathway that is dependent on MAPK/ERK activation. Although ligand-independent tyrosine phosphorylation (transactivation) of RTKs, such as the EGFR has been suggested to represent an essential event for MAPK/ERK activation by both G1- and G0-coupled receptors (69), we present evidence that the pathways leading to cyclin D1 promoter activation by Ang II are independent of EGFR transactivation in CHO-AT1A cells.

This is to our knowledge the first demonstration of the protein-tyrosine phosphatase SHP-2 acting downstream of a G protein-coupled receptor triggered to induce cyclin D1 expression involved in the control of cell cycle progression.

EXPERIMENTAL PROCEDURES

Materials—Acrylamide, salts, and other electrophoresis materials were purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA) unless otherwise specified. PD098059, PD158780, LY294002, and wortmannin were obtained from Calbiochem. [γ-32P]ATP was purchased from ICN Life Science Products. Prestained protein molecular mass markers were from Bio-Rad. Sodium vanadate was from Fisher. Protein A/G PLUS-agarose was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). LipofectAMINE-PLUS reagent was from Life Technologies, Inc. Ang II was purchased from Sigma. Monoclonal anti-phosphotyrosine antibodies were obtained from Transduction Laboratories (Lexington, KY) and Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-SHP-2 antibodies were purchased from Santa Cruz Biotechnology, Upstate Biotechnology, and Transduction Laboratories. Monoclonal anti-hemagglutinin (HA) antibody 12CA5 was from Roche Molecular Biochemicals; monoclonal anti-cyclin D1 antibody (HD11) was from Santa Cruz Biotechnology; polyclonal anti-p44/p42 ERK1/2 antibody was from New England Biolabs; and monoclonal phosphospecific MAPK antibodies were from Upstate Biotechnology. Ras K12 mutants were provided by Dr. F. Schweig. Rabbit anti-mouse IgG-peroxidase conjugate and goat anti-rabbit IgG-peroxidase conjugate were from BioSys (Compiègne, France). Myelin basic protein (MBP) and EGF were from Upstate Biotechnology.

cDNA Plasmids—HA-tagged p44/ERK1 was a kind gift from Dr. J. Pouyssegur (45). Dr. R. Müller provided the −973 base pair human cyclin D1 promoter fragment linked to a luciferase reporter gene (−973 CD1/LUC). The different SHP-2 and SHP-1 constructs were obtained and used as described (44, 70). The dominant negative Ras N17 and constitutive active Ras K12 mutants were provided by Dr. F. Schweig. Constitutive active Raf-1 BXB and dominant negative Raf-1 C4 mutants were gifts from Dr. Z. Luo (71). The plasmid Δp58α (a deletion mutant of the regulatory subunit of PI3K lacking 162 amino acids from residues 468 to 567 of the inter-SH2 domain that confers binding to the catalytic subunit p110) was kindly provided by Dr. A. Eder (72). All cDNA plasmids was prepared using the CONCERT high purity plasmid purification system from Life Technologies.

Cell Culture—Parental CHO-K1 cells and CHO-AT1A cells stably expressing a rat vascular AT1 receptor (73) were maintained in Ham’s F-12 medium containing 10% fetal calf serum, 100 µg/ml streptomycin, 100 units/ml penicillin, and 2 mM l-glutamine. CHO-AT1A cells were supplemented with 750 µg/ml Geneticin (G418). Cells in 100-mm dishes were grown to 75–85% confluence, washed once with serum-free Ham’s F-12 medium, and grown in serum-free Ham’s F-12 medium supplemented with 0.5 mg/ml bovine serum albumin for 48 h prior to use.

Cell Cycle Analysis—Cells (0.3 × 10⁶) in six-well culture dishes were maintained in serum-free medium as described above, treated with Ang II (10⁻⁷ m) or FCS (10%), and collected at various time points (0–44 h). Then cells were centrifuged at 1000 rpm for 5 min. The pellet was resuspended in 200 µl of PBS, fixed in 2 ml of ice-cold 70% ethanol, and stored at 4 °C. Cells were centrifuged at 1000 rpm for 5 min and resuspended in 400 µl of PBS containing RNase (100 µg) and propidium iodide (12 µg) for 30 min at 37 °C. The samples were analyzed by a fluorescence-activated cell sorter (ELITE, Beckman Coulter).

Measurement of DNA Synthesis—Incorporation of the thymidine analogue 5-bromo-2'-deoxyuridine (BrdUrd) was measured to determine the effect of Ang II on DNA synthesis. CHO-K1 or CHO-AT1A cells were placed in 96-well cell culture plates at a concentration of 10,000 cells/well. Twenty-four hours later, cells were growth-arrested in serum-free...
Ham’s F-12 medium (100 μl/well) for 72 h before adding either 10−7 m Ang II, 100 ng/ml EGF, or 10% fetal calf serum. After 18 h (FCS) and 42 h (Ang II and EGF), 10 μM BrdUrd was added to each well, and cells were incubated for an additional 6 h and then fixed. When inhibitors were tested, they were preincubated for 1 h in serum-free Ham’s F-12 before adding Ang II. Quantification of BrdUrd incorporation was performed using a commercially available detection kit (Roche Molecular Biochemicals).

