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Posttranscriptional Regulation of MEK-1 by Polyamines through the RNA–binding Protein HuR Modulating Intestinal Epithelial Apoptosis

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Abstract

Mitogen-activated protein kinase kinase 1 (MEK-1) is an important signal transducing enzyme that is implicated in many aspects of cellular functions. Here, we report that cellular polyamines regulate MEK-1 expression at the posttranscriptional level through the RNA-binding protein HuR in intestinal epithelial cells (IECs). Decreasing the levels of cellular polyamines by inhibiting ornithine decarboxylase (ODC) stabilized MEK-1 mRNA and promoted its translation through enhancement of HuR interaction with the 3′-untranslated region of MEK-1 mRNA, whereas increasing polyamine levels by ectopic ODC overexpression destabilized the MEK-1 transcript and repressed its translation by reducing [HuR/MEK-1 mRNA] complex; neither intervention changed MEK-1 gene transcription via its promoter. HuR silencing rendered the MEK-1 mRNA unstable and inhibited its translation, thus preventing increases in MEK-1 mRNA and protein in polyamine-deficient cells. Conversely, HuR overexpression induced MEK-1 mRNA stability and promoted its translation. Inhibition of MEK-1 expression by MEK-1 silencing or HuR silencing prevented the increased resistance of polyamine-deficient cells to apoptosis. Moreover, HuR overexpression did not protect against apoptosis if MEK-1 expression was silenced. These results indicate that polyamines destabilize the MEK-1 mRNA and repress its translation by inhibiting HuR association with MEK-1 transcript. Our findings indicate that MEK-1 is a key effector of the HuR-elicited antiapoptotic program in intestinal epithelial cells.

Keywords

3′-untranslated region; mRNA stability; translational regulation; ribonucleoprotein; ornithine decarboxylase

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Supplemental Data: Supplemental data include three Figures (Supplemental Figures A1-A3).

INTRODUCTION

The epithelium of mammalian intestinal mucosa is a rapidly self-renewing tissue in the body, and maintenance of its integrity depends on a dynamic balance between cell proliferation and apoptosis [1,2]. In response to stress, rapid changes in gene expression patterns in intestinal epithelial cells (IECs) control cell division and survival, thereby preserving the epithelial homeostasis [3]. Although gene expression is critically regulated at the transcription level in IECs, the essential contribution of posttranscriptional events, particularly altered mRNA turnover and translation, is becoming increasingly recognized [2,4-6]. The posttranscriptional fate of a given mRNA is primarily controlled by the interaction of specific mRNA sequences (*cis*-elements) with specific *trans*-acting factors such as RNA-binding proteins (RBPs) and noncoding regulatory RNAs (such as microRNAs) [7-10]. The most common *cis*-elements responsible for rapid regulation of mRNA decay and translation in mammalian cells are U- and UA-rich elements (AREs) located in the 3′-untranslated regions (3′-UTRs) of many mRNAs [11-14]. Ribonucleoprotein (RNP) associations either increase or decrease mRNA stability or/and translation depending on the particular mRNA sequence, cellular growth conditions, and the stimulus type [5,15,17]. Among the RBPs that regulate specific subsets of mRNAs are several RBPs that modulate mRNA turnover (HuR, NF90, AUF1, BRF1, TTP, KSRP) and RBPs that modulate translation (HuR, TIAR, NF90, TIA-1), collectively known as *t*ranslation and *t*urnover-*r*egulatory (TTR)-RBPs [16-18].

The Hu antigen R (HuR) protein is the ubiquitously expressed member of the *ELAV*-like family of TTR-RBPs. HuR has two N-terminal RNA-recognition motifs (RRMs), followed by a nucleocytoplasmic shuttling sequence, and a C-terminal RRM [19-22]. HuR is predominantly located in the nucleus in unstimulated cells, but it rapidly translocates to the cytoplasm, where it directly interacts with and regulates target mRNA stability and/or translation in response to specific stimuli [20,23]. Recently, HuR was shown to play an important role in the regulation of intestinal epithelial homeostasis by modulating IEC proliferation and apoptosis [6,24-27]. The subcellular localization of HuR and its binding affinity for specific target transcripts in IECs are tightly regulated by numerous factors, including cellular polyamines [6,26]. The natural polyamines spermidine, spermine, and their precursor putrescine are ubiquitous, small basic molecules that are intimately involved in the control of epithelial homeostasis [27-29]. Normal IEC proliferation in the mucosa depends on the supply of polyamines to the dividing cells in the crypts [2,4,5,30]; polyamines also regulate IEC apoptosis [31,32]. Decreasing cellular polyamines by inhibiting ornithine decarboxylase (ODC, the first rate-limiting step for polyamine biosynthesis) is found to increase cytoplsmic levels of HuR-stabilizing p53 and nucleophosmin (NPM) mRNAs, thus contributing to the inhibition of IEC proliferation [6,26]. Polyamines are also necessary for HuR phosphorylation in IECs, and polyamine depletion represses c-Myc translation by reducing [HuR/c-Myc mRNA] complex through inhibition of Chk2-dependent HuR phosphorylation [25]. Indeed, growing evidence shows that HuR is emerging as a pivotal posttranscriptional regulator essential for maintaining the intestinal epithelial integrity.

Mitogen-activated protein kinase (MAPK) kinase-1 (MEK-1) is a dual-specificity kinase that plays a role in the regulation of various cellular functions including proliferation, development, differentiation, migration, and apoptosis by activating MAPK/ERK signals [33-35]. While a single *MEK* gene is present in *C. elegans*, *Drosophila* and *Xenopus*, there are two MEK homologs, MEK-1 and MEK-2, in mammalian systems [33]. Studies analyzing the protein sequence differences between MEK-1 and MEK-2 suggest that MEK-2 diverged from MEK-1, most likely to achieve unique functions in mammalian [36,37]. Gene disruption of MEK-1 is lethal at early stages of embryonic development [38],

but MEK-2$^{-/-}$ mice are normal in their overall general behavior [39], suggesting that MEK-1 can compensate for the absence of MEK-2 protein, but MEK-2 fails to substitute for the important role of MEK-1. The cellular MEK-1 protein level and its kinase activity in IECs are highly regulated and shown to be crucial for maintaining epithelial homeostasis [40,41], but the exact mechanism involved in MEK-1 expression remains elusive. Here, we set out to study if polyamines regulate MEK-1 expression at the posttranscriptional level through the RBP HuR. The results presented here indicate that polyamine depletion increased the stability and translation of MEK-1 mRNA by inducing HuR association with MEK-1 mRNA, whereas elevating the cellular levels of polyamines reduced the abundance of [HuR/MEK-1 mRNA] complexes, thus decreasing the steady-state level of MEK-1. Furthermore, the HuR-elevated MEK-1 suppressed IEC apoptosis, as silencing MEK-1 or HuR sensitized IECs to apoptotic cell death.

MATERIALS AND METHODS

Chemicals and Supplies

Tissue culture medium and dialyzed fetal bovine serum were from Invitrogen (Carlsbad, CA, USA), and biochemicals were from Sigma (St. Louis, MO, USA). The antibodies recognizing HuR, MEK-1 and caspase-3 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the secondary antibody conjugated to horseradish peroxidase was purchased from Sigma. DFMO (α-difluoromethylornithine) was from Genzyme (Cambridge, MA, USA). L-[1-^{14}C]ornithine (sp. radioactivity 51.6 Ci/mmol) was purchased from NEN (Boston, MA, USA).

Cell Culture and Stable ODC Gene Transfection

The IEC-6 cell line, derived from normal rat intestinal crypt cells [42], was purchased from the American Type Culture Collection (Manassas, VA) at passage 13 and used at passages 15–20 [5,6]. Cells were maintained in DMEM supplemented with 5% inactivated fetal bovine serum, 10 μg/ml insulin, and 50 μg/ml gentamicin. ODC-overexpressing IEC-6 (ODC-IEC) cells were developed as described in our previous studies [30] and expressed a more stable ODC variant with full enzyme activity [43].

