Angiotensin II (AII) binds to specific G protein-coupled receptors and is mitogenic in adrenal, liver epithelial, and vascular smooth muscle cells. Since the cyclin D1 gene encodes the regulatory subunit of the cyclin D1-dependent kinase (CD1K) required for phosphorylation of the retinoblastoma protein (pRB), an essential and rate-limiting step in G1 phase progression of the cell cycle, we examined the effect of AII on cyclin D1 expression and CD1K activity in the human adrenal cell line H295R. AII (10^{-8} M) stimulated G1 phase progression within 12 h, with a maximal effect after 72 h. This action was antedated by the induction of cyclin D1 mRNA (3-fold), cyclin D1 nuclear protein abundance (4-fold), and CD1K activity (4-fold). All induced cyclin D1 promoter activity 4-fold, via the AT1 receptor through an enhancer sequence at -954 base pairs. c-Fos and c-Jun bound the cyclin D1 -954 enhancer sequence, and the abundance of c-Fos within this complex was increased by AII treatment. All induced extracellular signal-regulated kinase (ERK) activity 7-fold, and dominant-negative mutants of either p21^{ras} or ERK reduced AII-stimulated cyclin D1 promoter activity. These findings suggest that AII may stimulate mitogenesis by increasing CD1K activity through a p21^{ras}/ERK/activator protein 1 pathway.

The octapeptide angiotensin II (AII) binds to specific high affinity receptors present in the adrenal cortex, liver epithelial cells, and in vascular smooth muscle cells, where it elicits a vast array of biological effects. AII increases DNA synthesis, cell proliferation, and steroidogenesis in cultured adrenal cortical cells, whether the cells are derived from the adrenal fasciculata or glomerulosa cell layer (1, 2). AII also functions as a growth factor in cardiac fibroblasts, myocytes, and vascular smooth muscle cells (3–5). Many of the known biological actions of AII, including enhanced DNA synthesis, are mediated by stimulation of the AT1 receptor (1, 2).

The AT1 receptor is a member of the G protein-coupled seven-transmembrane spanning receptor family (6, 7). Binding of AII to the AT1 receptor activates phospholipase C, which initiates a rapid release of inositol trisphosphate and diacylglycerol from phosphatidylinositol 4,5-bisphosphate, causing intracellular calcium release (8). The intracellular transmission of signaling by Ca^{2+} and activation of cytosolic phospholipases involves, in part, sequential activation of p21^{ras} (9–11) and thereby protein kinases, including the mitogen-activated protein kinases (MAPKs) (7, 12). Previous studies showed that AII can stimulate phosphorylation of several intracellular signaling protein kinases at tyrosine residues including the extracellular signal-regulated kinases (ERKs) in vascular cells (13, 14) and the related Stress Activated Protein Kinases (SAPKs or Jun N-terminal Kinases in hepatic cells (15)). In addition, AII stimulates tyrosine phosphorylation of p44/p56^{SHC} (13), the Jak family proteins Jak2 and Tyk2 (16), and focal adhesion kinase (FAK) in vascular smooth muscle cells (13).

Although these pathways of AII-mediated signal transduction have been studied extensively, the steps critical for the proliferative action of this hormone are not fully elucidated. Several transcription factors have been implicated in the proliferative signaling pathway induced by AII. The STAT proteins, STAT1 and STAT2, are transcriptionally activated upon AII-induced tyrosine phosphorylation (16). The STAT proteins contribute to the transcription factor complex known as SIF (sis inducing factor), and SIF binding is stimulated by AII in cardiac fibroblasts (17). SIF binds to the sis-inducible element sequences found in the promoter region of the c-fos and other immediate-early genes (18). In cultured adenocortical and smooth muscle cells, AII increases the abundance of the mRNA encoding the early genes c-fos and c-jun (19–21), which contribute to activity of the AP-1 transcription factor complex (22). It is likely that AII may induce these immediate-early genes through a STAT/SIF pathway.

