The 3′-Untranslated Region of p21WAF1 mRNA Is a Composite cis-Acting Sequence Bound by RNA-binding Proteins from Breast Cancer Cells, Including HuR and Poly(C)-binding Protein*

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Despite promoting growth in many cell types, epidermal growth factor (EGF) induces growth inhibition in a variety of cancer cells that overexpress its receptor. The cyclin-dependent kinase inhibitor p21WAF1 is a central component of this pathway. We found in human MDA-468 breast cancer cells that EGF up-regulates p21WAF1 mRNA and protein, through a combination of increased mRNA stability and transcription. The decay rate of a hybrid luciferase reporter full-length p21WAF1 3′-untranslated region (UTR) mRNA was significantly faster than that of a control mRNA. Transfections with a variety of p21WAF1 3′-UTR constructs identified multiple cis-acting elements capable of reducing basal reporter activity. Short wavelength ultraviolet light induced reporter activity in constructs containing the 5′ region of the p21WAF1 3′-UTR, whereas EGF induced reporter activity in constructs containing sequences 3′ of the UVC-responsive region. These cis-elements bound multiple proteins from MDA-468 cells, including HuR and poly(C)-binding protein 1 (CP1). Immunoprecipitation studies confirmed that HuR and CP1 associate with p21WAF1 mRNA in MDA-468 cells. Over- and underexpression of HuR in MDA-468 cells did not affect EGF-induced p21WAF1 protein expression or growth inhibition. However, binding of HuR to its target 3′-UTR cis-element was regulated by UVC but not by EGF, suggesting that these stimuli modulate the stability of p21WAF1 mRNA via different mechanisms. We conclude that EGF-induced p21WAF1 protein expression is mediated largely by stabilization of p21WAF1 mRNA elicited via multiple 3′-UTR cis-elements. Although HuR binds at least one of these elements, it does not appear to be a major modulator of p21WAF1 expression or growth inhibition in this system. CP1 is a novel p21WAF1 mRNA-binding protein that may function cooperatively with other mRNA-binding proteins to regulate p21WAF1 mRNA stability.

Inhibition of human tumor cell growth is mediated by a variety of cell cycle-related proteins and tumor suppressors. p53, a well characterized tumor suppressor, activates transcription of a number of target genes, including p21WAF1 (wild-type p53 activated fragment-1) (1, 2), which encodes a protein of M, 21,000 (p21), also known as cyclin-dependent kinase-interacting protein 1. p21WAF1 inhibits cyclin-cyclin-dependent kinase activity, preventing phosphorylation of critical cyclin-dependent kinase substrates, blocking transition from G1 to S phase of the cell cycle (3), as well as inducing apoptosis (4). Recent evidence suggests that factors other than p53, such as EGF1 (16), can induce p21WAF1 expression in various cell types (p53-independent pathways). Because most human tumors lack p53 function (5), investigation of the mechanisms that regulate p21WAF1 expression through alternative growth factor-induced pathways has become an important focus in cancer research. In particular, a major goal is to devise approaches that would increase expression of p21WAF1 in tumors to reduce proliferation and tumor growth.

Although EGF is typically growth-proliferative in breast cancer cells (6), some cancer cells are growth-inhibited by EGF (e.g. MDA-468 breast (7, 8), A431 epidermoid (9, 10)). EGF-induced growth inhibition of these cells is associated with EGF receptor (EGFR) overexpression (11) and appears to be mediated by induction of p21WAF1 mRNA and protein (8). Multiple reports show conclusively that the regulation of p21WAF1 expression by growth factors and other ligands occurs predominantly at the level of mRNA stability (12–17). However, there is little understanding of the specific RNA-protein interactions involved in this process, particularly in breast cancer cells. Thus, the EGF-induced up-regulation of p21WAF1 mRNA provides an ideal system to investigate the mechanisms governing p21WAF1 mRNA decay.

The regulation of mRNA decay is a critical mechanism in the control of gene expression (reviewed in Hollams et al. (18)) that involves interactions between cis-acting sequences that confer instability to mRNA and the trans-acting protein factors that bind them. Many cis-acting sequences consist of AU-rich ele-

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Regulation of p21WAF1 mRNA Stability in Breast Cancer

...ments (AREs), most often located in the 3'-untranslated region (3'-UTR) of labile mRNAs. However, cis-acting elements are also found within the coding regions and 5'-UTRs of various mRNAs (e.g. c-fos, c-myc) (19). AREs often contain single or multiple repeats of pentamer (AUAUA) sequences, and inclusion of the AUUUA pentamer motif often targets the mRNA for rapid cytoplasmic degradation (20). One well characterized cis-acting element is the AU-rich sequence found within the 3'-UTR of granulocyte colony-stimulating factor mRNA, which is able to reduce the half-life of β-globin mRNA from many hours to less than 30 min (20). Other studies have shown the AUUUA pentamer motif to be more predictive of rapid mRNA decay than the AUUUA pentamer motif (21, 22). Several other RNA binding motifs have been identified, including the C-rich motif that is the target for the poly(C)-binding proteins (CPs) (23). To date, the cis-activity of the 3'-UTR of p21WAF1 has not been characterized extensively.