Reporter Plasmids and Luciferase Assays

The MEK-1-promoter sequence was predicted by online software *Genomatrix* (http://www.genomatix.de) and amplified by primers 5′-GACCTGCGTGCTAGAACCTC-3′ (sense) and 5′-TCTGGACGCTTGTAGCAGAG-3′ (antisense) using rat genomic DNA (Clontech, Mountain View, CA, USA) as template. PCR product was sequenced (see Supplemental Fig.-A1) to confirm that no mutations were introduced by PCR and then cloned into pGL3-basic Luciferase vector (Promega Madison, WI, USA). Transient transfections were performed using the Lipofectamine Reagent and performed as recommended by the manufacturer (Invitrogen). The promoter constructs were transfected into cells along with phRL-null, a Renilla luciferase control reporter vector from Promega, to monitor transfection efficiencies as described previously [44]. The transfected cells were lysed for assays of promoter activity using the Dual Luciferase Reporter Assay System (Promega) 48 h after the transfection. The levels of luciferase activity were normalized by Renilla-driven luciferase activity in every experiment. The chimeric firefly luciferase reporter construct of the MEK-1 3′-UTR was generated as described previously [25,45,46]. The 924-bp ARE fragment from the MEK-1 3′-UTR was amplified and subcloned into the pGL3-Luc plasmid (Promega) at the XbaI site to generate the chimeric pGL3-Luc-MEK1-3′UTR. The sequence and orientation of the fragment in the luciferase reporter were identified by DNA sequencing and enzyme digestion. Luciferase activity was measured using the Dual Luciferase Assay System following the manufacturers' instructions. To

measure translational changes (*translation efficiency*), the Firefly and Renilla luciferase activities were compared to Firefly and Renilla normalized for RNA levels.

Recombinant Viral Construction and Infection

Recombinant adenoviral plasmids containing human HuR were constructed by using the Adeno-X Expression System (Clontech, Mountain View, CA, USA) according to the protocol provided by the manufacturer. Briefly, the full-length cDNA of human wild-type HuR was cloned into the pShuttle by digesting the BamHI/HindIII and ligating the resulting fragments into the XbaI site of the pShuttle vector [24]. pAdeno-HuR (AdHuR) was constructed by digesting the pShuttle construct with PI-SceI/I-CeuI and ligating the resulting fragment into the PI-SceI/I-CeuI sites of the pAdeno-X adenoviral vector. Recombinant adenoviral plasmids were packaged into infectious adenoviral particles by transfecting human embryonic kidney (HEK)-293 cells using LipofectAMINE Plus reagent (Invitrogen). The adenoviral particles were propagated in HEK-293 cells and purified upon cesium chloride ultracentrifugation. Titers of the adenoviral stock were determined by standard plaque assay. Recombinant adenoviruses were screened for the expression of the introduced gene by Western blot analysis using anti-HuR antibody. pAdeno-X, which was the recombinant replication–incompetent adenovirus carrying no HuR cDNA insert (Adnull), was grown and purified as described above and served as a control adenovirus. Cells were infected with AdHuR or Adnull, and expression of HuR was assayed at 48 h after infection.

RNA Interference

The silencing RNA duplexes that were designed to specifically cleave HuR and MEK-1 mRNAs were synthesized and transfected into cells as described previously [6,24]. The sequences of small interfering RNA (siRNA) that specifically target the coding region of HuR and MEK-1 mRNA (siHuR) were AACACACTGAACGGCTTGAGG, CAGAAGGTGGGAGAGTTGAAGGATG, respectively, whereas the sequence of control siRNA (C-siRNA) was AAGTGTAGTAGATCACCAGGC. The siRNA was introduced into cells by transient transfection as mentioned previously [24]. For each 60-mm cell culture dish, 15 μl of the 20 μM stock duplex siHuR, siMEK-1, or C-siRNA was mixed with 500 μl of Opti-MEM medium (Invitrogen). This mixture was gently added to a solution containing 15 μl of LipofectAMINE 2000 in 500 μl of Opti-MEM. The solution was incubated for 20 min at room temperature and gently overlaid onto monolayers of cells in 4 ml of medium, and cells were harvested for various assays after 48 h later.

Analysis of Newly Translated Protein

New synthesis of MEK-1 was measured by incubating IEC-6 cells with 1 mCi L-[^{35}S]methionine and L-[^{35}S]cysteine per 60-mm plate for 20 min, whereupon cells were lysed using RIPA buffer [47]. Immunoprecipitations were carried out for 1 h at 4 °C using either a polyclonal antibody recognizing MEK-1 or IgG1 (BD Pharmingen). Following extensive washes in TNN buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 5 mM EDTA, 0.5% NP-40), the immunoprecipitated material was resolved by 10% SDS-PAGE, transferred onto PVDF filters, and visualized with a PhosphorImager (Molecular Dynamics).

Western Blot Analysis

Whole-cell lysates were prepared using 2% SDS, sonicated, and centrifuged (12,000 rpm) at 4°C for 15 min. The supernatants were boiled for 5 min and size-fractionated by SDS-PAGE (7.5% acrylamide). After transferring proteins onto nitrocellulose filters, the blots were incubated with primary antibodies recognizing MEK-1, HuR, or caspase-3. After incubations with secondary antibodies, immunocomplexes were developed by using chemiluminescence.

RT-PCR and Real-Time PCR Analysis

Total RNA was isolated by using RNeasy mini kit (Qiagen, Valencia, CA, USA) and used in reverse transcription and PCR amplification reactions as described [24]. PCR primers for rat MEK-1 were 5 ′-CAGAAGAAGCTGGAGGAGCT-3′ (sense) and 5′-GCTTCTCTCGTAGATATGTCAGG-3′ (antisense), yielding a 450-base pair fragment. Primers for GAPDH were 5 ′-TACTAGCGGTTTTACGGGCG-3 ′ and 5′-TCGAACAGGAGGAGCAGAGAGCGA-3′. Primers for luciferase were 5′-TCAAAGAGGCGAACTGTGTG-3 ′ and 5 ′-GGTGTTGGAGCAAGATGGAT-3′. The levels of β-actin PCR product were assessed to monitor the even RNA input in RT-PCR samples. Real-time quantitative PCR (Q-PCR) was performed using 7500-Fast Real-Time PCR Systems (Applied Biosystems, Foster City, CA Molecular Dynamics) with specific primers, probes, and software (Applied Biosystems). The levels of MEK-1 mRNA were quantified by Q-PCR analysis and normalized by GAPDH levels.

Preparation of Synthetic RNA Transcripts

cDNA from IEC-6 cells was used as a template for PCR amplification of the coding region (CR) and 3′-UTR of MEK-1. The 5′-primers contained the T7 RNA polymerase promoter sequence (T 7): 5 ′-CCAAGCTTCTAATACGACTCACTATAGGGAGA-3 ′ . To prepare the CR of MEK-1 (spanning position 27-1228), oligonucleotides (T7)5′-CAAGATGCCCAAGAAGAAGC-3′ and 5′-GCTTCCCAAAGGCTCAGAT-3′ were used. To prepare the MEK-1 3′-UTR template (spanning position 1215–2108), oligonucleotides (T7)5′-CTTTGGGAAGCAGCAGAGAG-3′ and 5′-ACTTTAACTTGATAGTATTTT-3′ were used. To prepare the MEK-1 3′-UTR fragments F-1, F-2, F-3, and F-7 (spanning position 1212-1359, 1327-1430, 1400-1686, and 1856-2136, respectively), the following oligonucleotides were used: (T7)5′-GTTGCTTTCAGGCCTCTCC-3 ′ and 5 ′-GACACAAGTACGATTTGGCACA-3′; (T7)5′-AGAACACAGCATGTGCCAAA-3′ and 5 ′-ACAAATAGCCCCAAGCACAA-3′; (T7)5′-AGTGGATTGGCTTTGTGCTT-3′ and 5 ′-CGTTCTGGTGCTCATTTCAG-3′; (T7)5′-AGTGGATTGGCTTTGTGCTT-3 ′ and 5 ′-TTTGATAAACATCTTGAGTAAAGTGG-3′. PCR-amplified products were used as templates to transcribe biotinylated RNAs by using T7 RNA polymerase in the presence of biotin-cytidine 5′-triphosphate as described [6,24]. Various short RNA probes for MEK-1 3′-UTR fragments, including F-2 (spanning position 1327–1430), F-3 (spanning position 1400–1686), F-4 (spanning position 1680–1790), F-5 (spanning position 1769–1833), F-6 (spanning positions 1861–1877), and F-7 (spanning position 1856–2136), were synthesized in the Biopolymer Laboratory at the University of Maryland Baltimore.