The retinoblastoma protein (pRB) plays a critical role as an intermediary protein in proliferative responses (23, 24). For instance, the vascular proliferation induced by intimal trauma was abrogated by the introduction of a dominant-negative mutant of the pRB protein, suggesting that inactivation of pRB was critical for normal proliferative signaling in vascular cells (25). Whether or not AII-mediated proliferative signaling involves inactivation of the pRB protein remains to be investigated. Inactivation of pRB is normally achieved by phosphorylation that is mediated by serine/threonine cyclin-dependent kinases (CDKs) (23, 24, 26). A regulatory subunit of the G1 phase CDKs, cyclin D1, forms a complex with the catalytic
partners CDK4 and CDK6 to form an active holoenzyme that phosphorylates pRB (23, 24, 26). Cyclin D1 is required for progression of the G1 phase and is, therefore, a critical target for proliferative signals in G1 (23, 24). Immunoneutralization and antisense studies have demonstrated that cyclin D1 is capable of shortening the G1 phase of the cell cycle, indicating that cyclin D1 is rate-limiting in fibroblast G1 phase progression (27–29). Cyclin D1 expression is induced by several different growth factors including colony stimulating factor (CSF-1) and epidermal growth factor (30–32). A role for cyclin D1 in AII signaling, to our knowledge, has not been examined. Several of the intermediary proteins implicated in AII signaling, including p21^{ras} and c-Jun, however, are capable of inducing either cyclin D1 mRNA levels or promoter activity in fibroblast cell lines (31, 33, 34). Accordingly, we reasoned that this hormone could stimulate cyclin D1 as well.

As AII induces cellular proliferation and DNA synthesis in adrenal cortical cells (1, 2), we used a human adrenal cell line to investigate a possible role of AII in regulating cyclin D1 expression. Because phosphorylation of the pRB protein appears to be necessary for its inactivation and this may be required for cell cycle progression, we examined the ability of AII to stimulate cyclin D1-dependent kinase activity using pRB as a substrate. Since we observed that AII induced cyclin D1 expression and promoter activity, we further examined the hypothesis that p21^{ras} and ERK may be involved in AII-mediated induction of the cyclin D1 promoter.

**MATERIALS AND METHODS**

**Construction of Plasmid Vectors**—The human cyclin D1 promoter was linked to the luciferase reporter gene using the vector pA3LUC (31). This vector includes the trimeredized SV40 poly(A) termination site, which abolishes transcriptional readthrough (35) and does not contain the AP-1-responsive vector backbone sequences (36). A series of 5′ promoter deletion constructions derived from this plasmid were described recently (31).

The human c-fos promoter from −361 to +157 was cloned by PCR using oligodeoxyribonucleotide probes to the published sequence and human genomic DNA and was subcloned into the pA3LUC reporter. The 5′ oligo primer sequence was 5′-GGT ACC CCC CCG CAG CAG TTC CCG-3′, and the 3′ oligo sequence was 5′-AAG CTG CCT CCG GGC TAG GCA AAG-3′. The vector pTP-LUX, which contains three collagenase AP-1 sites, was described previously (37, 38). Restriction enzyme analysis and dideoxy DNA sequencing using an Applied Biosystems 373 automated sequencer confirmed the integrity of these constructs.

**Expression Vectors**—The wild-type and dominant-negative human ERK expression vector pCMV-p41^{Euki} (ERKwt), pCMV-p41Ala^{h} Ala^{55}p90 (ERK h) (39), the Xenopus dominant-negative p42 ERK expression vector (ERK x) (40), and pGEM c-Fos and pGEM c-Jun were described previously (31, 37). The plasmids RSV Ras L61 (p21^{ras} mutated within the membrane targeting motif), and RSV Ras L61 S186 (p21^{ras} mutated within the membrane targeting motif), were expressed essentially as described previously (38). The human ribosomal protein L19 primers were included in each PCR reaction, and relative cyclin D1 mRNA abundance was expressed in arbitrary light units (38). The background activity from the parental empty expression vector to avoid spurious effects of the expression vector cassette. The fold effect was determined for 600 ng of expression vector. Luciferase was determined for 600 ng at room temperature using an Autolumat LB 953 (EGG & G Berthold). Luciferase content was measured by calculating the light emitted during the initial 30 s of the reaction, and the values are expressed in arbitrary light units (38). The background activity from cell extracts was typically <150 arbitrary light units/30 s. Statistical analyses were performed using the Mann-Whitney U test. Significant differences were established at p < 0.05.

