α1 Adrenergic Agonist Induction of p21waf1/cip1 mRNA Stability in Transfected HepG2 Cells Correlates with the Increased Binding of an AU-Rich Element Binding Factor

(Received for publication, November 3, 1999, and in revised form, January 19, 2000)

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Stimulation of transfected HepG2 cells (TFG2) with the α1-adrenergic agonist phenylephrine (PE) significantly activated p21waf1/cip1 gene expression without affecting p53 gene expression. Northern blotting and reporter assay demonstrated that this induction was due to PE stimulation of p21waf1/cip1 mRNA stability. To further define the underlying mechanism, we prepared a chloramphenicol acetyltransferase (CAT)-p21waf1/cip1 3′-untranslated region (3′-UTR) hybrid construct by inserting the 3′-UTR of p21waf1/cip1 mRNA just downstream from the CAT coding sequence and transfected it into TFG2 cells. PE treatment enhanced the activity of this construct by 6-fold. Deletion analyses indicated that an AU-rich element (AURE) located between 553 to 625 within the p21waf1/cip1 3′-UTR was required for this induction. RNA gel shift assays demonstrated that this AURE bound an RNA-binding protein. This protein has been purified 5000-fold from PE-treated TFG2 cells by heparin-Sepharose and RNA affinity chromatography. SDS-polyacrylamide gel electrophoresis, UV cross-linking, and Northern analyses indicated the molecular mass of this protein as 24 and 52 kDa. Finally, PE treatment markedly enhanced this RNA-protein binding by a p42/44 mitogen-activated protein kinase-dependent mechanism. These data suggest that the AURE located between 553 and 625 within the p21waf1/cip1 mRNA 3′-UTR, which binds an RNA-binding protein, is responsible for PE-induced p21waf1/cip1 mRNA stability.

α1-Adrenergic receptors (α1AR)1 are G-protein-coupled receptors that play an important role in key components of the sympatho-adrenal response to stress, such as peripheral vasconstriction, increased cardiac contractility, and hepatic glycogenolysis (1–3). In addition to such short term effects, activation of α1AR modulates the growth of a number of normal and malignant cells, including primary hepatocytes (4–7), cardiomyocytes (8, 9), smooth muscle cells (10, 11), and transfected Raf-1 fibroblasts (12). Interestingly, we have recently demonstrated that activation of α1AR significantly inhibited the proliferation of transfected HepG2 cells, which is mediated by a p21waf1/cip1-dependent mechanism (7).

p21waf1/cip1 is a cyclin-dependent kinase inhibitor that plays a critical role in mediating growth arrest in response to a variety of conditions associated with DNA damage, cell differentiation, or growth factor deficiency (13–16). The expression of the p21waf1/cip1 gene is controlled by transcriptional and posttranscriptional mechanisms (17). The transcriptional control of p21waf1/cip1 has been extensively investigated. It has been reported that the transcription of p21waf1/cip1 can be induced by p53 and other transcription factors, including E2F, AP2, Sp1, BRCA1, Smad, C/EBPα, STAT family, IRF-1, androgen receptor, RB-binding protein, etc. (for review, see Ref. 17, and references therein). Recent evidence suggests that posttranscriptional mechanisms also play an important role in the control of the p21waf1/cip1 gene expression. For example, posttranscriptional mechanisms have been implicated in the regulation of p21waf1/cip1 gene expression by epidermal growth factor (18), redox state (19), okadaic acid (20), retinoid acid (CD437) (21), phorbol myristate acetate (22), UV light (23), and genistein (24). However, the underlying mechanisms controlling p21waf1/cip1 mRNA stability are poorly understood. Here, we reported that activation of α1AR by phenylephrine (PE) significantly enhanced p21waf1/cip1 mRNA stability. Further studies demonstrated that an AU-rich element (AURE) within p21waf1/cip1 3′-untranslated region, which bind an AU-rich binding protein, was responsible for this induction.

