Angiotensin II-induced Transcriptional Activation of the Cyclin D1 Gene Is Mediated by Egr-1 in CHO-AT1A Cells*

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Cyclin D1 protein expression is regulated by mitogenic stimuli and is a critical component in the regulation of G1 to S phase progression of the cell cycle. Angiotensin II (Ang II) binds to specific G protein-coupled receptors and is mitogenic in Chinese hamster ovary cells stably expressing the rat vascular Ang II type 1A receptor (CHO-AT1A). We recently reported that in these cells, Ang II induced cyclin D1 promoter activation and protein expression in a phosphatidylinositol 3-kinase (PI3K)-, SHP-2-, and mitogen-activated protein kinase (MEK)-/extracellular signal-regulated kinase (MAPK/ERK)-dependent manner (Guillemot, L., Levy, A., Zhao, Z. J., Béréziat, G., and Rothhut, B. (2000) J. Biol. Chem. 275, 26349–26358). In this report, transfection studies using a series of deleted cyclin D1 promoters revealed that two regions between base pairs (bp) −136 and −96 and between bp −29 and +139 of the human cyclin D1 promoter contained regulatory elements required for Ang II-mediated induction. Mutational analysis in the −136 to −96 bp region provided evidence that a Sp1/early growth response protein (Egr) motif was responsible for cyclin D1 promoter activation by Ang II. Gel shift and supershift studies showed that Ang II-induced Egr-1 binding involved de novo protein synthesis and correlated well with Egr-1 promoter activation. Both U0126 (an inhibitor of the MAPK/ERK kinase MEK) and wortmannin (an inhibitor of PI3K) abrogated Egr-1 endogenous expression and Egr-1 promoter activity induced by Ang II. Moreover, using a co-transfection approach, we found that Ang II induction of Egr-1 promoter activity was blocked by dominant-negative p21 Ras, Raf-1, and tyrosine phosphatase SHP-2 mutants. Identical effects were obtained when inhibitors and dominant negative mutants were tested on the −29 to +139 bp region of the cyclin D1 promoter. Taken together, these findings demonstrate that Ang II-induced cyclin D1 up-regulation is mediated by the activation and specific interaction of Egr-1 with the −136 to −96 bp region of the cyclin D1 promoter and by activation of the −29 to +139 bp region, both in a p21 Ras/Raf-1/MEK/ERK-dependent manner, and also involves PI3K and SHP-2.

The control of mammalian cell proliferation by extracellular signals in G1 to S phase progression of the cell cycle is largely mediated by serine/threonine cyclin-dependent kinases CDK4 and CDK6, which interact with specific D-type cyclins. The CDK-D-type cyclin complexes induce phosphorylation of the retinoblastoma protein (pRb), thereby releasing the transcription factor E2F, which is required for the transcription of S phase-specific genes (1–4). Activation by mitogenic stimuli of D-type cyclins during the G1 phase appears to be an essential and rate-limiting step in G1 to S phase progression of the cell cycle (5–7). The cyclin D1 gene expression seems to be essentially regulated at the transcription level. The promoter region of the cyclin D1 gene contains multiple potential cis-regulatory elements including binding sites for AP1, E2F, Oct, Egr-1, Sp1, ATF/CREB, NF-κB, and signal transducers and activators of transcription (7–12). Transcriptional activation of the cyclin D1 gene occurs in a cell type- and mitogen-specific manner. For instance, it has been shown that the AP1 binding site was implicated in Ang II-induced cyclin D1 activation in the human adrenal cell line H295R (13), whereas signal transducers and activators of transcription binding sites were involved in cytokine-dependent growth of hematopoietic cells (12) and Sp1 and a cAMP-responsive element in serum-stimulated vascular endothelial cells (9). Numerous studies have shown that cyclin D1 gene activation by growth factors and by Ang II is dependent upon the Ras/extracellular signal-regulated kinase pathway (13–18).

Ang II, the major effector molecule of the renin-angiotensin system, has long been implicated in the pathobiology of hypertension. This octapeptide hormone exerts diverse biological effects including induction of cell hypertrophy and/or hyperplasia and stimulation of hormone synthesis and ion transport in the heart, kidney, and adrenal (19). Ang II functions as a growth factor in vascular smooth muscle cells, cardiac fibroblasts, and Chinese hamster ovary cells stably expressing the rat vascular Ang II type 1A receptor (CHO-AT1A) (20–22). Many of the known biological actions of Ang II are mediated by stimulation of the AT1 receptor subtype, a member of the G protein-coupled seven-transmembrane-spanning receptor family (23). Signal transduction through the AT1 receptor involves phospholipase C, phospholipase A2, phospholipase D, adenylate cyclase, and the release of intracellular calcium (24–27). Moreover, Ang II mediates many intracellular signaling pathways similar to those induced by classical growth factors and