Multiple proteins have been identified that can bind to AU- and U-rich regions (reviewed in Hollams et al. (18)). These include AUF1 (24), AUF1 (hnRNPI D) (25), Hel-N1 (26), hnRNPI C (27), hnRNPI A1 (27), AUH (28), HuR (29), HuD (30), tristetraprolin (31), and poly(A)-binding protein (32). Of the AU- and U-rich-binding proteins, only a few have been shown to definitively regulate mRNA stability: AUF1, HuR, and other Hu/ELAV proteins and tristetraprolin and its family members. HuR, a ubiquitously expressed member of the Hu/ELAV family, is involved in the shuttling of transcripts from the nucleus into the cytoplasm (33–35), as well as in the regulation of mRNA stability (17, 35–38). In RKO colorectal carcinoma cells, HuR mediates UVC-induced stabilization of p21WAF1 mRNA (17), and of interest, HuD, a neuron-specific member of the Hu/ELAV family (30), has been shown to bind to a 42-nt sequence within the 3'-UTR of p21WAF1 mRNA (39). It seemed possible, therefore, that HuR would play an important role in the regulation of p21WAF1 mRNA stability in breast cancer cells.

Here, we show that the 3'-UTR of p21WAF1 mRNA contains multiple cis-acting regions that reduce basal reporter activity and confer EGF- and UV-induced changes to reporter constructs in a region-specific manner. These 3'-UTR elements are targets for a number of RNA-binding proteins, including HuR and CP1, from MDA-468 breast cancer cells. Despite its role in the mediation of p21WAF1 mRNA stabilization and p21WAF1 expression by UVC in other cell systems, HuR does not appear to have a major role in EGF-induced p21WAF1 expression in MDA-468 breast cancer cells (EGFR overexpressed, mutant p53).

MATERIALS AND METHODS

Cell Culture—The MDA-468 (HTB 132) human breast cancer cell line was obtained from ATCC (Manassas, VA). Cells were routinely cultured in Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 10% fetal calf serum (Invitrogen). BING cells (40) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 2% fetal calf serum (Invitrogen). All cell lines were cultured in the presence of penicillin (50 units/ml) and streptomycin (50 μg/ml). Cells were utilized within 2 passages of the original stock received from ATCC for all experiments.

Cell Cycle Analysis—For cell cycle analysis, EGF-treated (25 ng/ml) MDA-468 cells were harvested by trypsinization and counted in a Coulter EPICS XL-MCL (Coulter Corp., Hialeah, FL), and cell cycle analysis was performed with MultiPlus AV MultiParameter data analysis software (Phoenix Flow Systems, San Diego, CA).

Plasmid Clones, DNA Probes, Riboprobes, Fusion Proteins, and Expression Clones—The p21WAF1 plasmid cDNA (pCPE-WAF1) (1) from Dr. B. Vogelstein) contained the 5'-UTR coding region, and 3'-UTR of p21WAF1 (see Fig. 1A; nucleotide sequence is in the GenBank™ data base under accession number U303106) (1) and was digested with EcoRI and NotI to liberate a 1-kb cDNA fragment, which for Northern analysis, was random prime-labeled using [α-32P]dCTP (~ 3000 Ci/mmol; Amersham Biosciences). A 1.1-kb 18 S rRNA cDNA probe was used as a loading control. Plasmids WAF1-1/7, WAF1-2/7, WAF1-6/7, WAF1-8/7, WAF1-15/12, and WAF1-1/8 (Fig. 1A) were constructed by cloning PCR-amplified sequences from the 3'-UTR of the p21WAF1 mRNA into either the XbaI site of pG3-L-control luciferase reporter vector (Promega) for transfection experiments or into the BsuHindIII-digested pBluescript II KS+ vector (Stratagene) for the generation of labeled riboprobes. The plasmid containing the HuD binding site (WAF1-Hud) was constructed by subcloning annealed 42-mer sense (nt 657–698, 5'-AAU UUA UUA UUA GTG UUU UAA UUU AAA CAC CUC CUG AUG-3') (39) and antisense oligonucleotides corresponding to this sequence (nt 698–738) into the BumHI site of pCPE-WAF1 3'-UTR (Fig. 1A). A and B indicate the HindIII sites of the pBluescript vector. The cDNA of Hu-R element that generates an unstable mRNA was also cloned into the XbaI site of pG3-L-control and used in transfection assays (22). pRL-SV40 (Promega) was utilized as a control for transfection efficiency in reporter assays. Some plasmids contained three AU-rich sequences (shown in Fig. 1, A and B) and are denoted A (nt 742–758, 5'-AAU UUA UUA AAC AAA AA-3'), B (nt 797–809, 5'-AAU UUA UUA UUA UUA-3'), and C (nt 811–824, 5'-AAU UAC UAA UUA AA-3'). For some RNA gel shifts (RNA electrophoretic mobility shift assays) the plasmid c-fos-Hud (5'-AAU UUA UUA UUA UUA UUA UUA UUA UUA UUA-3') was also used (Fig. 1B). All pBluescript plasmid clones were linearized with HindIII for transcription with T7 RNA polymerase (Invitrogen) in reaction containing [α-32P]UTP (3000 Ci/mmol; Amersham Biosciences), as described (41), to produce riboprobes with a specificity of ~ 2 × 106 cpm/μg RNA that included 66 nt of pBluescript, in addition to the corresponding portion of the p21WAF1 3'-UTR. Unlabeled RNA transcriptions were synthesized as above except with 2.5 mM NTPs, quantified by spectrophotometry and verified by PAGE. pGEX-2T-HuR (from Dr. H. Furneaux) generated a fusion protein (GST-HuR) that contained amino acids 2–326 of human HuR (29). pGEX-6P-cpC1 (from Dr. M. Kiledjian) generated a fusion protein (GST-CP1) that contained amino acids 13–347 of human CP1 (60). For HuR over-expression studies, the retroviral vector pABE puro (42) was used. The sequence of all plasmid constructs was confirmed by dye sequenase sequencing.