**Transient Transfection and Luciferase Assay**—The ~973 base pair human cyclin D1 promoter fragment linked to the luciferase reporter gene (~973 CD1/LUC) was transiently transfected in CHO-K1 or CHO-AT1α cells (640 ng/3.106 cells in 100-mm dishes) together with different amounts of relevant expression vectors (i.e. 2.5 μg of Ras N17 mutant, 2.5 μg of Raf-1 C4 mutant, 2.5 μg of Δp85α subunit, 5 μg of SHP-2 CS mutant, 5 μg of the SH2 domains of SHP-2, 5 μg of SHP-1 CS mutant or the corresponding empty vector), using LipofectAMINE-PLUS reagent following the protocol provided by the supplier. Twenty-four hours following transfection, cells were trypsinized and aliquoted into 12-well cell culture dishes. Six hours later, cells were serum-starved for 24 h in Ham’s F-12 medium supplemented with 0.5 mg/ml bovine serum albumin before preincubation with or without inhibitors for 1 h and then stimulated for the indicated times with Ang II at the concentrations indicated. Cells were lysed with 200 μl of passive lysis buffer (1×), and luciferase activity was assayed in a luminometer (Lumat LB9507 Berthold) using the kit from Promega (Madison, WI). Relative luciferase activity is expressed as the ratio of Ang II-stimulated to unstimulated samples.

**Assay of Transfected HA-tagged p44-ERK1**—The activity of transiently transfected HA-tagged p44-ERK1 was determined by MBP kinase assay as described (45). HA-tagged p44-ERK1 was transiently transfected in CHO-K1 or CHO-AT1α cells (0.5 μg/106 growing cells in 100-mm dishes) together with different amounts of relevant expression vectors (2.7 μg of Ras N17, 2.5 μg of Raf-1, 1.7 μg of Δp85α subunit, 2.5 μg of SHP-2 CS mutant, 5 μg of the SH2 domains of SHP-2, 5 μg of SHP-1 CS mutant or the corresponding empty vector). Twenty-four hours following transfection, cells were serum-starved for 24 h in Ham’s F-12 medium supplemented with 0.5 mg/ml bovine serum albumin before preincubation with or without inhibitors for 1 h and treatment with 10−7 m Ang II for the indicated times. When inhibitors were used, they were pretreated 1 h before Ang II stimulation. The reactions were terminated by aspirating the medium, washing the cells with ice-cold lysis buffer as described above, resuspended in SDS-sample buffer, and boiled for 5 min at 95 °C. Reaction mixtures were size fractionated by SDS-PAGE (8% gels) and transferred to nitrocellulose membranes. The blot was stained with Ponceau S to confirm equal loading of proteins and then probed with the indicated antibodies. When cyclin D1 protein expression was studied, nuclear extracts were prepared according to the method of Andrews and Faller (74), and equal amounts of proteins were loaded on 10% gels. Immunoblots were developed using appropriate secondary horseradish peroxidase-coupled antibodies and an enhanced chemiluminescence (ECL) kit (Pierce).

### RESULTS

**Ang II Stimulates G1 Phase Progression**—CHO-AT1α cells have been successfully used to investigate the action of Ang II on cell growth and division (73), compared with more physiologically relevant models such as VSMC or adrenal cells in culture. To determine whether Ang II stimulates cell cycle progression in CHO-AT1α cells, flow cytometric analysis was performed. Cells were treated with 10−7 m Ang II for 16–44 h, and the proportion of cells in G0-G1, S, and G2-M was determined at various time points as indicated in Table I. The relative proportion of cells in G0-G1 was reduced from 80.5 to 59.4% at 28 h, concomitant with an increase in cells in S phase (from 5.9 to 29.5%). The proportion of cells in G2-M increased from 13.2 to 27.5% at 44 h. These results indicate that Ang II decreases the proportion of cells in G1 and increases the proportion of cells in S phase and G2-M and that the effect persists up to 44 h.

For comparison, cells were also treated with 10% FCS for 12–24 h. The proportion of cells in S phase increased 68% after 16 h, concomitant with a decrease in the number of cells in G0-G1 phase (from 80.5 to 12.9%; Table I). The proportion of cells in G2-M increased from 13.2 to 48.3% at 20 h.

**Ang II-induced DNA Synthesis Is Blocked by both Inhibitors of MEK and PI3K in CHO-AT1α Cells**—Ang II-stimulated incorporation of BrdUrd into DNA was examined in quiescent CHO-AT1α cells and compared with parental CHO-K1 cells, which do not express AT1α receptors. Whereas 10−7 m Ang II was unable to stimulate BrdUrd incorporation in CHO-K1 cells, a 3.5-fold maximal stimulation was observed for CHO-AT1α cells (Fig. 1). As a control, 10% FCS stimulated 5-fold the incorporation of BrdUrd into CHO-K1 cells. There was no difference in the kinetics of FCS-dependent BrdUrd incorporation in CHO-AT1α cells compared with CHO-K1 cells (data not shown).