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The abbreviations used are: CDK, cyclin-dependent kinase; Ang II, angiotensin II; AT1A, angiotensin II type 1A receptor; bp, base pair; CHO, Chinese hamster ovary fibroblast cell line; CS, catecholamine to serine mutant; Egr, early growth response protein; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PI3K, phosphatidylinositol 3-kinase; SH2, Src homology 2; SHP-2, SH2 domain-containing protein-tyrosine phosphatase 2; WT, wild type; RASMC, rat aortic smooth muscle cell(s); SRE, serum response element; CHX, cycloheximide.
cytokines. Ang II induces rapid tyrosine phosphorylation and activation of phospholipase C-γ1 (28), the Janus kinase/signaling transducers and activators of transcription pathway (29), protein-tyrosine phosphatase SHP-2 (18, 30), MAPK/ERK (31, 32), and phosphatidylinositol 3-kinase (PI3K) (33). Ang II also activates c-Jun N-terminal kinase (34) and p38/MAPK (35). The array of genes activated by Ang II includes those encoding growth factor receptors (36–38), genes encoding extracellular matrix proteins (39–41), and several immediately early growth response genes such as c-fos, c-jun, c-myc, and the early growth response gene-1 (egr-1) (42–46).

\textit{egr-1} (also known as NGFI-A, Krox24, Tis8, and zig268) (47) is a member of the immediate early gene family that encodes an 80–82-kDa nuclear protein. Egr-1 is a DNA-binding protein containing three zinc finger motifs that regulates gene transcription by interacting with a consensus G+C-rich sequence 5'-GCG(T/G)GGGGCG-3' (48). The expression of the Egr-1 protein is rapidly and transiently induced by growth factors and other extracellular signals and is a critical upstream mediator of cell proliferation (49), differentiation (50), and apoptosis (51). In turn, Egr-1 regulates expression of many genes such as those for growth factors, cytokines, and adhesion molecules (52–54).

In a recent study (18), we established that Ang II-induced cyclin D1 protein expression and promoter activation required p21ras, Raf-1, MEK, PI3K, and also the catalytic activity of SHP-2 and its Src homology 2 (SH2) domains through the regulation of MAPK/ERK activity. However, the regulatory mechanisms underlying Ang II induction of the cyclin D1 promoter are not known in CHO-AT1A cells. Here we show that the Egr-1 transcription factor is largely implicated in Ang II-dependent activation of the cyclin D1 promoter and that induction of DNA binding activity and transcriptional activity via the Egr-1 site is mediated by the Ras/MEK/ERK-dependent pathway. We also demonstrate that Ang II induction of Egr-1 and cyclin D1 is modulated by PI3K and SHP-2 in CHO-AT1A cells. In addition, we found that the second region stimulated by Ang II and located between nucleotides −29 and +139 of the 5'-untranslated region of the promoter is also regulated by Ras/MEK/ERK, PI3K, and SHP-2.

**EXPERIMENTAL PROCEDURES**

**Materials**—All culture media, Ang II, poly(dI-dC)-poly(dI-dC), and cyclheximide were purchased from Sigma. Acrylamide, salts, and other electrophoresis materials were obtained from ICN Pharmaceuticals unless otherwise specified. U0126, wortmannin, and LY294002 were purchased from Calbiochem. \(10^{-6} M\) ATP was obtained from PerkinElmer Life Sciences. LipofectAMINE PLUS reagent was from Life Technologies, Inc. Monoclonal anti-Sp1 (1C6), polyclonal anti-Jun (C-18), and anti-AP2 (C-18) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-mouse IgG-peroxidase conjugate and goat anti-rabbit IgG-peroxidase conjugate were from BioSys (Compiègne, France).