RNA Isolation and Northern Analysis—MDA-468 cells were solubilized in 4 M guanidinium isothiocyanate, and total RNA was isolated using the method of Chomczynski and Sacchi (43). RNA (10–15 μg per sample) was size-fractionated on a 1% agarose-formaldehyde gel and transferred to Hybond-N (Amersham Biosciences). A 1.1-kb 18 S rRNA cDNA probe was used as a loading control. Plasmids WAF1 and 18 S rRNA cDNAs had been blotted. For some RNA gel shifts (RNA electrophoretic mobility shift assays) the plasmid c-fos-Hud (5'-AAU UUA UUA UUA UUA UUA UUA UUA UUA UUA-3') was also used (Fig. 1B). All pBluescript plasmid clones were linearized with HindIII for transcription with T7 RNA polymerase (Invitrogen) in reaction containing [α-32P]UTP (3000 Ci/mmol; Amersham Biosciences), as described (41), to produce riboprobes with a specificity of ~ 2 × 106 cpm/μg RNA that included 66 nt of pBluescript, in addition to the corresponding portion of the p21WAF1 3'-UTR. Unlabeled RNA transcriptions were synthesized as above except with 2.5 mM NTPs, quantified by spectrophotometry and verified by PAGE. pGEX-2T-HuR (from Dr. H. Furneaux) generated a fusion protein (GST-HuR) that contained amino acids 2–326 of human HuR (29). pGEX-6P-cpC1 (from Dr. M. Kiledjian) generated a fusion protein (GST-CP1) that contained amino acids 13–347 of human CP1 (60). For HuR over-expression studies, the retroviral vector pABE puro (42) was used. The sequence of all plasmid constructs was confirmed by dye sequenase sequencing.

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phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 2 μg/ml aprotinin (Roche Molecular Biochemicals). After 10 min on ice, the lysate was centrifuged at 750 × g for 10 min at 4 °C, after which the supernatant was recovered and stored at −85 °C. Total protein concentrations of lysates were determined using the Bio-Rad protein assay, and 10 μg of proteins were separated on 10% BisTris acrylamide gels (Invitrogen) in 1× MES SDS running buffer, pH 7.3 (Invitrogen), and transferred to polyvinylidene difluoride membranes (Osmonics) in 1× MES SDS transfer buffer. Membranes were blocked with 10% skim milk in TBS-T (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) at 22 °C for 1 h, prior to addition of either anti-p21WAF1 monoclonal antibody (1:1000) (15091A; BD Biosciences), anti-HuR monoclonal antibody 19F12 (1:15091A; BD Biosciences), or anti-actin monoclonal antibody (1:2000) (from Henry Furneaux), or anti-actin monoclonal antibody (1:1000) (Amersham Biosciences), and washed again with TBS-T, prior to detection with ECL Plus detection reagents (Amersham Biosciences) on ECL-Hyperfilm (7.5 μg/ml) (Sigma) for 0–4 h. Total MDA-468 RNA was harvested using TRIzol (Invitrogen). To generate cDNA, 2 μl of RNA (denatured at 70 °C for 10 min) was reverse-transcribed in a 20-μl reaction containing 5 mM MgCl2, 1× avian myeloblastosis virus reverse transcriptase buffer, 1 mM dNTPs, 20 units of RNasin, 10 units of avian myeloblastosis virus reverse transcriptase, and 250 ng of oligo(dT)12 primer at 42 °C for 30 min. PCR was then performed for both luciferase and β-actin cDNA (luciferase sense, 5′-TTG CAC CGG CTG CAT CGT CG-3′; luciferase antisense, 5′-GTC CAC CGG CTG CAT CGT CG-3′; β-actin sense, 5′-GCC AAC ACA GTG CTG TCT GG-3′; β-actin antisense, 5′-GCC AAC ACA GTG CTG TCT GG-3′) using a Bio-Rad iCycler q i real-time PCR detection system (Bio-Rad). Data were normalized using results obtained for β-actin and the ratio of luciferase mRNA remaining for pGL3-WAF1–1/7 and pGL3-c-fos-ARE expressed relative to pGL3-control as a function of time after ActD treatment.

Transfection and Luciferase Assays—MDA-468 cells (50% confluent) were transiently transfected with 8 μg of pGL3–1/7 and various p21WAF1 3′-UTR regions (see Fig. 1A) and 100 ng of pRL-SV40 as a control, using FuGENE 6 as above. Some cells were cultured following treatment with EGF (25 ng/ml) or UVC (254 nm, 20 J/m2), for 8 or 6 h respectively, prior to lysis extraction. Cells were washed in PBS, harvested by trypsinization, and lysed, and supernatant luciferase activity was measured using the dual luciferase reporter assay kit (Promega) and a Wallac Victor 1420 multilabel counter (Wallac Oy, Turku, Finland), according to the manufacturer’s instructions. Firefly luciferase (pGL3) activity was normalized against Renilla luciferase (pRL-SV40) activity to yield the relative luciferase activity.

Preparation of Cytoplasmic Extracts for RNA Gel Shift Assays—MDA-468 cells were grown to 70–80% confluence in 10-cm culture dishes. Cytoplasmic extracts were prepared as described previously (41). Briefly, cells were scraped from the culture dishes in chilled PBS,
was purified from bacterial pellets, lysed in 10 mM Tris, pH 7.8, 0.5 mM PMSF, and 10 mg/ml leupeptin, 2 mg/ml aprotinin. Lysates were cleared by centrifugation at 4 °C for 10 min at 12,100 × g, and the supernatant was snap-frozen in liquid nitrogen and stored at −80 °C. Protein concentrations were determined using the Bio-Rad protein assay kit.