RNA Protein-binding Assays

For biotin pull-down assays, biotinylated transcripts (6 μg) were incubated with 120 μg of cytoplasmic lysate for 30 min at room temperature. Complexes were isolated with paramagnetic streptavidin-conjugated Dynabeads (Dynal, Oslo, Norway) and analyzed by Western blot analysis. To assess the association of endogenous HuR with endogenous MEK-1 mRNAs, immunoprecipitations (IP) of HuR-mRNA complexes were performed as described [6]. Twenty million IEC-6 cells were collected per sample, and lysates were used for IP for 4 h at room temperature in the presence of excess (30 μg) IP antibody (IgG, or anti-HuR). RNA in IP materials was used in RT followed by PCR analysis to detect the presence of MEK-1 mRNA.

Immunofluorescence Staining

Immunofluorescence was performed as described [48] with minor changes [6]. Cells were fixed using 3.7% formaldehyde, and the rehydrated samples were incubated overnight at 4°C with primary antibody anti-MEK-1 diluted 1:300 in blocking buffer and then incubated

with secondary antibody conjugated with Alexa Fluor-594 (Molecular Probes, Eugene, OR, USA) for 2 h at room temperature. After rinsing, slides were incubated with 1 μM TO-PRO3 (Molecular Probes) for 10 min to stain nuclei, rinsed again, mounted, and viewed through a Zeiss confocal microscope (model LSM410). Images were processed using PhotoShop software (Adobe, San Jose, CA, USA). Annexin-V staining for apoptosis was carried out by using a commercial apoptosis detection kit (BD Biosciences, San Diego, CA, USA) and performed according to the protocol recommended by the manufacturer.

Assays for ODC enzyme activity and cellular polyamine content

ODC activity was determined by radiometric technique in which the amount of $^{14}CO_2$ liberated from L-[1-^{14}C]ornithine was estimated [30], and enzymatic activity was expressed as picomoles of CO_2 per milligram of protein per hour. The cellular polyamine content was analyzed by high-performance liquid chromatography analysis as described previously [4]. After 0.5 M perchloric acid was added, the cells were frozen at −80°C until ready for extraction, dansylation, and HPLC analysis. The standard curve encompassed 0.31-10 μM. Values that fell >25% below the curve were considered undetectable. The results are expressed as nanomoles of polyamines per milligram of protein.

Statistics

Values are means ± SE from three to six samples. Autoradiographic and immunoblotting results were repeated three times. The significance of the difference between means was determined by analysis of variance. The level of significance was determined using Duncan's multiple range test [49].

RESULTS

Polyamine depletion increases MEK-1 expression posttranscriptionally

To determine the role of cellular polyamines in the regulation of MEK-1 expression, we examined changes in the levels of MEK-1 mRNA and protein following polyamine depletion. Consistent with our previous studies [5,24] and works of others [40,41], inhibition of ODC activity by treatment with 5 mM DFMO almost completely depleted cellular polyamines in IEC-6 cells. The levels of putrescine and spermidine were undetectable at 6 days after treatment with DFMO, and spermine was decreased by ~60% (data not shown). As shown in Fig. 1A, depletion of cellular polyamines by DFMO increased the steady-state levels of MEK-1 mRNA and protein, but this induction was totally prevented by addition of exogenous putrescine (10 μM) given together with DFMO. Spermidine (5 μM) given together with DFMO had an effect equal to that of putrescine on levels of MEK-1 mRNA and protein expression when it was added to cultures that contained DFMO (data not shown). Immunofluorescence staining further revealed that the induced MEK-1 was predominantly located in the cytoplasm after polyamine depletion (Fig. 1B), whereas combined treatment with DFMO and putrescine completely prevented the increased MEK-1 immunostaining, rendering the subcellular localization patterns similar to those observed in control cells.

To define the mechanism by which decreasing the levels of cellular polyamines induces MEK-1 expression, three sets of experiments were performed. First, we examined the effect of polyamine depletion by DFMO on MEK-1 gene transcription. The MEK-1 promoter fragment was cloned from human genomic DNA, which contained several potential AP-1 and CREB sites (Supplemental Fig. A1); the MEK-1-promoter luciferase reporter was constructed as illustrated in Fig. 1Ca. Polyamine depletion by DFMO did not change MEK-1 gene transcription, because there were no significant differences in the levels of MEK-1-promoter luciferase reporter gene activity between control cells and cells exposed to

DFMO alone or DFMO plus putrescine (Fig. 1Cb, *left*). Analysis of the kinetics of adding exogenous putrescine or spermidine on MEK-1 promoter activity in control cells also revealed that exposure of normal IEC-6 cells (without DFMO) to 10 μM putrescine or 5 μM spermidine for 2 and 4 h similarly failed to alter the levels of MEK-1 promoter luciferase reporter gene activity (data not shown). On the other hand, ectopic overexpression of the AP-1 transcription factor c-Jun by infection with the adenoviral vector containing the human c-jun cDNA significantly increased the levels of MEK-1 promoter activity (Fig. 1Cb, *right*). These results indicate that polyamine depletion does not influence MEK-1 gene transcription via its promoter in IECs and that the increase in steady-state levels of MEK-1 mRNA following polyamine depletion is likely related to a mechanism different from its synthesis.

Second, we determined if the induction of MEK-1 mRNA levels by polyamine depletion was instead influenced by changes in mRNA turnover. As shown in Fig. 1D, depletion of cellular polyamines by DFMO increased the stability of MEK-1 mRNA. In control cells, the mRNA levels declined gradually after gene transcription was inhibited by the addition of actinomycin D, displaying an apparent half-life of ~247 min. However, the stability of MEK-1 mRNA was dramatically increased following polyamine depletion with a half-life of >480 min, which was totally prevented by exogenous putrescine given together with DFMO. The half-life of MEK-1 mRNA in cells exposed to DFMO plus putrescine was ~255 min, similar to that of control cells (without DFMO). These findings indicate that polyamines regulate the MEK-1 expression posttranscriptionally and that depletion of cellular polyamines induces MEK-1 mRNA levels primarily by enhancing MEK-1 mRNA stability.

Third, we examined the effect of polyamine depletion on MEK-1 translation. To directly investigate whether increased MEK-1 expression might also result from inducing MEK-1 translation following polyamine depletion, we compared the rate of new MEK-1 protein synthesis between control cells and cells treated with DFMO alone or DFMO plus putrescine. Cells were incubated in the presence of L-[^{35}S]methionine and L-[^{35}S]cysteine for 20 min, whereupon newly translated MEK-1 was visualized by IP. The brief incubation period was chosen to minimize the contribution of MEK-1 degradation in our analysis. As shown in Fig. 1E, newly synthesized MEK-1 was markedly increased in DFMO-treated cells, whereas exogenous putrescine given together with DFMO restored the rate of new MEK-1 protein synthesis to normal levels. Polyamine depletion did not affect nascent GAPDH translation, as the rates of newly synthesized GAPDH protein in control cells were similar to those in cells treated with DFMO alone or DFMO plus putrescine. To further confirm these findings and to examine whether the translational effect of polyamines was exerted through the ARE, we used a firefly luciferase reporter gene construct containing the MEK-1 3′-UTR ARE (pGL3-Luc-MEK1ARE) and the negative control vector pGL3-Luc (Fig. 1F). A plasmid expressing *Renilla* luciferase was also cotransfected as an internal control for normalization of firefly luciferase. To distinguish translational output from mRNA turnover, the luciferase assays were normalized to luciferase-reporter mRNA levels to obtain the translation efficiency [10]. As shown in Fig. 1G, polyamine depletion by DFMO induced MEK-1 translation via ARE within its 3′-UTR as indicated by an increase in MEK-1 ARE luciferase reporter gene activity. The combined DFMO and putrescine treatment prevented the increase in MEK-1 translation, rendering the rate of MEK-1 ARE-mediated translation similar to that observed in control cells. By contrast, no changes in the luciferase reporter gene activity were seen in response to polyamine depletion when testing a control construct without the MEK-1 ARE (data not shown). These results indicate that decreasing the levels of cellular polyamines also increases MEK-1 mRNA translation through the MEK-1 3′-UTR ARE. We also examined changes in the half-life of MEK-1 protein and demonstrated that MEK-1 protein stability (as measured by incubating cells with cycloheximide to block *de novo* protein synthesis) was not affected by polyamine depletion

(Supplemental Fig. A2), supporting the view that polyamine depletion increases MEK-1 expression by stabilizing MEK-1 mRNA and promoting its translation.