**DNA Plasmid Constructs**—Firefly luciferase reporter gene plasmids containing the full-length or various deletions (D1 O) of the −973 base pair human cyclin D1 promoter (−848, −543, −181, −136, −96, and −29) have been described previously (8). The different plasmids (HSV-TK) firefly luciferase reporter gene constructs were obtained from Dr. A. K. Rustgi (7). Dr. M. Bradnock kindly provided the −69 bp human Egr-1 promoter/reporter gene construct (pGGE) (55). The dominant negative Ras N17, the Raf-1 C4 mutants, and the different SH2 domains of SHP-2, 5 μg of the SH2 domains of SHP-2, 5 μg of the SHP-1 CS mutant, 5 μg of Egr-1 WT, 5 μg of Egr-1 RW mutant, or the corresponding empty vector) using LipofectAMINE PLUS reagent following the protocol provided by the supplier. To control transfection efficiency and to normalize firefly luciferase values in experiments using pT81 constructs, cells were co-transfected with 6 ng of the internal control vector pRL-TK (herpes simplex virus thymidine kinase promoter/luciferase expression). Twenty-four hours following transfection, cells were trypsinized and aliquoted into 12-well cell culture dishes (250,000 cells/well). 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 or not with inhibitors for 1 h and then stimulated for the indicated times with 10−7 M Ang II. Cells were lysed with 200 μl of passive lysis buffer (1×), and 20 μl aliquot was assayed for luciferase activity in a luminometer (Lumat LB9507, Berthold) using the kit from Promega.

**Preparation of Nuclear Extracts**—CHO-AT1A cells pretreated or not with inhibitors for 1 h and exposed to Ang II 10−7 M for the indicated times were washed once with ice-cold phosphate-buffered saline containing 1 mM Na2VO4 at 4 °C and then resuspended in 5 ml of cold phosphate-buffered saline. Cells were pelleted by centrifugation at 1300 rpm for 5 min at 4 °C. The pellet was resuspended in 500 μl of ice-cold hypotonic solution (buffer A) consisting of 5 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.2% Nonidet P-40, 50 mM NaF, 1 mM Na3VO4, 5 mM dithiothreitol, 0.1 mg/ml 4-(2-aminoethyl)-benzenesulfon fluoride, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin and repelleted by centrifugation at 5000 rpm for 4 min at 4 °C. The pellet was resuspended in 500 μl of ice-cold hypotonic solution (buffer A) consisting of 5 mM HEPES, pH 7.9, 25% glycerol, 0.5 M NaCl, 1.5 mM MgCl2, 0.5 mM EDTA, 50 mM NaF, 1 mM NaN3, 5 mM dithiothreitol, 0.1 mg/ml 4-(2-aminoethyl)-benzenesulfon fluoride, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin. The nuclear extract was clarified by centrifugation at 15,000 rpm for 30 min at 4 °C. The supernatant fraction was centrifuged at 3000 rpm for 10 min at 4 °C, and then nuclei were lysed for 30 min at 4 °C in 30 μl of ice-cold solution (buffer C) consisting of 20 mM HEPES, pH 7.9, 25% glycerol, 0.5 M NaCl, 1.5 mM MgCl2, 0.5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 5 mM dithiothreitol, 0.1 mg/ml 4-(2-aminoethyl)-benzenesulfon fluoride, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin. The nuclear extract was clarified by centrifugation at 15,000 rpm for 30 min at 4 °C. The supernatant fraction was immediately frozen on dry ice and stored at −80 °C prior to use. Protein concentrations were measured with the bicinchoninic acid assay from Pierce.

**Electrophoretic Mobility Shift Assay (EMSA)**—Binding reactions for gel shift assays were performed in 20 μl of 10 mM HEPES, pH 8, 0.1 mM EDTA, 50 mM NaCl, 50 mM KCl, 5 mM MgCl2, 4 mM spermidine, 2 mM dithiothreitol, 0.1 mM bovine serum albumin, 4% Ficoll, 4 μg of poly(dI-dC)-poly(dI-dC), 32P-labeled oligonucleotide probe (200 cpmp) and 7–8 μg of nuclear extract. The reaction was allowed to continue for 10 min at 4 °C. In supershift studies, 2 μg of the appropriate affinity-purified antibody was preincubated with the crude nuclear extract for 10 min at 4 °C before addition of the labeled probe. In competition experiments, the nuclear extract was incubated with a 100-fold molar excess of the appropriate unlabeled competitor oligonucleotides. Electrophoresis of the different samples was carried out on 6% nondenaturating polyacrylamide gels with 0.5x TBE (45 mM tris(hydroxymethyl)aminoethane, 45 mM boric acid, 1 mM EDTA, pH 8) and run at 15 V/cm for ~2 h. The gel was dried under vacuum and exposed to Fuji Super RX film overnight at room temperature.

**Oligonucleotides Used—**The sense strand of the double-stranded oligonucleotides were as follows: Oligo A (cyclin D1 promoter spanning nucleotides −117 to −99), 5′-GGCCCCCGCCCCCGCCCCC-3′; Oligo A-TA, 5′-GGGGGCCGCTTACGCCCACCC-3′; Sp1(101), 5′-TGAAGCGCGCCCCAAGCGGA-3′, and NF1(32), 5′-CATATCGGCTTCAATCCAAAA-3′.