Preparation of Whole Cell Extracts for RNA Gel Shift Assays—MDA-468 cells were grown to 70–80% confluence in 10-cm culture dishes. Medium was removed, the cell monolayer was washed twice in ice-cold PBS, and the cells were lysed in 0.5 ml of chilled lysis buffer (containing 50 mM Tris, pH 7.5, 5 mM EDTA, pH 8.5, 150 mM NaCl, 1% Triton X-100, 10 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM DTT, 5 mM NaVO₃, 50 mM NaF, and 10 mM Na₃MoO₄·2H₂O) on ice for 10 min. Cells were scraped and transferred to Eppendorf tubes, and lysates were then cleared by centrifugation at 4 °C for 10 min at 12,100 × g and stored at −80 °C. Protein concentrations were determined by Bio-Rad protein assay kit.

RNA Electrophoretic Mobility Shift Assay (REMSA)—Binding reactions were performed as described previously (41) with 5 μg of cytoplasmic extract or 200 ng of recombinant protein and 10² cpmp of ³²P-labeled RNA (∼2–5 pg). Briefly, binding reactions were incubated at 22 °C for 30 min, after which 0.3 units of RNAse T1 (Roche Molecular Biochemicals) was added for 10 min, followed by the addition of heparin, polyinosinic acid, and polyphosphonic acid (Sigma) for 10 min. Incubations were performed as described previously (41) with 5 μg of polyadenylic acid (hexadimethrine bromide; Sigma), the RNA-containing medium was incubated overnight with the target cells (MDA-468). Cells were selected in 1 μg/ml puromycin (Sigma) starting 48 h after infection. Pools of puromycin-resistant cells were analyzed by Western blotting to confirm transgene expression. All experiments were performed using pools of infected cells.

Retroviral Expression of HuR—Full-length HuR cDNA was cloned into the pBabe puro vector in the sense and antisense orientation and then transiently transfected into the retroviral packaging cell line BING (40) using FuGENE according to the manufacturer’s protocol. Retroviral-containing conditioned medium was collected from the BING cells at 48 h after transfection. Following filtration (0.45 μm) and the addition of 4 μg/ml polybrene (hexadimethrine bromide; Sigma), the retroviral-containing medium was incubated overnight with the target cells (MDA-468). Cells were selected in 1 μg/ml puromycin (Sigma) starting 48 h after infection. Pools of puromycin-resistant cells were analyzed by Western blotting to confirm transgene expression. All experiments were performed using pools of infected cells.

 Colony Formation Assays—Colony formation assays were performed as described previously (58). Briefly, MDA-468 sublines were plated in triplicate at a density of 5000 cells per 10-cm plate and then incubated overnight. A single dose of EGF (25 ng/ml) or PBS control was added at 24 h, and the cells were allowed to grow for a further 10 days. After fixation in methanol/acetic acid (3:1), colonies were stained with Giemsa (Fluka) and counted using a Quantimet 520 image analyzer (Leica).

Statistical Analysis—Transfection and luciferase mRNA decay data are shown as mean ± S.D. Statistical analysis was performed using Student’s t test, with a p value of <0.05 regarded as significant.

RESULTS

EGF Up-regulates p21WAF1 Expression in MDA-468 Cells—The MDA-468 breast cancer cell line, which contains mutant p53 and overexpresses the EGFR (7), provides an excellent model system to investigate the mechanisms underlying p21WAF1 gene expression and its regulation by EGF. To establish the validity of this cell line as a model of EGF-induced cell cycle arrest and growth inhibition, the cells were treated with EGF (25 ng/ml) for 8 h, and the proportion of cells in S phase of the cell cycle was determined using flow cytometry (see “Materials and Methods”). EGF treatment reduced the proportion of cells in S phase from 30 to 12.5% (data not shown). Furthermore, in a colony formation assay (see “Materials and Methods”), EGF treatment led to >98% reduction in the number of detectable colonies (data not shown).

We next examined the effect of EGF on endogenous p21WAF1 mRNA and protein levels in MDA-468 cells. EGF rapidly up-regulated p21WAF1 protein (Fig. 2A) and p21WAF1 mRNA (Fig. 2B) within 2 h. To determine whether this up-regulation of p21WAF1 mRNA occurred at the post-transcriptional level, we treated MDA-468 cells with EGF for 2 h and performed ActD chase studies. In the absence of EGF, the basal half-life of p21WAF1 mRNA was relatively short (2.7 h) (Fig. 2C). However, in the presence of EGF the half-life was increased to 11.5 h (Fig. 2C). Treatment of the cells with cycloheximide, a translational inhibitor, also stabilized p21WAF1 mRNA (Fig. 2C), suggesting that (i) ongoing translation is required for maintenance of the short basal half-life, and/or (ii) existing cellular proteins mediate the stabilization of p21WAF1 mRNA.

Nuclear run-on assays were employed to evaluate the effect of EGF on p21WAF1 transcriptional activity. After treatment of MDA-468 cells with EGF, p21WAF1 transcription increased by ∼2-fold (Fig. 2D). Taken together, these data suggest that the EGF-induced growth arrest in MDA-468 cells is associated...
with a rapid increase in p21WAF1 mRNA and protein, which results from a combination of increased mRNA stability and increased transcription.

Identification of cis-Acting Elements in the 3’-UTR of p21WAF1 mRNA—Based on the observation that the stabilization of p21WAF1 mRNA is a major contributor to the overall up-regulation of p21WAF1 protein expression in EGF-treated MDA-468 cells, we next sought to elucidate some of the mechanisms underlying this effect. In breast cancer cells, little is known of the cis-acting elements or trans-acting factors involved in the regulation of p21WAF1 mRNA stability. Transfection studies with ActD chase and real-time PCR demonstrated that WAF1–1/7 (entire p21WAF1 3’-UTR; see Fig. 1A) destabilized luciferase mRNA significantly, in a manner equivalent to that of the highly unstable c-fos ARE (Fig. 3). These data provided strong evidence that the p21WAF1 3’-UTR contains one or more cis-elements that confer basal mRNA instability and potentially contribute to the regulation of p21WAF1 mRNA stability.