EXPERIMENTAL PROCEDURES

Materials—p21waf1/cip1 and p53 antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). [γ-32P]ATP and [α-32P]UTP were obtained from NEN Life Science Products. HepG2 cells were stably transfected with α1bAR to generate TFG2 cells, as described previously (7).

Western and Northern Blotting Analyses—Western blotting and Northern blotting assays were described previously (25, 26).

Construction of Plasmids—The p21 promoter/CAT constructs (p2.4/CAT, p2.28/CAT, and p1.84/CAT) were kindly provided by Dr. Vogelstein (13, 15). The pCAT expression vector was prepared by subcloning the CAT coding region into pcDNA3 expression vector. The pCAT/p21b, pCAT/p21b1, and pCAT/p21b3 were prepared by inserting the p21waf1/cip1 3′-UTR region between +512 and +1924, +612 and +1999, and +512 and +708 (the A in the start codon was designed as +1) into the pCAT expression vector, respectively. Transient transfection and CAT assays were performed as described previously (26).

RNA Gel Mobility Shift Assay (RMSA)—TFG2 cells were resuspended in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 0.33 mM sucrose, 2 mM EDTA, 0.5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride and sonicated for 10 s, then spun at 100,000 × g for 1 h. The supernatant was collected as cytosol fraction and stored at −80 °C. The sense RNA probe used in RMSA was obtained by in vitro transcription of a polymerase chain reaction-generated DNA fragment designed to have a T7 RNA polymerase promoter at its 5′ end. For generating the
RNA probe corresponding the region from 553 to 625 in the 3′-UTR of the p21\textsuperscript{waf1/cip1} mRNA, the downstream primer was 5′-GGG TAT GGA CAT GAG GTG-3′, the upstream primer was 5′-TAA TAC GAC TCA TTA TAG GGA TCT TCT GCC TTA GTC TCA G-3′. The 20 nucleotides in boldface type correspond to the T7 promoter sequence, whereas the 3′ end of the probe corresponds to the sequence between 554 and 573 of the 3′-UTR of the p21\textsuperscript{waf1/cip1} mRNA. The RMSA analyses were similar to DNA gel mobility shift assays as described previously (25).

**RESULTS**

**PE Induction of p21\textsuperscript{waf1/cip1} Protein Is Due to Stabilization of the mRNA**—Our previous results demonstrated that activation of αβAR with PE for 24 h significantly increased p21\textsuperscript{waf1/cip1} protein expression in TFG2 cells (7). Here, the time course of PE induction of p21\textsuperscript{waf1/cip1} protein and mRNA expression was examined. As shown in Fig. 1, PE stimulation significantly increased the p21\textsuperscript{waf1/cip1} protein (Fig. 1A) and mRNA (Fig. 1B) expression, with a 3–4-fold induction noted at 4 h, whereas p53 protein (Fig. 1A) and mRNA (Fig. 1B) expression remained unaffected. To examine whether de novo protein synthesis is required for the PE-induced increase in p21\textsuperscript{waf1/cip1} mRNA in TFG2 cells, cycloheximide (40 μg/ml) was used to block protein synthesis. PE-induced p21\textsuperscript{waf1/cip1} mRNA expression was similar in the presence or absence of cycloheximide (data not shown). This indicates that PE-induced p21\textsuperscript{waf1/cip1} mRNA expression does not require the synthesis of new protein.

Next we examined the effect of PE on p21\textsuperscript{waf1/cip1} mRNA stability. As shown in the top panel of Fig. 1C, PE stimulation significantly increased the p21\textsuperscript{waf1/cip1} mRNA stability. The data from three independent experiments were plotted in the bottom panel of Fig. 1C; the half-life of the p21\textsuperscript{waf1/cip1} mRNA in control TFG2 cells was 62 min, while after incubation with PE for 2 h the half-life was 185 min. The effect of PE on p21\textsuperscript{waf1/cip1} gene promoter was also investigated in Fig. 1D; PE exposure for 24 h did not significantly enhance the activity of various p21\textsuperscript{waf1/cip1} promoter/CAT constructs. Taken together, these data suggest that PE-induced p21\textsuperscript{waf1/cip1} mRNA expression occurs mainly through stabilization of p21\textsuperscript{waf1/cip1} mRNA.