**Western Blotting**—Nuclear extracts prepared as described previously.
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RESULTS

Identification of Regions in the Human Cyclin D1 Promoter Required for Ang II-induced Gene Expression—To map cis-elements that were required for Ang II-induced transcriptional activity of the cyclin D1 promoter, we used a panel of luciferase promoter/reporter gene constructs in transient transfection experiments. Quiescent CHO-AT1A cells were transfected with the 5′-deleted constructs and were treated with 10⁻⁷ M Ang II for 24 h prior to lysis for luciferase assays. As shown in Fig. 1, Ang II stimulated cyclin D1 promoter/reporter gene activity (*) was observed for the D1Δ-96 and D1Δ-29 constructs compared with the D1Δ-136 construct (p < 0.05; Student’s t test).

FIG. 1. Characterization of the Ang II-inducible regions of the cyclin D1 promoter in CHO-AT1A cells. Quiescent CHO-AT1A cells transiently transfected with different luciferase (LUC) reporter gene constructs (D1Δ-973, D1Δ-848, D1Δ-543, D1Δ-181, D1Δ-136, D1Δ-96, or D1Δ-29) of the cyclin D1 promoter were treated or not with 10⁻⁷ M Ang II for 24 h and harvested for luciferase assays as described under “Experimental Procedures.” Luciferase activity is shown as the ratio of Ang II-stimulated to unstimulated samples. The data are presented as means ± S.E. (bars) from three independent experiments each performed in triplicate. A significant decrease in Ang II-stimulated promoter/reporter gene activity (*) was observed for the D1Δ-96 and D1Δ-29 constructs compared with the D1Δ-136 construct (p < 0.05; Student’s t test).

(18) were resuspended in SDS-sample buffer and boiled for 5 min at 95 °C. Reaction mixtures were size-fractionated by 10% denaturing SDS-polyacrylamide gel electrophoresis (10% gels), and transferred to nitrocellulose membranes. Blots were stained with Ponceau S to confirm equal loading of nuclear proteins and then probed with the indicated antibodies: monoclonal anti-Sp1 (1C8) or polyclonal anti-Egr-1 (C-19). Immunoblots were developed using appropriate secondary horseradish peroxidase-coupled antibodies and an enhanced chemiluminescence (ECL) kit (Pierce).

Ang II Induces the Specific Interaction of Nuclear Proteins with Cis-regulatory Elements between Nucleotides −136 and −96 of the Cyclin D1 Promoter—To determine whether Ang II induces a specific interaction between nuclear proteins from CHO-AT1A cells and the 3′ putative Egr/Sp1 motif, a 3²P-labeled double-stranded oligonucleotide (3²P-Oligo A) bearing the −117/−99 bp cyclin D1 promoter sequence (Fig. 2A) was incubated with nuclear extracts from CHO-AT1A cells exposed or not to 10⁻⁷ M Ang II for different times. Electrophoretic mobility shift assays revealed the formation of two major distinct nucleoprotein complexes (A and B) (Fig. 3A). However, only complex B was induced in response to Ang II. Time course experiments defined the transient nature with which this nucleoprotein complex formed. Complex B was totally absent in nuclear extracts from unstimulated cells but appeared within 24 h. Interestingly, induction of complex B seemed to be correlated with the earliest activation of the cyclin D1 promoter/reporter gene in CHO-AT1A exposed to Ang II (18).

The same experiment was performed with a double-stranded 3²P-labeled oligonucleotide (3²P-Oligo A-TA) bearing the mutated sequence of the 3′ putative Egr/Sp1 motif that corre-
sponds to the sequence found in mutant 1 and mutant 3 as described in the legend to Fig. 2A. Under these conditions, complex A normally present (Fig. 3B, lanes 1 and 2) in nuclear extracts from unstimulated cells or cells exposed to Ang II for 4 h was severely reduced when the mutated oligonucleotide was tested (Fig. 3B, lanes 3 and 4). Accordingly, Ang II was unable to induce the formation of complex B between this oligonucleotide and nuclear extracts of cells exposed to Ang II for 4 h (Fig. 3B, lane 4). These data are correlated with the previous observation using mutant 1 and mutant 3 in transient transfection experiments (Fig. 2B).