**Oligodeoxyribonucleotides and Electrophoretic Mobility Gel Shift Assays**—The AP-1 site of the cyclin D1 promoter, the wild-type AP-1 (CD1AP-1wt) site, and a mutant AP-1 (CD1AP-1mt) site were site synthetized as complementary oligodeoxyribonucleotide strands for electrophoretic mobility gel shift assays (EMSA). The antisense strands of these oligodeoxyribonucleotides were also used for transcriptional amplification of the promoter. For the sequence of the cyclin D1 promoter AP-1 site oligodeoxyribonucleotides, CD1AP-1wt was TCC ATT CTG ACT CAT TT TTT TTA, and CD1AP-1mt was TCC ATT CTG CCG CAT TT TTT TTA. The sequences of the wild-type collagenase AP-1 oligodeoxyribonucleotides (42) used as competitor in EMSA was AP-1wt 5′-CGC TTG ATG ATG CCG CCA GAA. The sequence of the 3′ CD1 antisense strand used in PCR transcriptional amplification of the promoter was 5′-TGG GCC TT CTC TGG GCA.

EMSA using nuclear extracts, in vitro translated proteins, or bacterially expressed c-Jun (Promega Corp., Madison, WI) were essentially as described previously (38). The cDNAs were transcribed in vitro and translated using the TNT-coupled reticulocyte lysate system according to the protocols of the suppliers (Promega). The synthesized lysates (5 μl) were incubated in a reaction mix (20 μl) consisting of 20 mM HEPES, pH 7.8, 50 mM KC1, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and 50 μg/ml poly(dI-dC) at room temperature for 15 min. γ^{32}P-Labeled oligonucleotides (50 fmol, 50,000 cpm) were added to the reaction and incubated at room temperature for an additional 15 min. The protein-DNA complexes were analyzed by electrophoresis through a 5% polyacrylamide gel, with 0.5 × Tris borate, EDTA buffer (TEB: 0.045 M Tris borate and 0.001 M EDTA) and 2.5% glycerol. For EMSA, 5–10 μg of nuclear extracts were used in binding buffer containing 20 mM HEPES, pH 7.4, 40 mM KC1, 1 mM MgCl2, 0.1 mM EDTA, and 0.1% Nonidet P-40, to which 0.5 ng of γ^{32}P-labeled probe and 2 μg of sonicated salmon sperm DNA were added. Supershifts were performed using antibodies referred to as c-Jun (KM-1) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and c-Fos antibody (Santa Cruz Biotechnology, Inc.). The reaction products were separated on 5% polyacrylamide gel run in 0.5 × Tris borate-EDTA at room temperature at 180 V for 2–4 h. The gels were dried and exposed to XAR5 (Kodak, Rochester, NY) radiographic film.

**Flow Cytometric Analyses**—Total RNA extracted from cells using Trizol reagent (Molecular Research, Inc., Cincinnati, OH), as described by the manufacturer, was subjected to reverse transcription-polymerase chain reaction with specific amplification of cyclin D1 mRNA. The cyclin D1 oligodeoxyribonucleotides were synthesized to anneal to sequences from exons 2 and 5 (44), spanning two introns, to give a 528-bp product. The 5′ primer sequence is 5′-GTC TGC GAG GAA CAG AAG-3′ and the 3′ sequence is 5′-GCC GGC CCG GAG CCA GCA GTG-3′. The primer sequences of the human ribosomal protein L19 (45) used as an internal standard for reverse transcription-polymerase chain reaction are 5′-CCA GTA TGC TCA GCC TTC and 3′-TGC TCT TAG ACC TGC GGC CCT and produced a 500-bp PCR product. Human ribosomal protein L19 primers were included in each PCR reaction, and relative cyclin D1 mRNA abundance was expressed as a ratio after specific transcript to internal standard. The PCR products were analyzed by electrophoresing a 20-μl aliquot on a 5% polyacrylamide gel run in 0.5 × Tris borate-EDTA at room temperature at 250 V for 3 h. Autoradiography was performed at −70°C with an intensifying screen using XAR5 film or a phosphorimagereader. Initial confirmation of the specificity of the 528-bp cyclin D1 PCR product was determined through subcloning and sequence analyses. No amplified products were detected.
Western Blots—The abundance of cyclin D1 protein was determined by Western blotting as described previously (37) using a monoclonal cyclin D1 antibody (HD-11) (Santa Cruz Biotechnology, Inc.) and an antimumose second antibody. Reactive proteins were visualized by the enhanced chemiluminescence system (Amersham, Arlington Heights, IL). Quantitation was performed by densitometry using a Fuji Bio Imaging Analyzer BAS 2000.