**Evidence for the Involvement of an AURE in PE Induction of p21\textsuperscript{waf1/cip1} mRNA Stability**—To further study the mechanism involved in the PE induction of p21\textsuperscript{waf1/cip1} mRNA stability, we prepared several CAT-p21\textsuperscript{waf1/cip1} 3′-UTR hybrid constructs by inserting various segments of the p21\textsuperscript{waf1/cip1} 3′-UTR downstream of the CAT cDNA, as described under “Experimental Procedures.” As shown in Fig. 2A, the pCAT construct, which does not contain the p21\textsuperscript{waf1/cip1} 3′-UTR, showed the highest activity, which was unaffected by PE treatment. After a 1.5-kilobase pair segment of the p21\textsuperscript{waf1/cip1} 3′-UTR was inserted into the pCAT, the CAT activity of the resulting pCAT/p21b construct was significantly declined by 90%; while PE treatment enhanced this activity by about 6-fold. Examination of the p21\textsuperscript{waf1/cip1} 3′-UTR sequence revealed an AURE located between 583 and 625. To identify the role of this element, we deleted it from pCAT/p21b to generate pCAT/p21b1. Surprisingly, deletion of this AU-rich element still inhibited the pCAT
activity by 90%, but PE treatment only stimulates the CAT/p21b1 activity by 2-fold. Inserting the AURE alone in the pCAT construct (pCAT/p21e5 construct) did not significantly inhibit the activity of CAT, which is consistent with a previous report (21). PE treatment did not significantly affect the pCAT/p21e5 activity.

To further examine whether PE induction of the CAT activity is due to stabilization of CAT mRNA, the pCAT, pCAT/p21b, and pCAT/p21b1 constructs were transiently transfected into TFG2 cells. As illustrated in Fig. 2B, PE treatment did not significantly affect CAT mRNA stability. Inserting the whole p21\textsuperscript{waf1/cip1} 3′-UTR (CAT/p21b2) significantly destabilized the CAT/p21b mRNA compared with pCAT construct, PE treatment markedly enhanced the CAT/p21b1 mRNA stability. Inserting the mutated p21\textsuperscript{waf1/cip1} 3′-UTR (CAT/p21b1) in which the AURE was deleted, still significantly destabilized the CAT/p21b1 mRNA, but PE treatment did not enhance the CAT/p21b1 mRNA stability. This suggests that the AURE between 583 and 625 within the p21\textsuperscript{waf1/cip1} 3′-UTR is responsible for PE induction of p21\textsuperscript{waf1/cip1} mRNA stability.

**Purification and Characterization of an AURE Binding Factor (AUBF) That Binds to the AURE Located at 583–625 within the p21\textsuperscript{waf1/cip1} 3′-UTR**—To further define how the AURE is involved in PE induction of p21\textsuperscript{waf1/cip1} mRNA stability, we performed EMSA to check whether any RNA-binding proteins specifically bind to this region. As shown in Fig. 3A, the radiolabeled RNA fragment e5 (see Fig. 2A) corresponding to the AURE 583–625 within the p21\textsuperscript{waf1/cip1} 3′-UTR binds a major complex specifically (lane 1), as it was competed away by the unlabeled RNA probe (lane 2). Next, we purified this protein by using heparin and RNA affinity column. TFG2 cells were treated with PE for 5 h (PE treatment significantly enhanced the binding; see below), after which crude cell extracts were prepared and applied to heparin-Sepharose column and eluted by a step gradient of KCl. Five-microliter aliquots of the 1-ml fractions collected were assayed by RMSA using \(^{32}\text{P}\)-labeled e5 RNA probe. As shown in Fig. 3B, the AUBF binding activity was eluted at 0.45–0.5 M KCl. This step enriched the AUBF binding 20-fold. The active fractions were pooled, diluted to 0.1 M KCl, and passed over an RNA affinity column (see “Experimental Procedures”). The column was eluted with 1 M KCl, and strong DNA binding activity could be detected in the eluted fraction (data not shown). This step resulted in a 250-fold purification, providing a 5,000-fold final purification over the crude extract. The fraction recovered from RNA affinity column was resolved by SDS-PAGE. Two major polypeptides of 24 and 52 kDa were identified by silver staining (Fig. 3C, lane 2).