A time course of BrdUrd incorporation into DNA was also analyzed after incubation for various periods of time with EGF (data not shown). Under these experimental conditions, 100 ng/ml EGF was unable to stimulate BrdUrd incorporation in CHO-AT1α cells up to 48 h (Fig. 1).

In order to investigate the pathways connecting Ang II to cell cycle progression into S phase, we measured the effect of LY294002, an inhibitor of PI3K, and PD98059, an inhibitor of MEK (the dual specificity kinase that activates p44/p42 MAPK/ERK by phosphorylation), on Ang II-induced BrdUrd incorporation in quiescent CHO-AT1α cells. Under these conditions, both inhibitors suppressed BrdUrd incorporation in a concentration-dependent manner. These results indicate that the proliferation of CHO-AT1α cells in response to Ang II is dependent

### Table I

|        | Untreated | Ang II (10−7 m) | FCS |
|--------|-----------|----------------|-----|
|        | G0-G1     | S              | G2-M |
| 16 h   | 80.5       | 7.49           | 13.2 |
| 20 h   | 6.26       | 23.6           | 15.8 |
| 28 h   | 59.4       | 29.5           | 13.0 |
| 36 h   | 5.94       | 22.9           | 11.7 |
| 40 h   | 54.7       | 21.8           | 22.9 |
| 44 h   | 54.9       | 17.3           | 27.5 |
| 12 h   | 67.9       | 23.8           | 8.9  |
| 16 h   | 12.9       | 73.8           | 13.3 |
| 20 h   | 22.3       | 29.2           | 48.3 |
| 24 h   | 25.7       | 22.2           | 19.4 |
Ang II-induced DNA synthesis is blocked by both inhibitors of MEK and PI3K in CHO-AT1A cells. Serum-starved cells were stimulated with 10^{-7} M Ang II or 100 ng/ml EGF for 48 h and with 10% FCS for 24 h. Incorporation of BrdUrd was measured using a detection kit as described under "Experimental Procedures." PD098059 and LY294002 were preincubated at the indicated concentrations 1 h before adding Ang II. The results are expressed as the percentage of stimulation over the basal value established as 100%. Each value is the mean ± S.E. (bars) from six wells in three independent experiments.

Ang II-induced Endogenous Cyclin D1 Protein Expression Is Dependent upon PI3K and MEK Activities—Induction of cyclin D1 protein expression represents one of the earliest effects of growth factors leading to cell cycle reentry, G1 phase progression, and the commitment of cells to enter S phase. In order to examine whether Ang II is able to induce expression of cyclin D1 protein, quiescent CHO-AT1A cells were treated with 10^{-7} M Ang II and harvested at different times. The level of cyclin D1 protein was analyzed by immunoblotting nuclear extracts with a monoclonal anti-cyclin D1 antibody (HD11). In unstimulated quiescent cells, cyclin D1 protein expression was low, barely detectable (Fig. 2A). Ang II stimulation led to an increase of cyclin D1 levels detectable at 16 h poststimulation and continued to increase until 32 h before declining by 48 h.

To investigate the role of MAPK/ERK and PI3K in this induction, we tested the effects of PD098059, LY294002, and wortmannin. As shown in Fig. 2B, PD098059 and LY294002 severely inhibited Ang II-induced cyclin D1 protein expression in a concentration-dependent manner. Wortmannin was less effective probably because the drug is more labile in living cells (75). These results indicate that PI3K and MAPK/ERK are both required for up-regulation of cyclin D1 protein expression in response to Ang II in CHO-AT1A cells.

Ang II Stimulated Cyclin D1 Promoter Activity in a Dose- and Time-dependent Manner—To determine whether Ang II was capable of inducing cyclin D1 promoter activity, we used the ~973 base pair human cyclin D1 promoter fragment linked to the luciferase reporter gene (~973 CD1/LUC). Quiescent CHO-AT1A cells transiently transfected with ~973 CD1/LUC were treated with Ang II at different concentrations (dose-response; Fig. 3A) and for different times (time course; Fig. 3B) as described. Ang II stimulated cyclin D1 promoter/reporter gene in a concentration-dependent manner, reaching a maximal increase (5–6-fold above basal level) at 10^{-7} M and 10^{-6} M for a 24-h stimulation (Fig. 3A). The specificity of this effect was tested using CHO-K1 cells in which Ang II at the highest concentration (10^{-6} M) was unable to stimulate cyclin D1 promoter/reporter gene activity (data not shown). The time course of Ang II-stimulated cyclin D1 promoter/reporter gene activity was also determined (Fig. 3B) and showed an increase that was significantly different from control at 8 h (1.8-fold), reaching a maximum at 32 h (6–7-fold) and remaining at this level at 48 h. The kinetic in cyclin D1 promoter/reporter gene activity induced by Ang II was exactly correlated with the increase in cyclin D1 protein expression until 32 h as shown in Fig. 2A. For longer times (48 h), cyclin D1 promoter/reporter gene activity was still elevated, but cyclin D1 protein expression decreased, possibly reflecting a difference in posttranscriptional modifications between luciferase and cyclin D1 genes.