AII Induces Cyclin D1 Kinase Activity

The effect of AII on nuclear cyclin D1 protein levels was measured by reverse transcription-polymerase chain reaction. Comparison of cyclin D1 mRNA abundance was made with the relative abundance of the human ribosomal protein L19 (45). Cyclin D1 mRNA was induced 4–5-fold at 6 h of exposure to AII (Fig. 1A). At 12 h, the abundance of cyclin D1 mRNA remained elevated 2–3-fold, although it started to decline and had returned to baseline at 24 h (Fig. 1A).

The effect of AII on nuclear cyclin D1 protein levels was determined using Western blot analysis and the cyclin D1 antibody, HD-11. AII induced cyclin D1 protein levels 2-fold within 3 h, with a 5-fold increase at 6 h (Fig. 1B). Cyclin D1 protein abundance remained elevated at 12 and 24 h of treat-

**TABLE I**

| Untreated | 12 h | 36 h | 48 h |
|-----------|------|------|------|
| G0-G1     | 73 ± 2 | 61.2 ± 4 | 60.2 ± 3.1 | 58 ± 7 |
| S         | 17.4 ± 0.6 | 12.3 ± 2.7 | 24.4 ± 2.6 | 26 ± 2 |
| G2-M      | 9.7 ± 1.6 | 25.4 ± 4.9 | 14.5 ± 3.5 | 15 ± 5 |

in PCR reactions that lacked reverse transcriptase or mRNA. Quantitation was performed by densitometry using a Fuji Bio Imaging Analyzer BAS 2000.

**p42ERK, p44ERK, and SAPK Immune Complex Assays—**Assays were performed as described previously (31, 37, 46) on cell extracts from H295R cells treated with AII (1 × 10⁻⁶ m) or vehicle. For assays, stage-specific protein A-Sepharose beads were incubated with either anti-MAPK antibody (C10) (Santa Cruz Biotechnology) or anti-SAPK antibody (46) for 1 h at 4 °C. The antibody and beads were washed once with RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 1% SDS, 50 mM Tris, pH 7.5, with 0.1 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin/ml, and 1 mM sodium orthovanadate (Sigma), 2 μM leupeptin, and 100 μM phenylmethylsulfonyl fluoride) and then incubated with cell lysates for 2 h at 4 °C. The immunoprecipitates were washed with RIPA buffer once, with LiCl/0.1 M Tris Base, pH 8.0 twice, and once in kinase buffer. The kinase reactions were performed at room temperature for 20 min in 30 μl of kinase buffer with 10 μCi of [γ-³²P]ATP (3000 Ci/mmol; 1 Ci, 37 GBq) and 34 μg of myelin basic protein or 4 μg of GST-c-Jun fusion protein. The samples were analyzed by SDS-polyacrylamide gel electrophoresis upon termination of the reaction with Laemmli buffer and boiling. The phosphorylation of myelin basic protein or GST-c-Jun was quantified by densitometry using a Fuji Bio Imaging Analyzer BAS 2000.