Increasing cellular polyamines represses MEK-1 expression by destabilizing MEK-1 mRNA and inhibiting its translation

To determine the effect of increasing the levels of cellular polyamines on MEK-1 expression, two clonal populations of intestinal epithelial cells stably expressing ODC (ODC-IEC) [30] were used in this study. As reported previously [6], ODC-IEC cells exhibited very high levels of ODC protein and greater than 50-fold increase in ODC enzyme activity. Consistently, the levels of putrescine, spermidine, and spermine in stable ODC-IEC cells were increased by ~12-fold, ~2-fold, and ~25% when compared with cells transfected with the control vector lacking ODC cDNA (data not shown). As shown in Fig. 2A, increasing cellular polyamines by ODC overexpression repressed MEK-1 expression as shown by a significant decrease in the levels of MEK-1 mRNA and protein in ODC-IEC cells as compared with those observed in cells infected with control vector. Since increasing the levels of cellular polyamines did not affect levels of the MEK-1-promoter activity (Fig. 2B), this repression in MEK-1 expression by ODC overexpression also occurs at the posttranscriptional level. In support of this possibility, the results presented in Fig. 2C further show that the half-life of MEK-1 mRNA was decreased in ODC-IEC cells when compared with that of cells infected with control vector. This inhibition of MEK-1 expression by increasing cellular polyamines was also partially due to a reduction in MEK-1 mRNA translation, because levels of newly synthesized MEK-1 protein (Fig. 2D) and the activity of MEK-1 ARE luciferase reporter gene (Fig. 2E) were significantly decreased in stable ODC-IEC cells. The inhibitory effects of ODC overexpression on MEK-1 expression were not simply due to clonal variation, since two different clonal populations, ODC-IEC-C1 and ODC-IEC-C2, showed similar responses. The decrease in MEK-1 expression in stable ODC-IEC cells did not result from a reduction in MEK-1 protein stability, since the half-life of MEK-1 protein was not significantly different in control cells when compared with cells stably overexpressing ODC (Supplemental Fig. A3). Together, these results indicate that increasing the levels of cellular polyamines destabilizes MEK-1 mRNA and represses its translation.

Polyamines inhibit HuR association with MEK-1 mRNA

Our previous observations [6,26] have demonstrated that polyamine depletion by DFMO enhanced the cytoplasmic abundance of HuR, whereas increasing cellular polyamines by ODC overexpression decreased cytoplasmic HuR, although neither intervention changed whole-cell HuR levels (data not shown). Given the predicted affinity of HuR for the 3′-UTR of the MEK-1 mRNA (Fig. 3A), we hypothesized that HuR bound to the MEK-1 3′-UTR and further postulated that this association could be regulated by cellular polyamines. Three sets of experiments were performed to test the hypothesis. First, we examined if MEK-1 mRNA associated with HuR in IECs by performing RNP-IP assays using anti-HuR antibody under conditions that preserved RNP integrity [50]. The interaction of MEK-1 mRNA with HuR was examined by isolating RNA from the IP material and subjecting it to reverse transcription (RT), followed by either conventional PCR or real-time quantitative (Q-PCR) analyses. MEK-1 PCR products were highly enriched in HuR-IP samples compared with control IgG1 samples (Fig. 3B and C, left-second lanes), indicating that MEK-1 mRNA is a target of HuR. The enrichment of p53 PCR product was also examined and served as a positive control (data not shown), since HuR is shown to bind to p53 mRNA [6]. The amplification of GAPDH PCR products, found in all samples as low-level contaminating housekeeping transcripts (not HuR targets), served to monitor the evenness of sample input as reported previously [51]. Second, we determined changes in the levels of [HuR/MEK-1 mRNA] complexes after altering the levels of cellular polyamines. As shown in Fig. 3B,

polyamine depletion significantly enhanced HuR-binding to the MEK-1 mRNA, as indicated by an increase in the levels of MEK-1 mRNA in the HuR-IP materials from DFMO-treated cells when measured by RT followed by conventional PCR and Q-PCR analyses, but this induction was prevented by putrescine given together with DFMO. In contrast, increased levels of cellular polyamines by ODC overexpression repressed the formation of [HuR/MEK-1 mRNA] complexes. The levels of MEK-1 mRNA were decreased in the HuR-IP materials from stable ODC-IEC cells as compared with those observed in control cells (Fig. 3C). In addition, the MEK-1 mRNA was undetectable in nonspecific IgG1 IPs in all treatment groups.

Third, we tested if polyamines regulate HuR association with the MEK-1 mRNA through HuR interaction with the MEK-1 3′-UTR, CR, or both by using biothinylated transcripts which spanned the MEK-1 mRNA regions shown in Fig. 4A. The MEK-1 3′-UTR transcript readily associated with cytoplasmic HuR, as detected by Western blot analysis of the pull down material (Fig. 4B***a***); the binding intensity increased significantly when using lysates prepared from cells that were treated with DFMO, but was reduced when cells had been treated with DFMO plus putrescine. This increase in the levels of HuR-binding to MEK-1 3′-UTR is specific, because transcripts corresponding to the MEK-1 CR did not bind to HuR in controls and cells exposed to DFMO alone or DFMO plus putrescine (Fig. 4B***b***). On the other hand, increasing the levels of cellular polyamines by ODC overexpression inhibited the HuR association with the MEK-1 3′-UTR as indicated by a decrease in the levels of HuR in the MEK-1 3′-UTR pull down materials from stable ODC-IEC cells (Fig. 4Ca). Consistently, HuR was undetectable in the MEK-1 CR pull-down materials from control cells and cells overexpressing ODC (Fig. 4Cb). To further examine whether HuR binding to the MEK-1 3′-UTR is mediated through the specific sites containing predicted hits of the HuR motif, partial biotinylated transcripts spanning the MEK-1 3′-UTR were prepared (Fig. 4D, schematic) and their association with HuR was tested in pull down assays. HuR was found to bind the F-3, F-4, F-6, and F-7, four transcripts which contained HuR motif hits, but it did not bind to the F-1, F-2, and F-5 (although they also contained potential HuR hits). Together, these findings indicate that cytoplasmic HuR specifically binds to the 3-′UTR of MEK-1 mRNA and that this binding is negatively regulated by cellular polyamines.

HuR silencing prevents the increased stability of MEK-1 mRNA and inhibits its translation in polyamine-deficient cells

To directly examine the putative role of HuR in the increased MEK-1 mRNA stability and its translation in polyamine-deficient cells, small interfering RNA (siRNA) targeting the HuR mRNA (siHuR) was used to reduce HuR levels. With >95% cells transfected (data not shown), siHuR potently and specifically silenced HuR expression in polyamine-deficient cells (Fig. 5A). As shown in Fig. 5B, silencing HuR completely prevented the increased MEK-1 mRNA levels in polyamine-deficient cells. This reduction in MEK-1 mRNA by HuR silencing resulted primarily from the destabilization of MEK-1 transcript, because the half-life of MEK-1 mRNA in DFMO-treated cells transfected with siHuR was similar to that measured in control cells (Fig. 5C). HuR silencing also abolished the polyamine depletion-induced MEK-1 translation as indicated by a decrease in levels of MEK-1 ARE luciferase reporter gene activity (Fig. 5D). Furthermore, in HuR-silenced populations, the increased in MEK-1 protein levels after polyamine depletion was also prevented (Fig. 5A, *middle*) and was reduced to the levels similar to that obtained from control cells. Transfection with C-siRNA had no effect on the MEK-1 mRNA stability and translation or the levels of MEK-1 protein in polyamine-deficient cells. These findings strongly suggest that the increased MEK-1 mRNA stability and its translation following polyamine depletion result from the enhanced interaction of HuR with the MEK-1 3′-UTR in intestinal epithelial cells.