Next we performed competition studies to examine the binding specificity of the factors constituting complexes A and B. A 100-fold molar excess of unlabeled Oligo A abrogated the formation of both complexes formed with 32P-Oligo A in presence of nuclear extracts from unstimulated and Ang II-stimulated cells for 4 h (Fig. 4A, lanes 2 and 6). The same molar excess of an unrelated NF1 consensus oligonucleotide (NF1cons) had no effect (Fig. 4A, lanes 4 and 8). However, the same molar excess of an unlabeled Sp1 consensus oligonucleotide (Sp1cons) abrogated the formation of complex A (Fig. 4A, lanes 3 and 7) but not that of complex B induced by Ang II (Fig. 4A, lane 7). These results suggest that, in contrast to complex A, nuclear factor from complex B is not related to Sp1. Finally, supershift analysis was performed in an attempt to identify all these complexes (Fig. 4B). Complex A was partially supershifted when nuclear extracts from unstimulated cells or cells exposed to Ang II for 4 h were preincubated with an anti-Sp1 antibody (Fig. 4B, lanes 2 and 4). Incomplete supershifts with Sp1 antibodies have been reported elsewhere (52). The inducible complex B was completely supershifted when nuclear extracts from Ang II-stimulated cells were preincubated with an anti-Egr-1 antibody (Fig. 4B, lane 5). As a control, we used an antibody directed against AP-2, another zinc-finger transcription factor that interacts with the G + C-rich sequence, 5'-CATGGGGTGGCC-3' (57, 58). Under these conditions, this antibody failed to supershift any complex (Fig. 4B, lane 6), although it was able to supershift AP-2 protein (data not shown). Taken together, these results show that Ang II induces binding of Egr-1 transcription factor to the cyclin D1 promoter in a specific and transient manner.

Overexpression of a Mutant Form of Egr-1 Inhibits Ang II-induced Cyclin D1 Promoter Activity—To further investigate the functional role of Egr-1, we co-transfected an expression vector encoding either a WT or a mutant form of Egr-1 (RW) together with the cyclin D1 promoter constructs pT81 WT or pT81 mutant 2 (see Fig. 2). Overexpression of Egr-1 RW almost completely inhibited Ang II-induced WT and mutant 2 promoter activities (Fig. 5, lanes 3 and 6). In addition, the induction of both cyclin D1 promoter constructs by Ang II was increased by Egr-1 WT overexpression (Fig. 5, lanes 2 and 5). Altogether, these results show that Ang II-induced cyclin D1 promoter activity is mediated by Egr-1.

Ang II-induced Egr-1 Binding Activity Requires de Novo Protein Synthesis—To determine whether the specific interaction of the Egr-1 transcription factor with the cyclin D1 pro-
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**Fig. 3.** EMSA reveals an Ang II-inducible interaction of nuclear proteins with the 3′ putative Egr motif of the cyclin D1 promoter. A, nuclear extracts from CHO-AT1A cells treated or not (control (C)) with 10⁻⁷ M Ang II for the indicated times were incubated with the double-stranded oligonucleotide probe (32P-Oligo A) bearing the 3′ putative Egr site, which overlaps two Sp1 consensus motifs (see Fig. 2). The arrows indicate the position of two major nucleoprotein complexes (A and B). B, nuclear extracts from CHO-AT1A cells treated (lanes 2 and 4) or not (C, lanes 1 and 3) with 10⁻⁷ M Ang II for 4 h were incubated with either 32P-Oligo A (wild type) (lanes 1 and 2) or 32P-Oligo A-TA-(mutant) probes (lanes 3 and 4). Electrophoretic mobility shift assays were performed in the same way as in A. Nucleotide sequences of Oligo A and Oligo A-TA- probes are 5′-GCGCCGCCCCGCCCTAGCCCCCC-3′ and 5′-GCGCCGCCCCTAAGCCCCC-3′, respectively.

The experiments were performed in the presence of cycloheximide, and even after pretreatment with cycloheximide for 1 h (Fig. 6, lane 4). However, the Egr-1 complex was absent after Ang II stimulation in the continued presence of cycloheximide (Fig. 6, lane 5), although the noninducible Sp1 complex was not affected by this treatment. These results probably indicate that Ang II-mediated induction of the Egr-1 complex is dependent on de novo protein synthesis.

**Ang II-induced Endogenous Egr-1 Protein Expression Is Dependent on PI3K and MEK Activities in CHO-AT1A Cells**—To further analyze the transduction pathway following Ang II stimulation, we first determined the kinetics of Egr-1 protein expression. Quiescent CHO-AT1A cells were treated with 10⁻⁷ M Ang II and harvested at different times. The level of the Egr-1 protein was analyzed by immunoblotting nuclear extracts with a polyclonal anti-Egr-1 antibody (C-19). As shown in Fig. 7A, in unstimulated quiescent cells, Egr-1 protein was undetectable. Ang II treatment led to an increase in the Egr-1 level detectable at 1 h poststimulation, reaching a maximum at 4 h before declining by 24 h. Interestingly, this time course of Egr-1 protein induction nicely correlated with binding to the 32P-Oligo A probe (Fig. 3A).