Ang II-stimulated Cyclin D1 Promoter Activity Is Dependent upon Ras/Raf-1/MEK/ERK and PI3K Activities—Previous work on the regulation of cyclin D1 gene transcription has shown that the promoter is growth factor-regulated and can be activated by oncogenic mutants of Ras (58, 76, 77). The role of Ras in Ang II-induced cyclin D1 promoter/reporter gene activity was examined in CHO-AT1A cells transiently transfected with ~973 CD1/LUC and an expression vector for the dominant negative Ras N17 mutant. Overexpression of Ras N17 reduced Ang II-induced ~973 CD1/LUC activity, reaching approximately 50% inhibition (Fig. 4A). This effect was only partial, suggesting that additional or parallel pathways might also be involved. To address this possibility, we tested the effects of LY294002 and wortmannin on Ang II-induced reporter gene activity. LY294002 at 50 μM and wortmannin at 200 nM partially inhibited Ang II-induced promoter activity, reaching approximately 50% inhibition. These results suggest that PI3K is also involved in Ang II-induced cyclin D1 promoter/reporter gene activity. To further explore the relationship between PI3K and Ang II-induced cyclin D1 promoter activity, a dominant-negative form of p85 (Δp85α) was used to inhibit the function of endogenous PI3K. As expected, Ang II-induced cyclin D1 promoter activity was partially inhibited (50%) when Δp85α subunit was coexpressed, therefore identifying the role of class IA PI3K in this pathway. We then also tested the effect of Ras N17...
overexpression in the presence of 50 μM LY294002, and under these conditions, the inhibition was complete (Fig. 4A).

The first Ras effectors to be identified in mammalian cells were the protein kinases of the Raf family. To test the involvement of Raf-1 in Ang II-induced promoter/reporter gene activity, CHO-AT1A cells were transiently transfected with −973 CD1/LUC and an expression vector for the dominant negative Raf-1 C4 mutant. Interestingly, this mutant completely inhibited Ang II-induced promoter/reporter gene activity. These results strongly suggest that the pathway(s) triggered by Ang II and leading to cyclin D1 promoter induction converges toward Raf-1.

Ang II Induced The Tyrosine Phosphorylation of Intracellular Proteins—It is now well known that seven-transmembrane domain-containing receptors functionally coupled to heterotrimeric G-proteins are devoid of intrinsic protein tyrosine kinase activity. However, it has been well documented that some of the intracellular signaling pathways mediated by the AT1 receptor are dependent upon stimulation of tyrosine kinase activity. To test our model, quiescent CHO-AT1A cells were treated for various times with 10−7 M Ang II. Whole cell lysates were prepared, and equal amounts of proteins were separated on SDS-PAGE, transferred to nitrocellulose membrane, and analyzed using monoclonal anti-phosphotyrosine antibodies. The results from Fig. 5A show that Ang II rapidly stimulated immunoreactive proteins with molecular masses of 120, 90, 75–85, 60–65, and 40–45 kDa. However, a different time course of induction was observed among the different species. For some proteins, the induction was seen as early as 2 min (90 kDa) and lasted up to 16 min. For others (40–45 kDa), the induction was seen as early as 2 min (90 kDa) and lasted up to 16 min. For others (40–45 kDa), the induction was seen as early as 2 min (90 kDa) and lasted up to 16 min. For others (40–45 kDa), the induction was seen as early as 2 min (90 kDa) and lasted up to 16 min. For others (40–45 kDa), the induction was seen as early as 2 min (90 kDa) and lasted up to 16 min. For others (40–45 kDa), the induction was seen as early as 2 min (90 kDa) and lasted up to 16 min. For others (40–45 kDa), the induction was seen as early as 2 min (90 kDa) and lasted up to 16 min.
Role of SHP-2 in Cyclin D1 Promoter Activation by Ang II

Inhibition of EGFR Tyrosine Kinase by PD158780 Does Not Block Ang II-induced MAPK/ERK and Cyclin D1 Promoter Activities—The family of MAPK/ERKs plays a central role in mitogenic signaling in response to a number of growth-stimulating agents. Tyrosine kinase activity of the single transmembrane receptor class has been implicated in MAPK/ERK activation by G protein-coupled receptors by recruiting signaling complexes containing Shc and GRB2. To clarify the role of EGF receptor tyrosine kinase in Ang II-induced signal transduction in CHO-AT1A cells, we tested the effect of the selective receptor tyrosine kinase inhibitor PD158780 on MAPK activity. As shown in Fig. 6A, at 100 nM, the compound did not affect Ang II-induced ERK1/2 activation. Increasing the concentration of inhibitor up to 1 μM also did not block (data not shown). On the other hand, EGF (at 100 ng/ml) had only a modest and transient effect on MAPK/ERK activation, probably because these cells do not express high levels of the receptor. These results suggest that EGFR is not involved in the pathway linking the Ang II receptor to MAPK/ERK activation.