**Cyclin D1 Immune Complex Assays—**Immunoprecipitation kinase assays were performed essentially as described previously (47). Cells were suspended at 1 × 10⁶/mlin immunoprecipitation (RIPA) buffer at 4 °C. Lysates were centrifuged at 10,000 × g for 5 min, and the supernatants were precipitated for 4 h at 4 °C with protein A-Sepharose beads precoated with saturating amounts of the cyclin D1 antibody (DCS-11; NeoMarker, Freeman, CA). Immunoprecipitated proteins on beads were washed twice with 1 ml of RIPA buffer and twice with kinase buffer (50 mM HEPEs, pH 7.0, 10 mM MgCl₂, 5 mM MnCl₂, 1 mM dithiothreitol, and 10 μM ATP). The beads were then suspended in 40 μl of kinase buffer containing the protein substrate (2 μl of soluble glutathione S-transferase-RB fusion protein, 2.5 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1 mM NaF, 20 μM ATP, and 10 μCi of [γ-³²P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq). The samples were incubated for 30 min at 30 °C with occasional mixing, and the samples were then run in boiling in polyacrylamide gel sample buffer containing sodium dodecyl sulfate and separated by electrophoresis. Phosphorylated proteins were visualized by autoradiography of the dried gels, and quantitation was performed by densitometry using a Fuji Bio Imaging Analyzer BAS 2000.

**RESULTS**

**AII Stimulates G₂ Phase Progression—**To determine whether AII stimulates cell cycle progression in H295R cells, flow cytometric analysis was performed. Cells were treated with AII (10⁻⁶ m) for 6–72 h, and the proportion of cells in G₁, S, and G₂-M was determined at 12, 36, and 48 h (Table I). In six separate experiments (Table I), the relative proportion of cells in G₁ was reduced from 73 to 58% (p < 0.05) at 48 h, concomitant with an increase in cells in S phase (from 17 to 26%, p < 0.05) (Table I). The proportion of cells in G₂-M increased from 9.7 to 15% (p < 0.05). At 72 h, the proportion of cells in G₁ phase was further reduced to 51% (data not shown). Together, these studies indicate that AII decreases the proportion of cells in G₁ and increases the proportion of cells in S phase and G₂-M, and that the effect persists after 48 h of treatment.

**AII Stimulates Cyclin D1 Expression and Kinase Activity—**To determine whether cyclin D1 expression is regulated by AII, the abundance of cyclin D1 mRNA was assessed using reverse transcription-polymerase chain reaction. Comparison of cyclin D1 mRNA abundance was made with the relative abundance of the human ribosomal protein L19 (45). Cyclin D1 mRNA was induced 4–5-fold at 6 h of exposure to AII (Fig. 1A). At 12 h, the abundance of cyclin D1 mRNA remained elevated 2–3-fold, although it started to decline and had returned to baseline at 24 h (Fig. 1A).

![Fig. 1. AII induces cyclin D1 protein and kinase activity in H295R cells. A, reverse transcription-polymerase chain reaction was performed using mRNA derived from the AII (10⁻⁶ m) treated H295R cells. Fold induction is shown with comparison to the human ribosomal protein L19 mRNA in the same samples. Data are shown for the mean of three separate experiments; bars, S.E. B, Western blot analysis of cyclin D1 protein levels, from H295R cells treated with AII (10⁻⁶ m) for the time points indicated, was conducted using the monoclonal cyclin D1 antibody HD-11. The control lane contains in-vitro translated cyclin D1. C, immune complex assays were conducted using extracts from H295R cells treated with AII for the time points indicated. Immunoprecipitation was conducted using the cyclin D1-specific antibody DCS-11, and phosphorylation of the GST-pRB substrate was performed as described in "Materials and Methods". The phosphorylated pRB band is indicated by the arrow (pRB).](http://www.jbc.org/doi/10.1074/jbc.M000000200)
ment at a time when mRNA levels were declining (Fig. 1, compare A and B).