HuR overexpression stabilizes MEK-1 mRNA and promotes its translation

To further define the role of HuR in regulating MEK-1 posttranscriptionally, we examined the effect of overexpressing wild-type HuR upon MEK-1 mRNA stability and its translation in control IEC-6 cells (without DFMO). The adenoviral vector containing the corresponding human HuR cDNA under the control of the human cytomegalovirus immediate early gene promoter (AdHuR) was previously described [24,25]. The adenoviral vectors used in this study infect intestinal epithelial cells with near 100% efficiency [30]; >95% of IEC-6 cells were positive when they were infected for 24 h with the adenoviral vector encoding GFP (data not shown). As noted in Fig. 6A, the levels of HuR protein increased with viral load and reached ~2-, ~6-, ~12-, and ~15-fold higher levels than control cells when AdHuR was used at 50, 100, 150, and 200 pfu/cell, respectively. A control adenovirus that lacked exogenous HuR cDNA (Adnull) failed to induce HuR. Transient infection with AdHuR (100 pfu/cell) for 48 h increased the levels of MEK-1 mRNA (Fig. 6B); this induction in MEK-1 mRNA was due to increased stabilization of MEK-1 mRNA, as indicated by a significant increase in its half-life in IEC-6 cells (Fig. 6C). In addition, HuR overexpression also enhanced MEK-1 translation as shown by an increase in the levels of MEK-1 ARE luciferase reporter gene activity (Fig. 6D). Consistently, the increased MEK-1 mRNA stability and its translation were associated with an increase in the steady-state levels of MEK-1 protein after the infection with AdHuR compared with those observed in control cells and cells infected with Adnull (Fig. 6A, *middle*). These results indicate that HuR overexpression enhances MEK-1 expression by stabilizing MEK-1 mRNA and enhancing its translation.

HuR-mediated MEK-1 expression plays a critical role in the regulation of IEC apoptosis

To investigate the biological consequences of inducing endogenous MEK-1 levels following polyamine depletion, we examined its possible involvement in regulating IEC apoptosis. Our previous studies [31,32] and the work of other groups [29,41] have shown that polyamines regulate apoptosis through multiple signaling pathways and that depletion of cellular polyamines promotes the resistance of normal IECs to apoptosis. The results presented in Fig. 7 further show that inhibition of MEK-1 expression by HuR silencing or MEK-1 silencing by transfection with siRNA targeting MEK-1 mRNA (siMEK-1) altered the susceptibility of polyamine-deficient cells to tumor necrosis factor-α (TNF-α)/cycloheximide (CHX)-induced apoptosis. As shown in Fig. 7B***a*** and C, control cells were exposed to TNF-α/CHX for 4 h, showed morphological features characteristic of programmed cell death. The assessments of apoptosis were confirmed by an increase in levels of active caspase-3 (Fig. Fig. 7D, *left*) after treatment with TNF-α/CHX. Consistent with our previous studies, exposure of polyamine-deficient cells to the same doses of TNF-α/CHX caused no apoptosis. In keeping with our earlier findings [31,32], there were no differences in the morphological features and percentages of apoptotic cells when comparing cells treated with DFMO alone with DFMO-treated cells exposed to TNF-α/CHX for 4 h (data not shown). This increased resistance to TNF-α/CHX-induced apoptosis was not affected when polyamine-deficient cells were transfected with C-siRNA (Fig. 7B***b***), but it was lost when MEK-1 expression was inhibited by HuR silencing (Fig. 7B***c***) or MEK-1 silencing (Fig. 7B***d***). The percentages of apoptotic cells (Fig. 7C) and the levels of the active caspase-3 protein (Fig. 7D) in DFMO-treated cells transfected with siHuR or siMEK-1 increased significantly when compared with those observed in DFMO-treated cells transfected with C-siRNA.

To further define the role of MEK-1 in the HuR-mediated anti-apoptotic effect in IECs, we also examined changes in TNF-α/CHX-induced apoptosis after ectopic HuR overexpression in MEK-1-silenced populations of cells. As shown in Fig. 8, HuR overexpression protected IEC-6 cells against TNF-α/CHX-induced apoptosis, but this protective effect was prevented

by MEK-1 silencing. Numbers of apoptotic cells (Fig. 8Bb and C) and the active caspase-3 protein levels (Fig. 8D) decreased remarkably in HuR-expressing cells when compared with those observed in cells infected with Adnull after exposure to TNF-α/CHX. Although this HuR-mediated protection was not altered when HuR-expressing cells were transfected with C-siRNA (Fig. 8B***c***), it was abolished by MEK-1 silencing (Fig. 8B***d***). The percentage of apoptotic cells (Fig. 8C) and active caspase-3 protein levels (Fig. 8D) in MEK-1-silenced cells infected with AdHuR increased significantly after exposure to TNF-α/CHX. These results implicate HuR-mediated MEK-1 expression in the regulation of intestinal epithelial apoptosis and indicate that the elevation in MEK-1 levels promotes an increase in resistance to apoptosis following polyamine depletion.

DISCUSSION

The regulation of MEK-1 activity through its phosphorylation has been extensively investigated [33,34,52,53], but little is known about the control of MEK-1 protein abundance, particularly at the posttranscriptional level. In the present study, we highlight a novel function of the RBP HuR and cellular polyamines in the regulation of MEK-1 mRNA stability and translation, thus advancing our understanding of the mechanisms that control the expression of MEK-1 and the biological function of cellular polyamines. Studies aimed at characterizing the molecular aspects of this process revealed that HuR bound to the MEK-1 mRNA by interacting with its 3′-UTR and affected the turnover and translation of the MEK-1 transcript. Depletion of cellular polyamines enhanced HuR association with the MEK-1 mRNA, thereby stabilizing the MEK-1 mRNA and promoting its translation. Conversely, increasing the levels of cellular polyamines decreased the abundance of the [HuR/MEK-1 mRNA] complex, leading to a reduction in the steady-state level of MEK-1 by destabilizing the MEK-1 mRNA and repressing its translation. The present studies also implicate HuR-mediated MEK-1 in the regulation of IEC apoptosis and therefore in intestinal epithelial homeostasis.

The results reported here show that polyamines negatively regulate expression of the MEK-1 gene posttranscriptionally. As observed in a range of tissues [33], expression of the MEK-1 gene is constitutive in IECs and its basal level is relatively high. Decreasing cellular polyamines by inhibiting ODC with DFMO led to additional increases in the steady-state levels of MEK-1 mRNA and protein in IECs (Figure 1), whereas increasing cellular polyamines by ectopic ODC overexpression repressed the expression of the MEK-1 (Figure 2). Our results further indicate that altering the levels of cellular polyamines did not influence MEK-1 gene transcription, because there were no significant differences in the levels of MEK-1 promoter activity between control cells, polyamine-deficient cells, and cells overexpressing ODC. Instead, depletion of cellular polyamines not only increased the half-life of MEK-1 mRNA but also enhanced its translation through AREs located in the MEK-1 3′-UTR. The increases in MEK-1 mRNA stability and translation in DFMO-treated cells were completely prevented by the addition of exogenous putrescine, indicating that the observed posttranscriptional changes in MEK-1 gene expression are due to the polyamine depletion rather than to nonspecific effects of DFMO. Consistent with our current findings, it has been reported that MEK-1 expression is increased by an induction in IL-4 following treatment with lysophosphatidic acid in human mast cells [54], but its expression is repressed during the process of postnatal heart development in rats [55]. However, the exact mechanism underlying the regulation of MEK-1 gene expression in response to specific stimuli remains elusive.