We then performed similar experiments on Ang II-induced cyclin D1 promoter/reporter gene activity. Fig. 6B shows that EGF (at 100 ng/ml) did not stimulate cyclin D1 promoter activity and that PD158780 at 50 and 100 nM did not influence the Ang II-induced effect. Due to all of these results, it is unlikely that Ang II-induced MAPK/ERK and cyclin D1 promoter activities are dependent on EGFR transactivation.

Ang II-induced MAPK/ERK Activity Is Inhibited by LY294002; Dominant Negative Ras N17, Raf-1 C4, SHP-2 CS Mutants and SH2 Domains of SHP-2—We have shown that cyclin D1 promoter induction by Ang II required MAPK/ERK activity. In order to corroborate the pathway(s) connecting to this activation, we tested the effects of both PI3K and MEK inhibitors (LY294002) and SHP-2 CS mutants (Ras N17, Raf-1 C4, SHP-2 CS mutant) on Ang II-induced cyclin D1 promoter activity. As shown in Fig. 6C, the PI3K inhibitor completely abolished Ang II-induced cyclin D1 promoter activity, whereas the MEK inhibitor had only a modest effect. These results are consistent with the notion that Ang II-induced cyclin D1 promoter activation is mediated primarily through the MAPK/ERK pathway.

In conclusion, these results demonstrate that Ang II-induced cyclin D1 promoter activation is mediated primarily through the MAPK/ERK pathway, and that this activation is required for Ang II-induced cyclin D1 promoter induction. However, it is possible that other signaling pathways may also contribute to Ang II-induced cyclin D1 promoter activation, particularly in the context of cell proliferation and differentiation.

Fig. 5. Implication of the protein-tyrosine phosphatase SHP-2 in Ang II signaling. A, serum-starved CHO-AT1A cells were either left untreated (0) or stimulated for various periods of time (indicated in min) with Ang II. Whole cell lysates containing equal amounts of total protein were subjected to SDS-PAGE (8% gels) and analyzed by Western blotting with monoclonal anti-phosphotyrosine antibodies. Molecular size standards are shown in kDa on the left. The arrows on the right denote tyrosine-phosphorylated proteins at 120, 90, 75–85, 60–65, and 40–45 kDa. Data are representative of independent experiments that were performed at least three times. B, serum-starved CHO-AT1A cells were either left untreated (0) or stimulated with 10^{-7} M Ang II for the indicated times. SHP-2 was immunoprecipitated (IP) with a rabbit polyclonal anti-SHP-2 antibody. Bound proteins were separated by SDS-PAGE (8% gels) and immunoblotted with monoclonal anti-phosphotyrosine (anti-P-Tyr) antibodies (upper panel). For loading controls, the blot was stripped and reprobed with a monoclonal anti-SHP-2 antibody (lower panel). Data are representative of three independent experiments. C, serum-starved cells were either left untreated (0) or stimulated for the indicated times with Ang II (10^{-7} M). Immunoblotting with antibody specific for active phosphorylated MAPK/ERK (anti-P-ERK1/2) was performed on whole cell lysates as described under “Experimental Procedures.” The arrowheads indicate tyrosine-phosphorylated ERK1/2 (upper panel). The amounts of protein were controlled by probing a duplicate blot with an anti-ERK1/2 antibody (lower panel). Data are representative of two independent experiments. D, quiescent CHO-AT1A cells were transiently transfected with 973 CD1/LUC and either the catalytically inactive SHP-2 CS mutant, the two SH2 domains of SHP-2, the catalytically inactive SHP-1 CS mutant, or the empty vector alone. Cells were stimulated with 10^{-7} M Ang II for 32 h, and luciferase activity was assayed as described previously. The data are presented as means ± S.E. (bars) from three independent experiments each performed in triplicate. * indicates significantly different compared with empty vector alone (p < 0.05; Student’s t test).
inhibitor (LY294002 and PD098059, respectively) on Ang II-stimulated MAPK/ERK activity in CHO-AT1A cells transiently transfected with HA-tagged p44-ERK1. The kinase was immunoprecipitated from lysates of cells stimulated with Ang II for different times (10 min and 2, 5, and 8 h), and enzymatic activity was determined using the MBP phosphorylation assay. As expected, 50 μM PD098059 completely inhibited Ang II-induced ERK1 activity (data not shown), whereas 50 μM LY294002 partially but significantly blocked this activation (Fig. 7A), confirming an involvement of PI3K in MAPK/ERK activation in CHO-AT1A cells. Control experiments performed in CHO-K1 cells transfected with the same amount of HA-tagged p44-ERK1 and treated with Ang II did not reveal phosphorylation of MBP (data not shown).