Cyclin D1-dependent kinase activity was assessed by the immunoprecipitation assay, using the cyclin D1 antibody DCS-11 (49) with the GST-pRB protein as a substrate (47). In H295R cells treated with AII for 3–24 h, cyclin D1 kinase activity increased 2-fold after 12 h of treatment with AII and 6-fold after 24 h of treatment (Fig. 1C). Thus, the increase in activity was delayed by several hours compared to the increase in cyclin D1 mRNA (Fig. 1A) and protein levels (Fig. 1B), which were observed after only 6 h of exposure to AII.

**ALL Stimulates Cyclin D1 Promoter Activity in an AT$_1$-dependent Manner**—To determine whether AII was capable of inducing cyclin D1 promoter activity, the −1745 bp human cyclin D1 promoter fragment linked to the luciferase reporter gene (−1745 CD1LUC) was transiently transfected into H295R cells. Cells were then treated with AII to determine whether this hormone induces the cyclin D1 promoter and whether such an effect is concentration-dependent. AII stimulated cyclin D1 promoter activity in a dose-dependent manner, reaching a maximal increase (4-fold above basal) at 10$^{-7}$ and 10$^{-6}$ M (n = 28; Fig. 2, A and B). Stimulation (2-fold) was observed at 10$^{-9}$ M AII. The 4-fold induction of the human cyclin D1 promoter by AII was completely inhibited by the AT$_1$ receptor antagonist L-158,809 (10$^{-6}$ M). A representative example from six separate experiments is shown in Fig. 2A).

To examine further the specificity of the AII-dependent induction of the cyclin D1 promoter, cells were transfected with other native or synthetic promoters with identical plasmid construction in which sequences resembling an AP-1 site within this region were mutated (−964 CD1AP-1mtLUC). Basal LUC activity of the −964 CD1LUC reporter was established as 100%. The basal activity of −964 CD1AP-1mtLUC was approximately 40% of the −964 CD1LUC reporter. The data are shown as the means (bars, S.E.).

![Figure 2](http://www.jbc.org/)

**Fig. 2. AII activation of the cyclin D1 promoter requires the AT$_1$ receptor.** A, dose-response curves were conducted with the −1745 CD1LUC reporter with AII alone or in the presence of L-158,908 (10$^{-6}$ M). A representative example from six separate transfections is shown. B and C, the effect of AII (10$^{-6}$ M) on −1745 CD1LUC and several other promoters was assessed in H295R cells; bars, S.E.

8-fold above basal at 10$^{-6}$ M. The induction of the c-fos LUC reporter by AII was also inhibited by the AT$_1$ receptor antagonist L-158,809 (10$^{-6}$ M) (Fig. 2C).

**A Distal Enhancer Sequence Is Required for AII-dependent Induction of the Cyclin D1 Promoter**—The DNA sequences required for AII-dependent induction of the cyclin D1 gene were investigated further using a series of 5' promoter deletions of the cyclin D1 promoter (Fig. 3A). Deletion of sequences from 1093 to −964 did not affect induction by AII, whereas deletion from −964 to −420 reduced induction from 3- to 1.3-fold (Fig. 3B). The region of the cyclin D1 promoter required for activation by AII was, therefore, located between −964 and −420. Within this region, sequences resembling an AP-1 site were identified. The effect of point mutations of these AP-1-like enhancer sequences was determined. The AP-1 site at −1093 was completely abolished by AII (1.4-fold, Fig. 3C).

![Figure 3](http://www.jbc.org/)