The 3’-UTR of p21WAF1 contains an AU-rich region at the 5’ end that spans ~250 nt, termed WAF1-1/6 (see Fig. 1, A and B), which contains at least one known HuR binding site (17). Within WAF1–1/6 is a 42-nt sequence, termed WAF1-HuD, which contains an imperfect consensus nonamer and is the target for HuD binding (39). The WAF1–1/6 region also contains several smaller stretches of AU-rich sequence, denoted A, B, and C (see Fig. 1, A and B, and see “Materials and Methods”).

**Fig. 2.** EGF increases p21WAF1 mRNA and protein levels and p21WAF1 mRNA stability and transcription in MDA-468 cells. A, Western blot analysis showing p21WAF1 protein levels in MDA-468 cells following treatment with EGF (25 ng/ml). Cell lysates (10 µg) were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with anti-p21WAF1 and actin antibodies. ECL-generated images were quantified using ImageQuant and p21WAF1 protein levels graphed over time. B, Northern blot analysis of total RNA extracted from MDA-468 cells after treatment with EGF (25 ng/ml) for the times indicated. Following hybridization with a 32P-labeled p21WAF1 cDNA probe, each blot was normalized using a 32P-labeled 18 S ribosomal RNA cDNA probe. Quantification was performed using a PhosphorImager and ImageQuant software, and p21WAF1 mRNA levels were graphed over time. C, ActD chase studies in MDA-468 cells. Cells were grown to 50% confluence and treated with 25 ng/ml EGF or 10 µg/ml cycloheximide (CHX) for 2 h and then 7.5 µg/ml ActD. Total RNA was extracted from the cells at 0, 2, 4, or 8 h after ActD treatment and analyzed by Northern blot as in B. p21WAF1 mRNA was normalized against 18 S rRNA (image not shown). Half-life of p21WAF1 mRNA was 11.5 h (EGF-treated cells) and 2.7 h (control cells). **, p < 0.01. D, transcription run-on analysis of MDA-468 cells after treatment with EGF (25 ng/ml) for 2 h. p21WAF1 transcription rates were measured in isolated nuclei by run-on transcription assays, and the results were analyzed by PhosphorImager and ImageQuant and shown in this figure relative to 18 S rRNA transcription levels. CON, control; EGF, 2-h EGF treatment.
and was therefore a candidate cis-acting sequence.

To further dissect the cis-activity of the p21WAF1 3′-UTR, we generated several reporter constructs containing portions of the 3′-UTR of p21WAF1 (Fig. 1A). In transfection assays using MDA-468 cells, the full-length 3′-UTR, WAF1–1/7, reduced basal reporter activity by ~85% (Fig. 4, A and B), supporting our ActD-luciferase mRNA data (Fig. 3). Subsequent analysis of the three major components of the 3′-UTR (WAF1–1/6, WAF1–879, WAF1–1512) showed that each reduced reporter activity but that the major effect was 3′ of the previously identified AU-rich region contained within WAF1–1/6 (Fig. 4B). In support of this observation, clones WAF1–2/7 and WAF1–6/7, which harbored deletions of the AU-rich regions, reduced reporter activity similarly to WAF1–1/7. Taken together, these results suggest that the WAF1–1/6 region is not the sole determinant of basal p21WAF1 mRNA stability in MDA-468 cells and that the four AU-rich regions (HuD; see Fig. 1, A–C) are not major contributors to basal turnover of p21WAF1 mRNA in MDA-468 cells. Furthermore, although WAF1–879 and WAF1–1512 each decrease reporter activity, neither is as effective as the combined sequence (WAF1–6/7) (Fig. 4B). This suggests the presence of multiple cis-elements within the 3′-UTR of p21WAF1 mRNA.

We next examined the effect of EGF on the reporter activity of each of these constructs in MDA-468 cells. EGF increased reporter activity by ~60% in the case of WAF1–1/7, with most of this effect being contained within the WAF1–6/7 region (Fig. 4C). Both the WAF1–879 and WAF1–1512 constructs conferred EGF-induced up-regulation of luciferase reporter activity, whereas the WAF1–1/6 construct appeared to be relatively EGF-unresponsive (Fig. 4C). Taken together, these results suggest that the predominant cis-element(s) within the 3′-UTR of p21WAF1 that are responsible for both basal mRNA instability and EGF-inducibility in MDA-468 cells reside downstream of the WAF1–1/6 sequence.

To compare these results with another regulator of p21WAF1 mRNA stability, we tested the effect of UVC treatment on reporter activity using the same constructs in transfection experiments with MDA-468 cells. The full-length 3′-UTR (WAF1–1/7) increased reporter activity by ~70% after UVC treatment (Fig. 4D). Further analysis revealed that the UVC-mediated up-regulation of reporter activity occurred predominantly through sequences contained within the WAF1–1/6 construct, with a lesser contribution from sequences downstream of WAF1–1/6 (Fig. 4D). This result suggests that the WAF1–1/6 region is the major 3′-UTR determinant of UVC-induced stabilization of p21WAF1 mRNA in MDA-468 cells. These data illustrate that even within the one cell type, different stimuli may lead to the preferential regulation of different and specific cis-elements within the p21WAF1 3′-UTR, presumably via different sets of RNA-protein interactions.