We further studied the effects of Ras N17, Raf-1 C4, SHP-2 CS, and SH2 domains of SHP-2 on Ang II-induced MAPK/ERK1 activation after 10 min and after 2 h of stimulation. These times were chosen because MAPK/ERK activities were elevated compared with 5 and 8 h. Overexpression of the dominant negative Ras N17 mutant was ineffective at 10 min but partially inhibited at 2 h, whereas overexpression of the dominant negative Raf-1 C4 mutant completely inhibited Ang II-induced ERK1 activation at each time tested (Fig. 7B). As a positive control, constitutively active Raf-1 BXB or Ras K12 mutants were each able to activate ERK1 in the absence of Ang II stimulation (data not shown). In addition, overexpression of dominant negative Ras N17 and Raf-1 C4 mutants could both inhibit constitutively active Ras K12-induced ERK1 activity, confirming that both constructs effectively behave as dominant negative forms in these assays. These results show that Ang II-induced ERK1 activity is dependent on Raf-1 activity, independent of Ras at 10 min, and partially dependent on Ras at 2 h. In addition, when dominant negative SHP-2 CS mutant and SH2 domains were tested, they were both able to partially inhibit Ang II-induced ERK1 activity at each time tested (Fig. 7C). These results highlight the importance of the intrinsic phosphatase activity and the SH2 domains of SHP-2 in Ang II-stimulated MAPK/ERK activity in CHO-AT1A cells.

**DISCUSSION**

In CHO-AT1A cells, the Ang II receptor is coupled to more than one G protein (i.e., G, and possibly G, subunits) responsible for inducing an early phase and a second sustained increase in MAPK/ERK activity (79). The early phase of MAPK/ERK activation by Ang II is mediated through a pertussis toxin-insensitive G protein via phospholipase C and/or protein kinase C activation, while the sustained phase of activation may be mediated by G, as well as by a pertussis toxin-sensitive G protein. It has been proposed that the latter phase is an obligatory event for growth factor-induced cell cycle progression and
Role of SHP-2 in Cyclin D1 Promoter Activation by Ang II

**Fig. 7.** Ang II-induced MAPK/ERK activity is inhibited by LY294002, dominant negative Ras N17, Raf-1 C4, SHP-2 CS mutants, and SH2 domains of SHP-2. A, quiescent CHO-AT1A cells transiently transfected with HA-tagged p44-ERK1 were pretreated with 50 μM LY294002 (+) or left untreated (−) and were stimulated or not with Ang II for different periods of time as indicated. Cells were lysed, and ERK1 was immunoprecipitated with a monoclonal anti-HA antibody. Its activity was assessed using the MBP phosphorylation assay as described under “Experimental Procedures.” B and C, CHO-AT1A cells were transiently transfected with HA-tagged p44-ERK1 plus (+) either dominant negative Ras N17 or Raf-1 C4 mutants (B), catalytic inactive SHP-2 CS mutant, SH2 domains of SHP-2 (C), or empty vector alone (−). Quiescent cells were stimulated or not (0) with Ang II for the different times indicated and lysed. ERK1 was immunoprecipitated, and its activity was assayed as described previously. Results are expressed as fold increase over basal activity (unstimulated samples) set at 1. The data shown are representative autoradiograms from two (A and B) or three (C) experiments with similar results.

In this study, we also investigated the role of the protein-tyrosine phosphatase SHP-2 during Ang II-induced cyclin D1 promoter activation. Little is known about the participation and regulation of SHP-2 in pathways activated by G protein-coupled receptors that lack intrinsic tyrosine kinase activity. We found that a catalytic inactive SHP-2 CS mutant inhibited Ang II-induced cyclin D1 promoter/reporter gene activity (Fig. 5D), suggesting that the catalytic domain of SHP-2 plays an important role in pathways leading to cell cycle progression. In order to act as a positive regulator, SHP-2 must be able to dephosphorylate phosphotyrosines that negatively regulate key signaling components such as members of the Src family of protein-tyrosine kinases, known to activate the Ras/Raf-1/MEK/ERK pathway (81). The possibility that SHP-2 interacts with insulin-related substrate-1, resulting in PI3K activation, has also been suggested in insulin-stimulated Rat-1 fibroblasts (82). However, additional protein-tyrosine kinases may also be involved in this regulation. Marrero et al. (83) presented evidence that both the Jak/STAT and the Ras/MAPK cascades are important components in Ang II- and PDGF-mediated VSMC proliferation. They also showed that SHP-2 can associate with the Ang II type 1 receptor and serve as a docking partner for the protein-tyrosine kinase Jak2. These interactions are a prerequisite for Jak2 phosphorylation and activation (83), leading to Raf-1 phosphorylation and activation of MAPK/ERK. Therefore, Jak2 represents an additional target for SHP-2 to exert a positive regulation on Ang II-mediated mitogenic signaling. In this study, we also found that transfection with the SH2 domains of SHP-2 inhibited Ang II-induced cyclin D1 promoter/reporter gene activity (Fig. 5D), implying that the adapter function of SHP-2 via its SH2 domains plays an important role in this induction. But the participation of SH2 domains inter-
acting with the Ang II type 1 receptor has not been demonstrated in contrast to their association with activated PDGF and EGF receptors. Indeed, the two SH2 domains allow SHP-2 to interact with tyrosine-phosphorylated proteins, and it has been reported that ligation of these domains contributes to activation of the phosphatase activity (84).