**Fig. 3. AII activation of the cyclin D1 promoter requires a distal enhancer region.** A, schematic representation of the cyclin D1 5' promoter sequences linked to the LUC reporter gene. Sequences resembling enhancer binding sites are shown schematically. B, the effect of AII (10$^{-6}$ M, 12 h) on the 5' promoter deletions of the cyclin D1 promoter was determined in H295R cells. The mean data (bars, S.E.) of N separate transfections, as indicated in the figure, is shown. C, the effect of AII on the −964 CD1LUC reporter was compared with a construction in which sequences resembling an AP-1 site within this region were mutated (−964 CD1AP-1mtLUC). Basal LUC activity of the −964 CD1LUC reporter was established as 100%. The basal activity of −964 CD1AP-1mtLUC was approximately 40% of the −964 CD1LUC reporter. The data are shown as the means (bars, S.E.).
immune complex assays were performed on extracts derived from H295R cells. To determine whether AII induces ERK activity in H295R cells, expression vectors encoding the dominant-negative (Ras Leu 61/Ser 186, which is incapable of insertion in the plasma membrane) were transfected with either the empty expression vector cassette. Ras N17 reduced AII-induced cyclin D1 promoter activity required sequences within the −954 region AP-1 site, EMSA was performed with nuclear extracts from H295R cells to characterize the nature of the complex binding this site (Fig. 6A). Comparison was made with the binding of nuclear protein to the collagenase AP-1 site probe. The cyclin D1 AP-1 site bound a complex with similar electrophoretic mobility to the complex binding the wild-type collagenase AP-1 site probe. The cyclin D1 AP-1 site bound a complex with similar electrophoretic mobility to the complex binding the wild-type collagenase AP-1 site probe (Fig. 6A, lanes 1 versus 8). Binding to the cyclin D1 AP-1 site was competed by 100-fold molar excess of cyclin D1 AP-1 site probe or 100-fold molar excess of the collagenase AP-1 site probe (Fig. 6A, lanes 2 and 4). Mutant AP-1 sequences did not inhibit binding of the nuclear complex to the cyclin D1 −954 region (Fig. 6A, lane 3). The c-Jun antibody supershifted nuclear protein complex binding to the cyclin D1 −954 region (Fig. 6A, lane 5), whereas addition of the c-Fos antibody supershifted most of the complex binding to the cyclin D1 −954 region (Fig. 6A, lane 6). Equal amounts of control serum neither inhibited nuclear protein binding to the probe nor induced a supershift, indicating the specificity of the supershift observed (Fig. 6A, lane 7).

The effect of AII treatment on the nature of the complex binding to the cyclin D1 AP-1 site was examined using nuclear extracts from H295R cells treated with AII (10−8 m) for 1 to 24 h (Fig. 6B). When equal amounts of total nuclear protein were incubated with the cyclin D1 AP-1 site probe (Fig. 6B) or the collagenase AP-1 site (data not shown), the amount of protein binding increased with time of exposure to AII. The increase in binding was observed within 1 h and continued to increase to 24 h (Fig. 6B). Supershift assays were also performed using the c-Jun or c-Fos antibodies. The amount of c-Fos supershifted from the nuclear complex binding the cyclin D1 AP-1 site also increased with AII treatment (Fig. 6B). The amount of c-Fos supershifted from complexes bound to the collagenase AP-1 site also increased in nuclear extracts from cells treated with AII (data not shown). The c-Jun antibody efficiently shifted most of the complex binding the cyclin D1 AP-1 site at each of the time points studied, suggesting that c-Jun remained an important component of the complex binding this region in AII-treated cells.

**DISCUSSION**

Cyclin D1, the regulatory subunit of several CDKs, is required for and is capable of shortening the G1 phase progression of the cell cycle (27–29). The induction of cyclin D1 serine/threonine kinase activity promotes cell cycle progression and cellular proliferation by phosphorylating and inactivating the
activity occurs through temporally sequential induction and complex assembly. The induction of CDK activity followed an increase in protein abundance (26, 47, 51). The mechanisms responsible for the brief delay in AII-enhanced CDK activity after cyclin D1 protein levels had increased substantially (5-fold at 6 h) are unknown. The induction of CDK activity may require a greater amount of cyclin D1 protein or may require the association of CDK with other cofactors, such as the cyclin-associated kinase (26, 47, 51).