We previously demonstrated the important role that MAPK/ERK and PI3K proteins play in Ang II-induced cyclin D1 protein expression (18). To investigate whether these proteins are also involved in Ang II-induced Egr-1 protein expression, we tested the effects of U0126, an inhibitor of MEK (the dual specific kinase that activates p44/p42 MAPK/ERK by phosphorylation), and wortmannin, an inhibitor of PI3K. Quiescent CHO-AT1A cells were pretreated or not for 1 h with U0126 at 50 μM or wortmannin at 200 μM. Then 10⁻⁷ M Ang II was added for an additional 4 h incubation period. Both drugs severely inhibited Ang II-induced Egr-1 protein expression (Fig. 7B, lanes 3 and 4). Similar results were obtained with the second PI3K inhibitor, LY294002 at 50 μM (data not shown). These results indicate that MAPK/ERK and PI3K are both required for up-regulation of Egr-1 protein expression in response to Ang II in CHO-AT1A cells, as previously observed for cyclin D1 protein expression. In contrast, the Sp1 protein was detectable in unstimulated quiescent cells (Fig. 7C, lane 1) but was not induced by Ang II (Fig. 7C, lane 2). Moreover, U0126 and wortmannin had no effect on Sp1 protein expression (Fig. 7C, lanes 3 and 4), and similar results were obtained with LY294002 (data not shown).

Altogether, these results indicate that Ang II regulates Egr-1 protein expression and demonstrate that this transcription factor can be considered as the modulator of cyclin D1 expression in CHO-AT1A cells.

**Ang II-induced Egr-1 Promoter Activity Is Dependent upon Ras/Raf-1/MEK/ERK, PI3K, and the Protein-tyrosine Phosphatase SHP-2**—To determine whether Ang II was able to induce Egr-1 promoter activity, we used the −697 bp human Egr-1 promoter fragment linked to the firefly luciferase reporter gene (pGLE) (55). Quiescent CHO-AT1A cells transiently transfected with pGLE were treated with 10⁻⁷ M Ang II for different times before luciferase assay measurements. As shown in Fig. 8A, Ang II induced Egr-1 promoter/reporter activity in a time-dependent manner, reaching 6.5-fold at 4 h, and this time was chosen for subsequent experiments. This
kinetics correlated exactly with the Ang II-induced increase in the Egr-1 protein expression shown in Fig. 7A.

We have previously demonstrated that p21ras, Raf-1, PI3K, and also the catalytic activity of SHP-2 and its SH2 domains were required for cyclin D1 promoter activation by Ang II through the regulation of the MAPK/ERK activity (18). To confirm the possible relationship between Ang II-induced Egr-1 protein expression and cyclin D1 promoter activity, we first tested the effects of the dominant negative Ras N17 mutant on Ang II-induced Egr-1 gene promoter activity. Quiescent CHO-AT1A cells transiently co-transfected with the Egr-1 promoter/luciferase construct and Ras N17 were treated with 10⁻⁷ M Ang II for 4 h before luciferase activity measurements. As shown in Fig. 8B, overexpression of Ras N17 reduced Ang II-induced Egr-1 gene promoter activity by ~50% (lane 2). Likewise, pretreatment of quiescent CHO-AT1A cells with 200 nM wortmannin partially inhibited the activity of the Egr-1 promoter by ~50% (Fig. 8B, lane 3). When the effect of Ras N17 overexpression in the presence of 200 nM wortmannin was tested, the inhibition was complete (Fig. 8B, lane 4). Similar results were obtained with 50 μM LY294002 (data not shown).

Total inhibition was observed when CHO-AT1A cells were transiently co-transfected with the dominant negative Raf-1 C4 mutant (Fig. 8B, lane 5) or when cells were pretreated with 50 μM U0126 (Fig. 8B, lane 6).

To further investigate the possible role of SHP-2, the dominant negative SHP-2 CS mutant and its SH2 domains were transiently co-transfected. Under these conditions, both forms strongly prevented the stimulation of the Egr-1 promoter/luciferase construct by Ang II (Fig. 8C, lanes 2–6). The catalytically inactive form of the closely related tyrosine phosphatase SHP-1 (SHP-1 CS) had no effect (Fig. 8C, lane 4), and co-transfection with the wild type SHP-2 did not affect the activity of the construct (data not shown).