In the case of growth factor receptors, SHP-2 becomes rapidly tyrosine-phosphorylated upon ligand stimulation, creating a binding site for the Grb2-Sos complex and therefore facilitating activation of the Ras/ERK cascade. Moreover, an EGFR-dependent transactivation model has been recently described to mediate Ang II-induced ERK activation (85, 86). However, in our cell system, such a mechanism is unlikely because Ang II-induced MAPK/ERK and cyclin D1 promoter/reporter gene activities are not affected by PD15870, a highly specific inhibitor of EGFR tyrosine kinase. Moreover, EGF by itself was unable to stimulate a sustained MAPK/ERK activation or to induce cyclin D1 promoter activity and BrdUrd incorporation in CHO-AT1a cells. Our results show that SHP-2 becomes rapidly tyrosine-phosphorylated (Fig. 5B) and more precisely that both the SH2 domains and the catalytic activity of the phosphatase are important for Ang II-induced MAPK/ERK activity (Fig. 6C). We conclude that SHP-2 plays an important role downstream of the mitogenic signaling pathway induced by the G protein-coupled Ang II receptor independently of EGF receptor transactivation.

Depending on the cells and agonists that have been studied, tyrosine phosphorylation of SHP-2 correlates either with an inhibition (45) or a stimulation (34, 78) of its phosphatase activity. On the other hand, it may be difficult to address the influence of tyrosine phosphorylation on the enzymatic activity, since SHP-2 is subject to an autodephosphorylation mechanism (45) or a stimulation (34, 78) of its phosphatase activity. To further elucidate the mechanisms by which the phosphatase acts to regulate MAPK/ERK activity under in vitro phosphatase assay conditions. Moreover, insulin activates SHP-2 without inducing its tyrosine phosphorylation (40), suggesting that SHP-2 phosphorylation is not essential for its activation. Neither the precise mechanisms by which the phosphatase is regulated in CHO-AT1a cells nor the mechanisms by which the phosphatase acts to regulate MAPK/ERK pathays have been investigated in detail. However, our results demonstrate that Ang II-induced MAPK/ERK activation is modulated by PI3K, Ras, Raf-1, and SHP-2 (Fig. 7) and that it is independent of EGF receptor transactivation. Further studies are necessary to depict more precisely the exact mechanism of action of the phosphatase.

Taken together, our results suggest that in CHO-AT1a cells, SHP-2 plays a crucial role in the Ang II-induced initiation of the mitotic cyclin D1 promoter activity, leading to cell cycle progression and proliferation. This is to our knowledge the first demonstration for an SH2 domain-containing protein-tyrosine phosphatase to regulate a gene activated by a G-protein-coupled receptor pathway.

Acknowledgments—We are grateful to Dr. Rolf Müller for providing our cell system, such a mechanism is unlikely because Ang II-induced MAPK/ERK and cyclin D1 promoter activity, leading to cell cycle

REFERENCES

1. Griendling, K. K., and Alexander, R. W. (1993) Semin. Nephrol. 13, 558–566
2. Marrero, M. B., Schieffer, B., Paxton, W. G., Duff, J. L., Berk, B. C., and Bernstein, K. E. (1995) Cardiovasc. Res. 30, 530–536
3. Beirre, M. J. (1987) Biochim. Biophys. Acta 907, 33–45
4. Nishizuka, Y. (1988) Nature 334, 661–665
5. Huckle, W. R., and Earp, H. S. (1994) Prog. Growth Factor Res. 5, 177–194
6. Inagaki, T., Guo, D. F., and Kitami, Y. (1995) J. Biol. Chem. 270, 526–530
7. Naftilan, A. J., Gilliland, G. K., Elledge, C. S., and Kant, A. S. (1990) Mol. Cell. Biol. 10, 530–540
8. Naftilan, A. J., Pratt, R. E., and Dzau, V. J. (1995) J. Clin. Invest. 83, 1419–1424
9. Molloy, C. J., Taylor, D. S., and Weber, H. (1993) Mol. Cell. Biol. 13, 2396–2405
10. Naftilan, A. J., Pratt, R. E., and Dzau, V. J. (1995) J. Clin. Invest. 83, 1419–1424
11. Zohn, I. E., Yu, H., Li, X., Cox, A. D., and Earp, H. S. (1995) Mol. Cell. Biol. 15, 6160–6168
12. Kawahara, Y., Sunako, M., Tsuda, T., Fukuzaki, H., Fukumoto, Y., and Takai, Y. (1988) Biochem. Biophys. Res. Commun. 150, 52–59
13. Taubman, M. B., Berk, B. C., Izumo, S., Tsuda, T., Alexander, R. W., and Nadal-Ginard, B. (1989) J. Biol. Chem. 264, 526–530
14. Naftilan, A. J., Gilliland, G. K., Elledge, C. S., and Kant, A. S. (1990) Mol. Cell. Biol. 10, 530–540
15. Naftilan, A. J., Pratt, R. E., and Dzau, V. J. (1995) J. Clin. Invest. 83, 1419–1424
16. Nishizuka, Y., and Tanaka, N. K. (1997) J. Biol. Chem. 272, 11017–11024
17. Turner, C. E., Pietras, K. M., Taylor, D. S., and Molloy, C. J. (1995) J. Cell. Biol. 128, 333–342
18. Mauro, L. J., and Dixon, J. E. (1997) Trends Biochem. Sci. 22, 335–336
19. Berg, B. C., and Corson, M. A. (1997) Mol. Cell. Biol. 17, 1171–1178
20. Brinson, A. E., Harding, T., Diliberto, P. A., He, Y., Li, X., Hunter, D., Herman, B., Earp, H. S., and Graves, L. M. (1998) J. Biol. Chem. 273, 1711–1718
21. Turner, C. E., Pietras, K. M., Posner, B. I., and Chretien, P. (1991) Nature 352, 736–739
22. Yi, T., and Ihle, J. N. (1996) Mol. Cell. Biol. 15, 3350–3358
23. Duff, J. L., Berk, B. C., and Corson, M. A. (1992) Biochem. Biophys. Res.
Role of SHP-2 in Cyclin D1 Promoter Activation by Ang II