MDA-468 Cells Contain Proteins That Bind Specifically to p21WAF1 mRNA—To investigate whether the cis-acting p21WAF1 mRNA 3′-UTR elements were a target for cytoplasmic RNA-binding proteins from MDA-468 cells, we tested each region (WAF1–1/6, WAF1-HuD, WAF1–879, WAF1–1512) with REMSA. Cytoplasmic proteins from MDA-468 cells bound specifically to these probes (Fig. 5A, lanes 2, 6, 8, and 10), whereas no RNA-protein complexes were observed with 32P-labeled vector control (pBluescript; see Fig. 5A, lane

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**Fig. 4.** The 3′-UTR of p21WAF1 mRNA contains elements that modulate basal luciferase reporter activity and confer UVC and EGF inducibility to reporter constructs. A, MDA-468 cells were transfected with 8 µg of pGL3 ± p21WAF1 3′-UTR elements and 100 ng of pRL-SV40 as a control. Cells were cultured in the presence or absence of EGF (25 ng/ml) or UVC (20 J/m²) treatment. Firefly and Renilla luciferase activity was measured as described under "Materials and Methods." B, luciferase reporter activity for MDA-468 cells transfected with pGL3 ± p21WAF1 3′-UTR elements expressed relative to pGL3-control. *, p < 0.002. C, luciferase reporter activity for EGF-treated MDA-468 cells transfected with pGL3 ± p21WAF1 3′-UTR elements. Values were expressed relative to untreated cells transfected with the same cis-element (untreated self). *, p < 0.002; **, p < 0.03. D, luciferase reporter activity for UVC-treated MDA-468 cells transfected with pGL3 ± p21WAF1 3′-UTR elements. Values were expressed relative to untreated cells transfected with the same cis-element (untreated self). *, p < 0.001; **, p < 0.02. The graphs are representative of at least three separate experiments, each performed in triplicate. All values were normalized using Renilla luciferase activity.
compared to 14C molecular mass markers or WAF1-HuD (lane 3)–468 cell extracts and WAF1 (extracts in the absence of competitor RNA with WAF1-HuD riboprobe and MDA-468 self RNA (MDA-468 cytoplasmic extract). lane 4 trol RNA (vector control (pBluescript) riboprobes, and REMSA with MDA-468 Riboprobe and MDA-468 extracts in the absence of competitor RNA (lane 2) and in the presence of unlabeled self RNA (lane 3) or unlabeled vector control RNA (lane 4). C, UVXL with MDA-468 cell extracts and WAF1–1/6 (lane 1) or WAF1-HuD (lane 3) riboprobes, and compared to 14C molecular mass markers (lane 2). D, UVXL with MDA-468 cell extracts and WAF1–879 (lane 2) and WAF1–1512 (lane 3) riboprobes; 14C molecular mass markers (lane 1).

Furthermore, addition of ~100-fold molar excess unlabeled pBluescript competitor RNA did not diminish the formation of the RNA-protein complexes significantly (WAF1-HuD; see Fig. 5B, lane 4) (data not shown for the other riboprobes). However, addition of excess unlabeled self RNA (WAF1-HuD; see Fig. 5B, lane 3) (data not shown for the other riboprobes) virtually abolished RNA-protein complexes in all cases, demonstrating the specificity of the interaction.

We next utilized UVXL assays to characterize the individual p21WAF1 RNA-binding proteins. We found that multiple proteins targeted the WAF1–1/6, WAF1-HuD, WAF1–879, and WAF1–1512 probes (Fig. 5C, lanes 1 and 3; Fig. 5D, lanes 2 and 3, respectively). A similar set of RNA-protein complexes (RPCs) was identified with the WAF1–1/6 and WAF1-HuD probes, but the relative intensities of most of the RPCs was significantly different between the two probes (Fig. 5C, lanes 1 and 3). This suggested that the majority of these proteins bind to the WAF1-HuD region, which is contained within WAF1–1/6. Interestingly, a smaller range of RPCs was detected with the WAF1–879 and WAF1–1512 probes (Fig. 5D, lanes 2 and 3), and again the relative intensities of the RPCs differed between the two probes.

HuR and CP1 Bind to p21WAF1 mRNA—Previous studies in other cell types have demonstrated that the WAF1-HuD region within p21WAF1 mRNA is a target for members of the ELAV RNA-binding protein family (e.g. HuD) (39). Wang et al. (17) showed that increased binding of HuR to the WAF1–1/6 element mediated the UVC-induced stabilization of p21WAF1 mRNA (17). This suggested that the ~36-kDa band observed in UVXL studies with the WAF1-HuD and WAF1–1/6 probes and using MDA-468 breast cancer extracts (Fig. 5C, lanes 1 and 3) contained HuR. In addition, a preponderance of potential CP1 binding sites within the p21WAF1 3′-UTR (see Fig. 1A), together with the observed RPCs at ~42 kDa using the p21WAF1 3′-UTR riboprobes (Fig. 5C, lanes 1 and 3; Fig. 5D, lanes 2 and 3), suggested that CP1 protein might target p21WAF1 mRNA.

To investigate the association among HuR, CP1, and p21WAF1 mRNA in MDA-468 cells, we utilized an immunoprecipitation–RT-PCR assay with primers that target the p21WAF1 coding region (see “Materials and Methods”). Using HuR and CP1 antibodies, we were able to co-immunoprecipitate p21WAF1 mRNA from MDA-468 cells (Fig. 6A, lanes 2 and 3). However, no p21WAF1-specific PCR product was identified when using an unrelated antibody (EGFR) (Fig. 6A, lane 4) or with no antibody (beads alone) (Fig. 6A, lane 1). Controls were used routinely in these assays: positive (assay of supernatant following immunoprecipitation; see Fig. 6A, lane 5 and plasmid p21WAF1 DNA; see Fig. 6A, lane 6) and negative (H2O; see Fig. 6A, lane 7). These data provide definitive evidence that HuR and CP1 interact closely with p21WAF1 mRNA in MDA-468 cells.