To further verify the 24- and 52-kDa polypeptides represent the proteins that bind to RNA probe e5, UV cross-link and Northwestern analyses were performed. First, heparin-affinity-purified protein was cross-linked to \(^{32}\text{P}\)-labeled e5 RNA probe, followed by digesting with RNase T1 and A and resolving on SDS-polyacrylamide gel. As shown in Fig. 3D, a 24-kDa band was detected, which is identical to the 24-kDa band in the affinity-purified protein in SDS-polyacrylamide gel (Fig. 2C, lane 2). The 52-kDa band shown in the affinity-purified protein was not detected in UV cross-link analysis. The reason for this is not clear. It is possible that UV treatment did not link the 52-kDa proteins to the e5 probe well. Second, heparin-affinity purified protein was resolved on SDS-polyacrylamide gel and followed by transferring onto a nitrocellulose membrane and hybridizing with a \(^{32}\text{P}\)-labeled e5 RNA probe. As shown in Fig. 3E, both 24- and 52-kDa bands were detected, which is consistent with the sizes of proteins detected in the RNA affinity-purified sample in Fig. 3C. Those results suggest that the AURE (e5 probe) within the p21\textsuperscript{waf1/cip1} 3′-UTR binds both 24- and 52-kDa polypeptides (p24/52\textsuperscript{AUBF}).

**The PE-induced Protein That Binds to the AURE Located at 583–625 within the p21\textsuperscript{waf1/cip1} 3′-UTR Is Distinct from the Elav-like Protein HuR**—It has been shown that Elav-like mRNA-stabilizing proteins (HuD, HuC, HelN1, and HuR) can bind to the AU-rich element within the 3′-UTR of p21\textsuperscript{waf1/cip1} (27). We wondered whether the PE-induced protein in TFG2 cells is such a protein. Since among the various Elav-like proteins only HuR is expressed in the liver (27), we tested whether HuR is involved by using an RNA gel supershift assay. As shown in Fig. 4A, incubation of purified HuR protein with a radiolabeled RNA fragment corresponding to the region 553–625 within the p21\textsuperscript{waf1/cip1} 3′-UTR formed a strong complex, which was completely supershifted in the presence of the HuR antibody. This confirms the binding of HuR to the conserved AU-rich element within p21\textsuperscript{waf1/cip1} 3′-UTR. Incubation of crude extracts from PE-treated TFG2 cells with the same labeled RNA fragment also caused a strong shift; however, the position of this band remained unchanged in the presence of preimmune serum or HuR antibody, i.e., no supershift was observed (Fig. 4B). This indicates that the HuR protein is not involved in the major complex formed with crude extracts. Furthermore, no HuR protein was detectable in Western blots using crude extracts of PE-treated TFG2 cells (data not shown). These findings indicate that the protein induced by PE in TFG2 cells that binds to the AURE within the p21\textsuperscript{waf1/cip1} 3′-UTR is distinct from HuR.

**PE Treatment Enhances p21\textsuperscript{waf1/cip1} mRNA Stability and Increased AURE Binding**—Above data showed that the AURE within p21\textsuperscript{waf1/cip1} 3′-UTR is responsible for PE induction of
p21$^{\text{waf1/cip1}}$ mRNA stability, and a p24/52$^{\text{AUBF}}$ binding, we wondered whether PE was able to stimulate this p24/52$^{\text{AUBF}}$ binding. TFG2 cells were treated with PE for various time periods, then the cell extracts were prepared and subjected to RMSA. As shown in Fig. 5A, PE treatment significantly stimulated the p24/52$^{\text{AUBF}}$ binding. This suggests that PE induction of p21$^{\text{waf1/cip1}}$ mRNA stability is due to enhancement of binding of p24/52$^{\text{AUBF}}$ to the AURE within the p21$^{\text{waf1/cip1}}$ 3' UTR.