61. Gille, H., and Downward, J. (1999) J. Biol. Chem. 274, 22033–22040
62. Stephens, L. R., Jackson, T. R., and Hawkins, P. T. (1993) Biochim. Biophys. Acta 1179, 27–75
63. Varticovski, L., Harrison-Findik, D., Keeler, M. L., and Susa, M. (1994) Biochim. Biophys. Acta 1226, 1–11
64. McIlroy, J., Chen, D., Wjasow, C., Michaeli, T., and Backer, J. M. (1997) Mol. Cell. Biol. 17, 248–255
65. Cross, D. A., Alessi, D. R., Vandenheede, J. R., McDowell, H. E., Hundal, H. S., and Cohen, P. (1994) Biochem. J. 303, 21–26
66. Hawes, B. E., Luttrell, L. M., van Biesen, T., and Lefkowitz, R. J. (1996) J. Biol. Chem. 271, 12133–12136
67. Duckworth, B. C., and Cantley, L. C. (1997) J. Biol. Chem. 272, 27665–27670
68. Saward, L., and Zahradka, P. (1997) J. Biol. Chem. 272, 22033–22040
69. Luo, Z., Zhang, X., Rapp, U., and Avruch, J. (1995) J. Biol. Chem. 270, 23681–23687
70. Eder, A. M., Dominguez, L., Franke, T. F., and Ashwell, J. D. (1998) Mol. Endocrinol. 6, 845–854
71. Kimura, K., Hattori, S., Kabuyama, Y., Shibazaki, T., Takayama, J., Nakamura, S., Toki, S., Matsuda, Y., Onodera, K., and Fukui, Y. (1994) J. Biol. Chem. 269, 18961–18967
72. Filmus, J., Robles, A. I., Shi, W., Weng, M. J., Colombo, L. L., and Conti, C. J. (1994) Oncogene 9, 3627–3633
73. Herber, B., Truss, M., Beato, M., and Muller, R. (1994) Oncogene 9, 1295–1304
74. Ali, M. S., Schieffer, B., Delafontaine, P., Bernstein, K. E., Ling, B. N., and Marrero, M. B. (1994) J. Biol. Chem. 272, 12373–12379
75. Wen, Y., Nadler, J. L., Gonzalez, N., Scott, S., Clasper, E., and Natarajan, R. (1996) Am. J. Physiol. 271, C1212–C1220
76. Meloche, S., Seuwen, K., Pages, G., and Pouyssegur, J. (1992) Mol. Endocrinol. 6, 845–854
77. Ugi, S., Maegawa, H., Kasahara, A., Adachi, M., Olsensky, J. M., and Kikkawa, R. (1996) J. Biol. Chem. 271, 12595–12602
78. Marrero, M. B., Schieffer, B., Li, B., Sun, J., Harp, J. B., and Ling, B. N. (1997) J. Biol. Chem. 272, 24684–24690
79. Lechleider, R. J., Sugimoto, S., Bennett, A. M., Kashishian, A. S., Cooper, J. A., Shoelson, S. E., Walsh, C. T., and Neel, B. G. (1993) J. Biol. Chem. 268, 21478–21481
80. Li, X., Lee, J. W., Graves, L. M., and Earp, H. S. (1998) EMBO J. 17, 2574–2583
81. Eguchi, S., Numaguchi, K., Iwaki, H., Matsumoto, T., Yamakawa, T., Utsunomiya, H., Motley, E. D., Kawakatsu, H., Owada, K. M., Hirata, Y., Marumo, F., and Inagami, T. (1998) J. Biol. Chem. 273, 8890–8896
82. Stein-Gerlach, M., Kharchenko, A., Vogel, W., Ali, S., and Ulrich, A. (1995) J. Biol. Chem. 270, 24635–24637
The Protein-tyrosine Phosphatase SHP-2 Is Required during Angiotensin II-mediated Activation of Cyclin D1 Promoter in CHO-AT 1A Cells
Laurent Guillemot, Arlette Levy, Zhizhuang Joe Zhao, Gilbert Béréziat and Bernard Rothhut

J. Biol. Chem. 2000, 275:26349-26358.
doi: 10.1074/jbc.M001614200 originally published online June 7, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M001614200

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 87 references, 58 of which can be accessed free at
http://www.jbc.org/content/275/34/26349.full.html#ref-list-1