Our current studies also indicate that the MEK-1 mRNA is a target of the RBP HuR and that altering the levels of cellular polyamines affects HuR association with the MEK-1 mRNA. Although the precise mechanisms responsible for HuR in posttranscriptional regulation

remain to be uncovered, an increasing body of evidence shows that HuR-mediated transcript stabilization and translation are closely linked to its cytoplasmic presence [23,56]. We [6,26] have recently demonstrated that depletion of cellular polyamines increases the cytoplasmic abundance of HuR by inhibiting the AMPK-regulated phosphorylation and acetylation of importin-α1, whereas increased levels of polyamines decrease cytoplasmic HuR content by activating the AMPK-driven nuclear import via importin-α1. The results presented in Figure 3 further show that polyamine depletion increased HuR-binding to the MEK-1 mRNA, but the levels of [HuR/MEK-1 mRNA] complex decreased in the presence of elevated cellular polyamines in cells overexpressing ODC. Our current studies also find that the cytoplasmic HuR bound to the 3′-UTR of MEK-1 mRNA rather than its CR and that this binding affinity is primarily mediated through four specific regions containing hits of the HuR signature motif (Figure 4). These findings are consistent with observations in other studies showing that HuR binds to AREs commonly located at the 3′-UTRs of labile mRNAs and in particular mRNAs bearing a specific RNA motif in HuR target transcripts [7,21,24,45,47]. However, the sequence spanning positions 1212-1430 (F-1 and F-2) and 1769-1833 (F-5) of the MEK-1 3′-UTR also contain the computationally predicted HuR hits, but failed to bind to HuR in polyamine-deficient cells, suggesting that perhaps these sequences were inaccessible to HuR, possibly because they were targeted by other RBPs which had greater binding affinity than HuR, as measured by the biotin pulldown assays.

Increased HuR association with the MEK-1 mRNA in polyamine-deficient cells stabilizes MEK-1 mRNA and enhances its translation in IECs. As shown in Figure 5, depletion of cellular polyamines by DFMO increased the half-life of MEK-1 mRNA and enhanced its translation, but these effects were completely abolished in cells in which HuR expression was silenced, which in turn resulted in a marked reduction of MEK-1 protein. Consistent with our current findings, increased cytoplasmic HuR is also shown to interact with and stabilize p53, NPM, ATF2, and XIAP mRNAs in polyamine-deficient cells [6,24,47]. On the other hand, ectopic HuR overexpression stabilized MEK-1 mRNA and induced its translation, thereby increasing the steady-state level of MEK-1 protein (Figure 6). Although the exact mechanisms whereby cytoplasmic HuR alters posttranscription of the MEK-1 gene after changes in levels of cellular polyamines are still unknown, several studies suggest that HuR acts by protecting the body of the mRNAs from degradation, rather than slowing the rate of deadenylation [7,8]. Ongoing experiments are testing if the stimulatory effects of HuR on the MEK-1 mRNA and translation depend on HuR phosphorylation, which was shown to regulate both HuR subcellular localization and its binding affinity for specific target mRNAs [51,56,57].

The HuR-mediated MEK-1 expression plays an important role in the regulation of IEC apoptosis and is thus implicated in maintaining homeostasis of the intestinal epithelium. The epithelium of the intestinal mucosa is continuously renewed from the proliferative zone within the crypts, and this dynamic process is counterbalanced by apoptosis [58]. Apoptosis occurs in the crypt area, where it maintains the critical balance in cell number between newly divided and surviving cells, and at the luminal surface of the intestinal mucosa, where differentiated cells are lost. Our previous studies [31,32] and studies from other laboratories [29] have demonstrated that polyamines are crucial for the maintance of epithelial homeostasis and that depletion of cellular polyamines promotes the resistance of IECs to apoptosis through multiple signaling pathways. For example, polyamines negatively regulate NF-κB and Akt signals, and polyamine depletion increases NF-κB transcriptional activity [31,59] and activates Akt kinase [32]. Polyamines also inhibit the expression of ATF-2 and XIAP genes at the posttranscriptional level, as decreasing the levels of cellular polyamines induces the steady-state levels of ATF-2 [24] and XIAP [27] through stabilization of their mRNAs. Our current studies further show that increased levels of endogenous MEK-1 by HuR in polyamine-deficient cells also contribute to the increased resistance of IECs to

apoptosis, since this tolerance was significantly abrogated by silencing MEK-1 or HuR (Figure 7). In support of this finding, the anti-apoptotic influence induced by ectopic HuR overexpression was also blocked by MEK-1 silencing in normal IEC-6 cells (Figure 8). These results suggest that MEK-1 is a critical downstream effector of the prosurvival program elicited by HuR.

In summary, these results indicate that polyamines down-regulate MEK-1 expression at the posttranscriptional level through HuR-mediated events. Depletion of cellular polyamines increases MEK-1 expression by inducing the half-life of MEK-1 mRNA and enhancing its translation without affecting its gene transcription, whereas increasing the levels of cellular polyamines reduces the steady-state level of MEK-1 by destabilizing MEK-1 transcript and repressing its translation. Experiments searching for mechanism underlying polyamines in this process further show that increases in the cytoplasmic HuR abundances following polyamine depletion were linked to the increased HuR binding to the MEK-1 3′-UTR through specific RNA regions containing hits of the HuR motif and to increased stability and translation of the MEK-1 mRNA. Our current studies also indicate that MEK-1 is a novel downstream effector of HuR-mediated anti-apoptotic effect and that increased levels of endogenous MEK-1 via HuR promote the survival of IECs and elevate their resistance to apoptosis after polyamine depletion. These findings suggest that polyamine-regulated MEK-1 expression through HuR is of physiological significant and is crucial for maintaining intestinal epithelium homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

MEK-1	mitogen-activated protein kinase kinase 1
ODC	ornithine decarboxylase
UTRs	untranslated regions
RBPs	RNA-binding proteins
AREs	AU-rich elements
DFMO	D,L-α-difluoromethylornithine
RNP	ribonucleoprotein
HuR	Hu antigen R
pfu	plaque-forming units
IECs	intestinal epithelial cells
ODC-IEC	IEC stably expressing ODC
Q-PCR	real-time quantitative PCR
siRNA	small interfering RNA

CHX	cycloheximide
NPM	nucleophosmin

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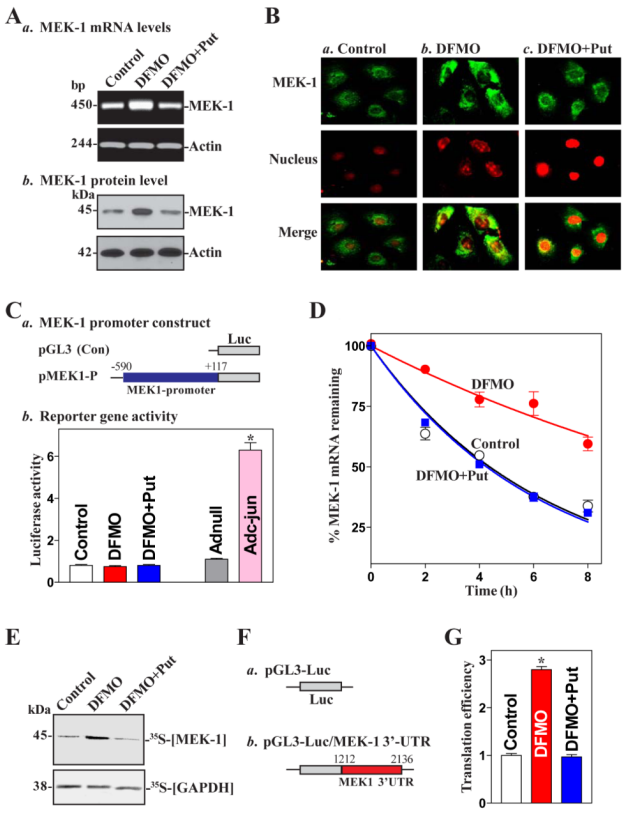

Fig. 1.