Taken together, these results reveal that the Ras/Raf-1/MEK/ERK pathway, PI3K, and also the catalytic activity of SHP-2 and its SH2 domains are required to connect Ang II to cyclin D1 promoter activation by inducing Egr-1 transcription factor in CHO-AT1A cells.

**Ang II-induced Cyclin D1 Proximal Promoter Region Activity Is Dependent upon Ras/Raf-1/MEK/ERK, PI3K, and SHP-2**—We have shown in Fig. 1 that another Ang II-sensitive
region seemed to be located between nucleotides −29 and +139 of the cyclin D1 promoter. To gain insight into the transcriptional control of this region, we tested the expression of the dominant negative Ras N17 mutant on Ang II-induced D1A-29 promoter/reporter gene activity. Quiescent CHO-AT1A cells transiently co-transfected with D1A-29 and Ras N17 constructs were treated with $10^{-5}$ M Ang II for 24 h and then analyzed by luciferase activity assays. As shown in Fig. 9A, overexpression of Ras N17 inhibited the activity of the D1A-29 promoter/reporter gene construct by ~50% (lane 2). In the case of cells pretreated with 50 μM LY294002, the activity of this promoter construct was reduced by ~50% (Fig. 9A, lane 3). When the effect of Ras N17 overexpression was tested in the presence of 50 μM LY294002, the inhibition was complete (Fig. 9A, lane 4). Similar results were obtained with 300 nM wortmannin (data not shown). Complete inhibition was also observed when CHO-AT1A cells were transiently co-transfected with D1A-29 and the dominant negative Raf-1 C4 mutant (Fig. 9A, lane 5) or when CHO-AT1A cells were pretreated with 50 μM U0126 (Fig. 9A, lane 6).

The dominant negative SHP-2 CS mutant and the SH2 domains of SHP-2 were also transiently co-transfected with D1A-29 promoter/reporter gene construct. Under these conditions, Ang II induction was greatly inhibited (Fig. 9B, lanes 2 and 3). No effect was observed when SHP-1 CS (Fig. 9B, lane 4) or SHP-2 WT (data not shown) were used instead.

Taken together, these data indicate that Ang II-induced cyclin D1 proximal promoter region activity is dependent on the Ras/Raf-1/MEK/ERK pathway and PI3K activity but also on the catalytic activity of SHP-2 and its SH2 domains.

DISCUSSION

Cyclin D1 protein expression is regulated by mitogenic stimuli, and its assembly with cyclin-dependent kinases CDK4 or CDK6 is a rate-limiting step in the G1/S phase progression of the cell cycle. The aim of our study was to examine the transcriptional regulation of the cyclin D1 gene by Ang II in CHO-AT1A cells employing serial cyclin D1 promoter deletion constructs fused to the luciferase reporter gene. It was previously
reported that Ang II-induced cyclin D1 promoter activity relies on the binding of c-Fos and c-Jun only to the AP-1-responsive element located at −954 bp in the human adrenal cell line H295R (13). However, in CHO-AT1A cells, deletion of this putative AP-1 site did not reduce Ang II-induced promoter activity (Fig. 1). By using a similar approach, we also found an AP-1 independent regulation of cyclin D1 promoter in cultured rat aortic smooth muscle cells (RASMC). This effect has also been reported by others in transforming growth factor-α-treated esophageal squamous carcinoma cells (7) in estrogen-treated (10) and pp60<sup>v-src</sup>-expressing (59) human mammary carcinoma cells MCF-7, in cytokine-activated hematopoietic cells (12), and in serum-stimulated vascular endothelial cells (9). These observations argue in favor of a cell type- and agonist-specific effect. In CHO-AT1A cells, we have mapped a key region located between nucleotides −11002 and −96 that contributes to the activation of the cyclin D1 promoter by Ang II.

It was also reported that the ATF/cAMP-responsive element-binding protein-binding site in the cyclin D1 promoter was implicated in transcriptional activation of the gene (9, 10, 59). However, results from Fig. 1 reveal that further deletion of this site located between nucleotides −96 and −29 did not affect the

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2 L. Guillemot, A. Levy, and B. Rothhut, unpublished data.
Egr motif located at 74. We have previously reported that in CHO-AT1A cells, Ang factor binding activity with a cis-regulatory element in the induction of the early growth response (Egr-1) transcriptional cyclin D1 gene by Ang II is mainly mediated through the /H11002/tween nucleotides therefore identify another Ang II-responsive region located be- /H11032/tween nucleotides with plasmids containing a mutation in the 3/glycosylation of Sp1 leading to an increased susceptibility to proteasome degradation (65).