To definitively map the binding site of HuR to WAF1-HuD we performed a UVXL assay using MDA-468 extracts and immunoprecipitated the resultant RPCs with HuR antibody. We identified a single major RPC with a molecular mass of ~36 kDa that was not present when the RPCs were precipitated with GST antibody or with beads alone (Fig. 6B, lanes 3–5). Similar results were obtained with the WAF1–1/6 probe (Fig. 6B, lanes 8–10). These findings provided strong evidence that the 36-kDa RPC detected in UVXL in Fig. 5C represents HuR bound to the WAF1-HuD and WAF1–1/6 probes. When tested, thrombin-cleaved recombinant GST-HuR bound to the WAF1-HuD and c-fos HuD probes (Fig. 6C, lanes 1 and 3), and in each case, the RPC could be supershifted with HuR antibody (Fig. 6C, lanes 2 and 4).

To investigate the binding of CP1 to the WAF1–1/6 element, we performed a UVXL assay with thrombin-cleaved recombinant GST-CP1. CP1 protein bound to the WAF1–1/6 riboprobe (Fig. 6C, lane 8); binding could be displaced using poly(C) ribohomopolymer (Fig. 6C, lanes 9 and 11) but not using poly(A) ribohomopolymer (Fig. 6C, lanes 10 and 12), confirming the specificity of this protein species for C-rich sequences. These data suggest that CP1 may target one or more motifs within the UVC-responsive WAF1–1/6 element.

We next used MDA-468 whole cell extracts treated with EGF or UV in UVXL experiments with WAF1-HuD and WAF1–1/6 probes. In each case, UV up-regulated binding of HuR (~36 kDa) to the probe, whereas EGF did not (WAF1-HuD; see Fig. 6D, lanes 1–5 and WAF1–1/6; see Fig. 6D, lanes 6–10). A similar UVC-induced increase in binding of HuR to p21WAF1...
mRNA has been observed in RKO colorectal carcinoma cells (17). However, no significant change was seen in the pattern of binding for any of the other p21WAF1 RNA-binding proteins. These data suggest an important role for HuR in the UVC-induced up-regulation of p21WAF1 mRNA stability and implication of the other p21WAF1 RNA-binding proteins.

To determine the functional role of HuR in the regulation of p21WAF1 expression and control of cell cycle in MDA-468 cells, we used retroviral vectors to generate stable pools of MDA-468 cells expressing antisense or sense HuR. HuR protein levels varied significantly between the antisense and sense MDA-468 sublines. Despite this, no significant difference was observed in p21WAF1 or actin protein levels following EGF treatment (Fig. 7A). We also used flow cytometry to examine the effect of HuR levels on the cell cycle profile. However, no difference was observed in the proportion of cells in S phase unaffected by increasing (sense) or decreasing (antisense) HuR levels. The EGF-induced reduction in S phase content was also unaffected by HuR levels (Fig. 7B).

Similarly, in a colony formation assay (not shown), EGF induced >98% reduction in the number of colonies with each subline. Taken together, these data suggest that although HuR binds to the WAF1-HuD sequence of p21WAF1 mRNA (within the context of the larger WAF1–1/6 region) in MDA-468 cells, modification of cellular HuR levels has little or no effect on the regulation of p21WAF1 protein levels or progression of cells through the cell cycle.

**Discussion**

p21WAF1 plays a central role in various models of growth inhibition, although the molecular mechanisms that regulate p21WAF1 expression in breast cancer cells are not well understood. Here we have shown that EGF-induced growth inhibition in MDA-468 breast cancer cells (mutant p53) is associated with a rapid increase in p21WAF1 protein and mRNA expression, which results from the combination of increased transcription and stabilization of p21WAF1 mRNA. Significantly, we established that the 3′-UTR of p21WAF1 contains cis-acting...
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Our data provides definitive mRNA decay evidence implicating the p21WAF1 3′-UTR in cis, supporting the findings of Liu et al. (48). Previous studies into the effect of shorter elements, such as WAF1-HuD or WAF1–1/6, demonstrated that the WAF1-HuD region did not destabilize a CAT reporter RNA in transfected HepG2 cells significantly (48). Similarly, Li et al. (48) found that the ARE motifs present within the p21WAF1 3′-UTR (HuD; A, B, and C in Fig. 1, A and B) did not contribute substantially toward message instability in breast cancer cells (14). Instead, they found that the predominant basal instability sequences were contained downstream of the WAF1–1/6 region thought responsible for UVC-induced stabilization of p21WAF1 mRNA. Together with our data, these findings suggest that the p21WAF1 3′-UTR is a composite cis-acting sequence, with contributions to basal turnover from each of the WAF1–1/6, WAF1–879, and WAF1–1512 regions, but that the majority of the effect is because of sequences downstream of WAF1–1/6 (cf. with Li et al. (14)). Interestingly, EGF and UVC augmented reporter activity preferentially via different components of the 3′-UTR. In particular, EGF-induced up-regulation of reporter activity occurred predominantly via a combination of WAF1–879 and WAF1–1512, with little effect via WAF1–1/6. In contrast, UVC augmented reporter activity predominantly through WAF1–1/6, consistent with the findings of Wang et al. (17), together with smaller, yet significant, up-regulation via WAF1–879 and WAF1–1512 sequences. Further analysis of each of these three components of the 3′-UTR will be required in different cell types and with different stimuli to develop a definitive understanding of the mechanisms underlying p21WAF1 mRNA turnover.