Activation of p42/44 MAP kinase has been implicated in controlling p21$^{\text{waf1/cip1}}$ mRNA stability (26). We wondered whether PE induction of p24/52$^{\text{AUBF}}$ binding is mediated by a p42/44 MAP kinase-dependent mechanism. As shown in Fig. 5B, treatment of TFG2 cells with PD98059 not only significantly attenuated basal levels of p24/52$^{\text{AUBF}}$ binding (lanes 2 and 3 versus lane 1) but also antagonized PE-induced p24/52$^{\text{AUBF}}$ binding (lane 4 versus lane 5). These findings suggest that activation of p42/44 MAP kinase is involved in PE induction of p24/52$^{\text{AUBF}}$ binding.

To further confirm the role of p42/44 MAP kinase in PE induction of p24/52$^{\text{AUBF}}$ binding, TFG2 cells were infected with a dominant negative MEK1 recombinant adenovirus to block the activation of p42/44 MAP kinase. As shown in Fig. 5C, infection with MEK1 dominant negative adenovirus but not with control virus not only significantly inhibited the basal p24/52$^{\text{AUBF}}$ binding but also markedly attenuated PE induction of p24/52$^{\text{AUBF}}$ binding, which further suggests that p42/44 MAP kinase is involved.

**DISCUSSION**

The control of p21$^{\text{waf1/cip1}}$ gene transcription has been extensively studied, with more than 15 transcription factors that bind to the p21$^{\text{waf1/cip1}}$ gene promoter identified (reviewed in Ref. 17). Posttranscriptional mechanisms have been also implicated in the regulation of p21$^{\text{waf1/cip1}}$ gene expression by a number of conditions listed in the Introduction (18–24). However, the mechanisms underlying such posttranscriptional regulation remain unknown. Here, we have demonstrated that PE treatment markedly enhances p21$^{\text{waf1/cip1}}$ mRNA stability in
TGF2 cells, and an AURE located between 583 and 625 within the p21waf1/cip1 3′-UTR is involved in this induction. We have also demonstrated that an RNA-binding protein specifically binds to this AURE and the binding is significantly induced by PE treatment, which correlates with PE induction of p21waf1/cip1 mRNA stability. It is generally accepted that AURE plays an important role in controlling mRNA half-life (see review in Ref. 29). It has been shown that many AUREs can function as mRNA-destabilizing signals. For example, the c-fos mRNA AURE has been shown to be a potent destabilizing element. Deletion of this AURE significantly increased c-fos gene expression by 20-fold (30, 31). Examination of sequences revealed that the AURE region located at 583–625 within p21waf1/cip1 3′-UTR contains one AUUUA and two AUUUA motifs (13). Surprisingly, deletion of this AURE did not significantly enhance the reporter CAT mRNA stability, and inserting of this AURE alone did not significantly destabilize the reporter CAT mRNA stability in TGF2 cells. The latter result is consistent with the data reported by Li et al. (21), who demonstrated that inserting this AURE alone did not cause a dramatic reduction in reporter luciferase activity in breast cancer MDA-MB-468 cells. Taken together, these data suggest that the AURE in p21waf1/cip1 3′-UTR does not play a significant role in controlling the basal p21waf1/cip1 mRNA stability, which may be due to weak AUBF binding to this element in the control cells detected in RNA gel mobility shift assay (Fig. 5). Interestingly, deletion of the AURE significantly abolished PE-induced stabilization of CAT mRNA, and RNA gel mobility shift assay demonstrated that PE treatment markedly enhanced the p24/52AUBF binding to this AURE. These data strongly suggest that the AURE is responsible for PE induction of p21waf1/cip1 mRNA stability by binding p24/52AUBF. In preliminary experiments we found that treatment of TGF2 cells with phorbol myristate acetate or retinoic acid, which are also known to increase p21waf1/cip1 mRNA stability, similarly increased the binding of this RNA-binding protein, while treatment with cisplatin or 5-fluorouracil, which are known to increase p21waf1/cip1 protein expression by a p53-dependent mechanism, did not stimulate the AUBF binding. This suggests that the binding of p24/52AUBF to the AURE within the p21waf1/cip1 3′-UTR may be a universal signal involved in the control of p21waf1/cip1 mRNA stability by many stimuli.