Using a functional approach, we show that cells transfected with plasmids containing a mutation in the 3’ Egr motif (mutant 1 and mutant 3) abolished Ang II-induced promoter/ reporter gene activity, while mutation in the 5’ Egr-like site (mutant 2) did not abolish this effect (Fig. 2B). Although several reports have suggested a role for Sp1 sites in the transcriptional activation of the cyclin D1 gene (9, 66), in CHO-AT1A cells, Sp1 does not seem to play a central role in Ang II-mediated cyclin D1 promoter activity but rather seems to be responsible for the basal expression of cyclin D1. Occupancy of the site may prevent the formation of a repressive chromatin structure (67). This could then explain the previously observed data with mutant 1 and mutant 3 in luciferase assays (Fig. 2B). Basal activities were severely diminished due to the possible inability of the mutated promoter sequences to bind the Sp1 protein, as also suggested in EMSAs (Fig. 3B, lanes 3 and 4). In addition, using RASMC, we found that the 3’ Egr motif on the cyclin D1 promoter was also regulated by Ang II. Indeed, with mutant 1 and mutant 3, Ang II-induced promoter activity was completely abolished.2 These results argue in favor of CHO-AT1A cells to represent a valid model with which to characterize Ang II-dependent gene activation.

egr-1 belongs to a family of genes termed immediate early genes, including c-fos, c-myc, and c-jun that are rapidly induced by signals stimulating mitogenesis and differentiation (68). The MAPK/ERK activation pathway has been implicated in the induction of egr-1 expression by urea (69), endothelin-3 (70), growth hormone (71), cellular stress (55, 72, 73), and Ang II (74). We have previously reported that in CHO-AT1A cells, Ang II-induced cyclin D1 promoter activity was dependent upon Ras/Raf-1/MEK/ERK, PI3K, and SHP-2 activities (18). Here we demonstrate that this induction is partly mediated by Egr-1 transcription factor synthesis and binding to an Egr response element of the cyclin D1 promoter in a specific and time-de- pendent manner. We show that Egr-1 is rapidly and tran- siently induced by Ang II and that this activation required de novo protein synthesis. In addition, U0126 completely inhibited Ang II-induced Egr-1 promoter activity and endogenous protein expression, while an inhibitor of PI3K and a dominant negative mutant (SHP-2 CS) or SH2 domains of SHP-2, which partially inhibited Ang II-induced ERK phosphorylation (18), also attenuated this effect. Taken together, these results suggest that Ang II-dependent activation of the Ras/MEK/ERK pathway contributes to Ang II-stimulated cyclin D1 expression through induction of Egr-1 and raise the possibility that PI3K and SHP-2 may regulate transcriptional activation of Egr-1. In addition, we found that the 29 bp proximal region of the cyclin D1 promoter is also dependent upon Ras/MEK/ERK, PI3K, and SHP-2 activities.

In CHO-AT1A cells, Ang II-responsive sequences of the Egr-1 promoter and interacting proteins have not yet been identified. The human Egr-1 promoter contains five serum response elements (SREs) organized into a downstream and an upstream cluster. It has been shown that transcriptional activation of this gene in response to human granulocyte-macrophage colony-stimulating factor, mouse interleukin-3, urea, and antigen cross-linking (69, 75, 76) is invariably mediated through SREs that are regulated by complexes composed of serum response factor and Ets protein family members. However, other functional cis-acting elements have been reported (i.e. EBS (Egr-1 binding site) (48) and CAMP response elements (77)). SRE/Ets binding sites on the Egr-1 promoter are occupied by multiprotein complexes that are similar to the ternary complex described for the c-fos SREs. Possible candidates of MAPK/ERK activation could be the Ets domain-containing proteins Elk1, Sap-1a, and Fli-1, members of the ternary complex factor family of transcription factors. Indeed, a ternary complex of serum response factor and Elk-1 or Sap-1a bound to SRE has been shown to mediate growth hormone-induced transcription of Egr-1 (78). Both Elk-1 and Sap-1a can be activated by ERKs through phosphorylation of serine residues (79–81). Elk-1 but not c-Jun activity is involved in Egr-1 activation by fluid shear stress (55), and the Ets protein Fli-1 and serum response factor form a complex on the SRE of the Egr-1 promoter, which is required for egr-1 gene transcription (82).

SHP-2 has been recently implicated as a positive regulator of the Egr-1 promoter by leptin receptor stimulation via activation of the MAPK/ERK pathway (83). Here we demonstrate for the first time that G-protein-coupled receptor induction of cyclin D1 is mainly mediated by Egr-1 and that the pathways connecting Ang II to this activation are dependent on Ras/MEK/ERK, PI3K, and SHP-2 activities.

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