AII was further shown to stimulate the cyclin D1 promoter, and the dominant-negative p21mutant N17 mutant antagonized a component of AII-induced transcriptional induction of the cyclin D1 promoter. Because AII is involved in the normal proliferative response to intimal trauma (52–54) and dominant-negative p21mutants inhibited part of the vascular proliferative response induced by vascular injury in vivo (55), it is likely that p21mutants conveys an important component of AII signaling in vivo. Both p21mutants and pp60src have complementary roles in several different signal transduction pathways. A component of phospholipase C-1 activation by AII requires the pp60src in rat aortic smooth muscle cells (56). Thus, both p21mutants and pp60src have been implicated in AII signaling. Interestingly, overexpression of either p21mutants or pp60src was capable of inducing cyclin D1 expression and G1 phase progression in fibroblast cell lines (34). In our studies, constitutively active p21mutants induced cyclin D1 promoter activity. In addition, pp60src is also capable of activating the cyclin D1 promoter in a robust manner.2 Whether the residual p21mutants-independent component of AII-mediated induction of the cyclin D1 promoter involves pp60src remains to be determined.

Previous studies investigating the signal transduction pathway conveying the mitogenic action of AII demonstrated the rapid phosphorylation of intermediary kinases at tyrosine residues (13, 14, 16). The induction of tyrosine phosphorylation is a common feature of many other G protein-coupled receptors. AII activates phospholipase C and Ca++ pathways in cultured glomerulosa cells (8, 57), and induction of these secondary messengers has been shown to induce ERK (9–11). AII was also shown previously to stimulate tyrosine phosphorylation of ERK (13, 14). Our study demonstrates a requirement for AII in the induction of the cyclin D1 promoter by AII as the dominant-negative ERK mutants reduced AII-mediated cyclin D1 promoter activity. This finding is consistent with studies performed in other cell types in which the induction of ERK activity by AII was associated with cellular proliferation. Our studies provide a mechanism by which the induction of ERK activity by AII may be linked directly to proliferative signaling through inducing cyclin D1 expression and pRB phosphorylation.

Overexpression of ERK is capable of inducing cellular proliferation (58, 59), and suppression of ERK activity antagonizes cellular proliferative responses (60). ERK induction may impart genotypic cues that may vary with the cell type (60). The sustained induction of ERK activity is associated with a proliferative response in fibroblasts, whereas PC12 cells undergo differentiation (58–60). In the studies described herein, the induction of cell cycle progression by AII in H295R cells was associated with a rapid and sustained activation of ERK. The mechanisms by which activation of ERK triggers cellular proliferation are unknown: however, genes capable of modulating cell cycle progression, such as cyclin D1, represent likely targets.

The induction of AP-1 binding to the cyclin D1 AP-1 site by AII is likely mediated through a mechanism that involves the induc-

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2 G. Watanabe, R. J. Lee, and R. G. Pestell, unpublished data.
tion of c-Fos. All treatment increased c-Fos abundance within the AP-1 complex, and the induction of c-Fos enhanced the affinity of AP-1 proteins for AP-1 sites (61). Furthermore, the c-fos promoter was induced by AII in a p21wax and ERK-dependent manner (Fig. 2). Both ERK and SIF, which can be induced by AII (17), have been shown to induce the c-fos promoter (18, 62); ERK2 phosphorylates and potentiates the activation of TCF/Elk1 and thereby induces c-Fos expression (63, 64). At least a significant component of the TCF/Elk1-mediated activation is p21wax-dependent (62). Induction of ERK activity phosphorylates inhibitory DNA binding domains of c-Jun, thereby enhancing c-Jun binding to AP-1 sequences (61, 65).

AII is well known to activate through a G protein-coupled receptor signaling pathway (6, 7). The induction of SAPK activity by AII demonstrated in this study is consistent with a recent study in which overexpression of activating mutants of the ras gene product in vascular smooth muscle cells following angioplasty, which plays an important role in restenosis (52, 53). Because AII induces c-Fos and c-Jun in vascular smooth muscle cells (19, 20), it will be of interest to determine whether AII induces AP-1 activity, cyclin D1 expression, and thereby mitogenesis in these cells. Furthermore, constitutive activation of G protein-coupled receptors is associated in some circumstances with tumor formation, though the molecular targets involved remain to be elucidated (72). Since overexpression of cyclin D1 is associated with a variety of tumors and cyclin D1 overexpression in the breast of transgenic animals has been shown to induce breast tumor formation (73), it will be of interest to determine whether AII induces AP-1 activity, cyclin D1 expression, and thereby mitogenesis in these cells.

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