The identity of the AUBF in the present paper is not yet clear. In the past few years, at least 10 AUBFs have been isolated. They include AUF1 (32), 3-oxoacyl-CoA thiolase (33), glyceraldehyde-3-phosphate dehydrogenase (34), heteronuclear ribonucleoproteins A1 and C (35), AUH with enoyl-CoA hydratase activity (36), and members of the Elav family (37, 38). Among them, only the Elav family of RNA-binding proteins has been well characterized (37, 38), and these proteins have been reported to bind the AURE in the p21waf1/cip1 mRNA 3′-UTR and to enhance p21waf1/cip1 mRNA stability (27). However, three lines of evidence suggest that the p24/52AUBF reported here is not an Elav protein. First, anti-HuR antibody did not induce any supershifted bands in RNA gel mobility supershift assays (Fig. 4). Second, we were unable to detect HuR protein in TGF2 crude cell extracts in Western blots, which confirms that TGF2 cells express little if any HuR protein. Third, the molecular mass of HuR is 30 kDa, which is distinct from 24 and 52 kDa reported here.

PE induction of p21waf1/cip1 mRNA is rapid, its peak occurring at 2 h (Fig. 1), and does not require new protein synthesis, which is consistent with the rapid induction of p24/52AUBF binding in RNA mobility shift assay (Fig. 5). Rapid induction of p24/52AUBF binding by PE is probably due to phosphorylation of this protein. We have earlier reported that PE induction of p21waf1/cip1 protein expression is mediated by a p24/44 MAP kinase-dependent mechanism (7), and activation of p42/44 MAP kinase has been implicated in the control of p21waf1/cip1 mRNA stability (28). Here we provided two lines of evidence to support that PE induction of p24/52AUBF binding requires activation of p42/44 MAP kinase (Fig. 5). First, specific inhibition of MEK1 and p42/44 MAP kinase activity by PD98059 markedly inhibited both basal and PE induction of p24/52AUBF binding (Fig. 5B). PD98059 has been reported to be specific for MEK1/2 and p42/44 MAP kinase (39, 40). We have shown that 25–50 µM PD98059 markedly suppressed PE-activated p42/44 MAP kinase but did not suppress PE-activated p38 MAP kinase or JNK in TGF2 cells. The inhibition of p24/52AUBF binding by PD98059 is not a nonspecific or toxic effect because this drug did not affect nuclear factor 1-DNA binding. Second, more convincingly, infection of a dominant negative MEK1 markedly suppressed both basal and PE-induced p24/52AUBF binding. It is generally believed that p42/44 MAP kinase is the specific substrate for MEK1 (41). Therefore, it is plausible that PE activation of p42/44 MAP kinase is followed by phosphorylation of the p24/52AUBF, resulting in its enhanced binding to and stabilization of the p21waf1/cip1 mRNA. However, whether there is a direct correlation between activation of p42/44 MAP kinase, induction of p24/52AUBF binding, and p21waf1/cip1 mRNA stability awaits further purification, cloning, and characterization of the p24/52AUBF. 
Acknowledgments—We thank Dr. B. Vogelstein for providing the p21waf1/cip1 cDNA and promoter/CAT construct vectors and Dr. Henry Furneaux for providing the anti-HuR antibody and for helpful discussions.

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*J. Biol. Chem.* 2000, 275:11846-11851.
doi: 10.1074/jbc.275.16.11846

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