Polyamine depletion increases MEK-1 expression. (***A***) Levels of MEK-1 mRNA and protein in cells exposed to DFMO (5 mM) alone or DFMO plus putrescine (Put, 10 μM) for 6 days: ***a***) changes in MEK-1 mRNA as measured by RT-PCR analysis; and ***b***) representative immunoblots of MEK-1 protein by Western analysis. The first-strand cDNAs, synthesized from total cellular RNA, were amplified with the specific sense and antisense primers, and PCR-amplified products displayed in agarose gel for MEK-1 (~450 bp) and β-actin (~244 bp). To measure levels of MEK-1 protein, 20 μg of total proteins were applied to each lane, and immunoblots were hybridized with the antibody specific for MEK-1 (~45 kDa). Actin (~42 kDa) immunoblotting was performed as an internal control for equal loading. (***B***) Cellular distribution of MEK-1: ***a***) control; ***b***) DFMO-treated cells; and ***c***) cells treated with DFMO plus Put. Cells were permeabilized and incubated with the anti-MEK-1 antibody and then with anti-IgG conjugated with Alexa Fluor. Nuclei were stained with the TO-PRO3. Green, MEK-1 signals; red, nuclei. Original magnification, ×1,000. (***C***) Levels of MEK-1-promoter activity in cells described in ***A***: ***a***) schematic MEK-1-promoter luciferase (Luc) reporter construct (pMEK1-luc); ***b***) levels of luciferase reporter activity after polyamine depletion. After cells were treated with DFMO or DFMO plus Put for 4 days, they were transfected with the pMEK1-luc or control vector (pGL3) using LipofectAMINE technique; luciferase activity was examined 48 h after transfection. In a separate study, cells were cotransfected with the pMEK1 and the c-jun expression vector (Adc-jun) or control vector (Adnull), and luciferase activity was assayed 48 h thereafter. Data were normalized by Renilla-driven luciferase activity and expressed as means ± SE of data from 3 separate experiments. * $P < 0.05$ compared with controls and cells infected with Adnull. (***D***) Half-life of MEK-1 mRNA in cells described in ***A***. After cells were incubated with actinomycin D for the indicated times, total cellular RNA was isolated, and the levels of remaining MEK-1 and GAPDH mRNAs were measured by Q-PCR analysis. Values are the means ± SE from

triplicate samples. (**E**) Newly translated MEK-1 protein in cells described in **A**. MEK-1 translation was measured by incubating cells with L-[^{35}S]methionine and L-[^{35}S]cysteine for 20 min, followed by immunoprecipitation by using anti-MEK-1 antibody, resolving immunoprecipitated samples by SDS/PAGE, and transferring for visualization of signals by using a PhosphorImager. The translation of housekeeping control GAPDH was measured similarly. (**F**) Schematic of plasmids: ***a)*** control (pGL3-Luc); ***b)*** chimeric firefly luciferease (Luc)-MEK-1 3′UTR (pGL3-Luc-MEK1ARE). (**G**) Changes in MEK-1 translation efficiency as measured by using pGL3-Luc-MEK1ARE reporter assays in cells described in **A**. The pGL3-Luc-MEK1ARE or pGL3-Luc (negative control) was cotransfected with a *Renilla* luciferase reporter. Twenty-four hours later, firefly and *Renilla* luciferase activities were assayed. Luciferase values were normalized to the mRNA levels to obtain translation efficiencies and expressed as means ± SE of data from 3 separate experiments. * $p < 0.05$ compared with controls and cells treated with DFMO plus Put.

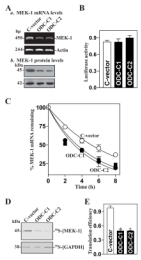

Fig. 2.

Increasing cellular polyamines represses MEK-1 expression. (**A**) Changes in MEK-1 mRNA and protein in clonal (C) populations of ODC-IEC cells (ODC) and control cells (C-vector). IEC-6 cells were infected with either the retroviral vector containing the sequence encoding mouse ODC cDNA or control retroviral vector lacking ODC cDNA. Clones resistant to the selection medium containing 0.6 mg/ml G418 were isolated and screened for ODC expression. The levels of MEK-1 mRNA and protein were assessed by RT-PCR analysis and Western immunoblotting, respectively. (**B**) Levels of MEK-1-promoter activity in cells described in ***A***. Luciferase activity was examined 48 h after transfection with pMEK1-luc or the control vector (pGL3). Data were normalized by Renilla-driven luciferase activity and expressed as means ± SE of data from 3 separate experiments. (**C**) Half-life of MEK-1 mRNA in cells described in ***A***. After cells were incubated with actinomycin D for the indicated times, total cellular RNA was isolated, and the levels of remaining MEK-1 and GAPDH mRNAs were measured by Q-PCR analysis. Values are the means ± SE from triplicate samples. (**D**) Newly synthesized MEK-1 protein in cells described in ***A***. After cells were incubated with L-[^{35}S]methionine and L-[^{35}S]cysteine for 20 min, cell lysates were prepared and immunoprecipitated by using anti-MEK-1 antibody, resolved by SDS/PAGE, and transferred for visualization of signals by using a PhosphorImager. The translation of housekeeping control GAPDH was measured similarly. (**E**) Changes in MEK-1 translation efficiency as measured by using pGL3-Luc-MEK1ARE reporter assays in cells described in ***A***. The pGL3-Luc-MEK1ARE or pGL3-Luc (negative control) was cotransfected with a *Renilla* luciferase reporter, and firefly and *Renilla* luciferase activities were assayed 24 h thereafter. Luciferase values were normalized to the mRNA levels to obtain translation efficiencies and expressed as means ± SE of data from 3 separate experiments. * $p < 0.05$ compared with controls.

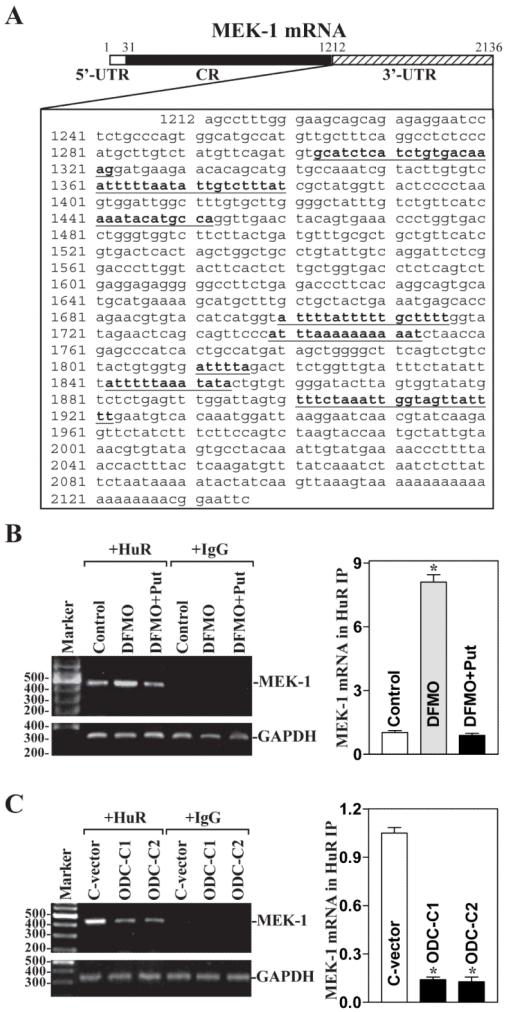

Fig. 3.