We have produced several lines of evidence in support of the association of HuR and CP1 with p21WAF1 mRNA in MDA-468 breast cancer cells. These include the immunoprecipitation of endogenous HuR bound to the WAF1–HuD and WAF1–1/6 ribonucleoproteins and the immuno-purification of p21WAF1 mRNA from MDA-468 cell extracts using HuR and CP1 antibodies. These assays (UVXL-IP and IP-RT-PCR) provide the first definitive evidence for a close association of HuR and CP1 with p21WAF1 mRNA in MDA-468 cells.

Based on these observations and the findings of others, we presumed there would be a significant role for HuR in the EGF-induced regulation of p21WAF1 mRNA stability in breast cancer cells. For example, HuR and other members of the ELAV protein family have been shown to stabilize AU-rich mRNAs in several other cell systems (35). These include the stabilization of VEGF mRNA (36), GLUT-1 mRNA (49), and p21WAF1 mRNA in UVC-treated colorectal carcinoma cells (17). In the latter report, the shortest riboprobe that the authors used was equivalent to our WAF1–1/6 probe (see Fig. 1A). HuR was one of only two proteins detected by UVXL, and HuR antibody produced a partial supershift in cell extracts. Furthermore, antisense HuR-expressing clones (with a 4- to 6-fold reduction in HuR levels) demonstrated a decrease in both basal and UV-induced p21WAF1 expression (mRNA stability and protein levels). However, we observed that the WAF1–1/6 region did not have a major role in modulating EGF-induced reporter activity in MDA-468 cells. Moreover, we found that treatment of MDA-468 cells with UVC, but not EGF, regulated the binding of HuR to the WAF1–HuD and WAF1–1/6 ribonucleoproteins. Thus, we were not surprised to find that modification of HuR expression in MDA-468 cells had no detectable effect on EGF-induced p21WAF1 protein levels, cell cycle, or growth. Taken together, these observations suggest that in these cells, HuR plays a relatively minor role in the regulation of basal and EGF-induced expression of p21WAF1.

In this context, Liu et al. (48) found that non-HuR RNA-binding proteins (24 and 52 kDa) mediated the induction of p21WAF1 mRNA stability by the α1 adrenergic agonist, phenylephrine (48). They did not observe binding of cellular HuR to their riboprobe, which was identical to our WAF1–HuD ribo-

Fig. 7. HuR does not regulate EGF-induced p21WAF1 expression or cell cycle profile in MDA-468 cells. A, Western blot analysis of HuR, p21WAF1, and actin protein levels in pools of retrovirally infected MDA-468 cells that stably express sense (lane 2) or antisense (lane 3) HuR and were treated for 4 h with EGF (25 ng/ml). Analysis of parental MDA-468 cells is included for comparison (lane 1). AS, antisense. B, MDA-468 sublines indicated in A, as well as vector control–infected cells (Puro), were incubated ± EGF (25 ng/ml) for 16 h and then subjected to flow cytometry analysis (as described under “Materials and Methods”) to determine % of cells in S phase.
probe. We therefore presume that the role of HuR in regulating p21WAP1 expression varies according to the mode of stimulus and is dependent upon cell type. It also emphasizes that RNA-binding proteins other than HuR can regulate p21WAP1 mRNA turnover.

The poly(C)-binding proteins, CP1 and CP2, are members of the hnRNPA K-homology domain family of RNA-binding proteins (50) and regulate the stability of a variety of transcripts, including globin, tyrosine hydroxylase and erythropoietin (51–55), as well as regulating translation of 15- lipoxigenase and human papillomavirus (56, 57). Co-immunopurification of p21WAP1 mRNA from MDA-468 cells using CP1 antibody suggests that CP1 protein binds to one or more of the motifs distributed throughout the p21WAP1 3′-UTR (see Fig. 1A). UVXL analysis of WAF1–1/6, WAF1–879, and WAF1–1512 demonstrates the presence of RPCs at −42 kDa, which may contain CP1 and/or CP2. CP1 may therefore play a role in the regulation of p21WAP1 mRNA stability in MDA-468 cells through interactions with sequences within and/or downstream of WAF1–1/6.

We have identified a U- and C-rich cis-element in the 3′-UTR of the human androgen receptor mRNA that is the target for simultaneous, co-operative binding of HuR and CP1/CP2 (47). The UVXL assays presented herein with recombinant CP1 suggest that CP1 may bind to the UVC-responsive WAF1–1/6 element. The close proximity of HuR and CP1 binding sites within WAF1–1/6 might allow both proteins to participate in coordinated mRNA decay. It also emphasizes the need to examine the functional role of CP1 in p21WAP1 mRNA turnover in MDA-468 cells.

In summary, EGF increases p21WAP1 mRNA expression in p53 mutant breast cancer cells through a combination of mRNA stabilization and transcriptional up-regulation. We have identified cis-elements within the p21WAP1 3′-UTR that are distinctively EGF- or UVC-inducible in MDA-468 cells. This implies that different stimuli can regulate p21WAP1 mRNA stability via independent cis-elements. HuR binding modulates p21WAP1 expression in mRNA-treated RKO cells but not in EGF-treated MDA-468 cells. This indicates that there is an HuR-independent, cell type-specific mechanism through which EGF induces p21WAP1 expression via stabilization of p21WAP1 mRNA. CP1 and other RNA-binding proteins associate with p21WAP1 mRNA in MDA-468 cells and may direct its turnover. The cloning and characterization of these proteins are the subject of further investigation.

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