Changes in HuR-binding to the MEK-1 mRNA after altering the levels of cellular polyamines. (**A**) Schematic representative of the MEK-1 mRNA and the predicted hits of the HuR signature motif in its 3′-UTR as indicated by underline. (**B**) Levels of association of endogenous HuR with endogenous MEK-1 mRNA after polyamine depletion. IEC-6 cells were exposed to DFMO alone or DFMO plus putrescine for 6 days. After immunoprecipitation (IP) of RNA-protein complexes from cell lysates using either anti-HuR antibody (Ab) or control IgG1, RNA was isolated and used in RT reactions. *Left panel*: representative RT-PCR products visualized in ethidium bromide-stained agarose gels; low-level amplification of GAPDH (housekeeping mRNA, which is not HuR targets) served as negative controls. *Right panel*: fold differences in MEK-1 transcript abundance in HuR IP compared with IgG IP, as measured by Q-PCR analysis. Values were means ± SE from triplicate samples. * $p < 0.05$ compared with controls and cells treated with DFMO plus Put. (**C**) Changes in levels of MEK-1 mRNA in HuR IP from clonal (C) populations of ODC-IEC cells (ODC) and control cells (C-vector). *Left panel*: representative RT-PCR products of MEK-1 and GAPDH mRNAs in HuR or IgG1 IP. *Right panel*: fold differences in MEK-1 mRNA in HuR IP compared with IgG IP as measured by Q-PCR analysis. Values were means ± SE from triplicate samples. * $p < 0.05$ compared with controls.

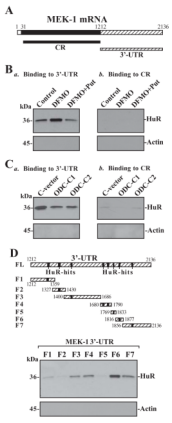

Fig. 4.

HuR-binding to the 3′-UTR and coding region (CR) of MEK-1 mRNA in cells described in Fig. 3. (**A**) Schematic representation of the MEK-1 biotinylated transcripts (CR and 3′-UTR) used in this study. (**B**) Representative HuR immunoblots using the pulldown materials by different fractions of MEK-1 mRNA after polyamine depletion: ***a***) HuR-binding to 3′-UTR; and ***b***) HuR-binding to CR. Cytoplasmic lysates prepared from control cells and cells exposed to DFMO alone or DFMO plus Put for 6 days were incubated with 6 μg of biotinylated MEK-1 3′-UTR or CR for 30 min at 25 °C, and the resulting RNP complexes were pulled down by using streptavidin-coated beads. The presence of HuR in the pull-down material was assayed by Western blotting. β-actin in the pull-down material was also examined and served as a negative control. (**C**) Representative HuR immunoblots using the pulldown materials in clonal (C) populations of ODC-IEC cells (ODC) and control cells (C-vector): ***a***) HuR-binding to 3′-UTR; and ***b***) HuR-binding to CR. (**D**) Representative HuR immunoblots in the material pulled down by different biotinylated fractions of the MEK-1 mRNA 3′-UTR. *Top panel*, schematic representation of the MEK-1 3′-UTR biotinylated transcripts used in this study. Three experiments were performed that showed similar results.

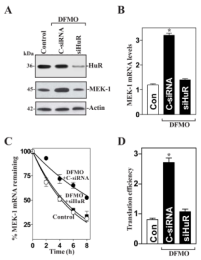

Fig. 5.

Effect of HuR silencing on MEK-1 mRNA stability and its translation in polyamine-deficient cells. (**A**) Representative HuR and MEK-1 immunoblots. After cells were cultured in the presence of DFMO for 4 days, they were transfected with either siRNA targeting the HuR mRNA coding region (siHuR) or control siRNA (C-siRNA), and whole-cell lysates were harvested 48 h thereafter. The levels of HuR and MEK-1 proteins were measured by Western blot anaylsis, and equal loading was monitored by β-actin immunoblotting. (**B**) Levels of MEK-1 mRNA in cells that were processed as described in panel ***A***. Total RNA from each group was harvested, and levels of MEK-1 mRNA were measured by RT + Q-PCR analysis. The data were normalized to the amount of GAPDH mRNA and the values represented as the means ± SE of data from triplicate experiments. * $P < 0.05$ compared with controls and cells transfected with C-siRNA. (**C**) Half-life of the MEK-1 mRNA in cells that were transfected and treated as described in ***A***. Total cellular RNA was isolated at the indicated times after administration of actinomycin D, and the remaining levels of MEK-1 and GAPDH mRNAs were measured by RT + Q-PCR analysis. Values are means ± SE from triplicate samples. (**D**) Changes in MEK-1 translation efficiency as measured by using pGL3-Luc-MEK1ARE reporter assays in cells described in ***A***. The pGL3-Luc-MEK1ARE or pGL3-Luc (negative control) was cotransfected with a *Renilla* luciferase reporter, and firefly and *Renilla* luciferase activities were assayed 24 h thereafter. Luciferase values were normalized to the mRNA levels to obtain translation efficiencies and expressed as means ± SE of data from 3 separate experiments. * $p < 0.05$ compared with control cells and DFMO-treated cells transfected with siHuR.

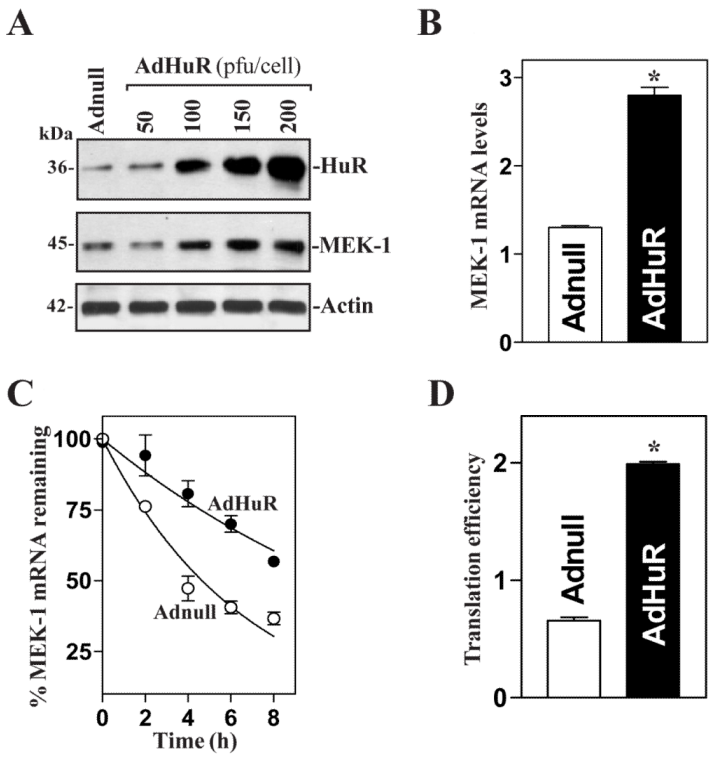

Fig. 6.

Changes in MEK-1 mRNA stability and translational efficiency after ectopic HuR overexpression. (**A**) Representative immunoblots of HuR and MEK-1 proteins after ectopic HuR expression. Cells were infected with the recombinant adenoviral vector encoding HuR cDNA (AdHuR, prepared as explained in Materials and Methods) or adenoviral vector lacking HuR cDNA (Adnull) at a multiplicity of infection of 50-200 plaque-forming units (pfu)/cell; the expression of HuR and MEK-1 proteins was analyzed 48 h after the infection. (**B**) Levels of MEK-1 mRNA as measured by RT-qPCR analysis in cells infected with AdHuR or Adnull at the concentration of 100 pfu/cell for 48 h. Data were normalized to amount of GAPDH mRNA, and values are means ± SE of data from triplicate experiments. * $P < 0.05$ compared with cells infected with Adnull. (**C**) Half-life of the MEK-1 mRNA as measured by RT-qPCR analysis by using actinomycin D in cells described in ***B***. Values are the means ± SE from triplicate samples. (**D**) Changes in MEK-1 translation efficiency as measured by using pGL3-Luc-MEK1ARE reporter assays in cells that were processed as described in ***B***. Twenty-four h after cells were transfected with the pGL3-Luc-MEK1ARE or pGL3-Luc (negative control), the levels of luciferase activity were examined and normalized to the mRNA levels to obtain translation efficiencies. Values were expressed as means ± SE of data from 3 separate experiments. * $p < 0.05$ compared with cells transfected with Adnull.

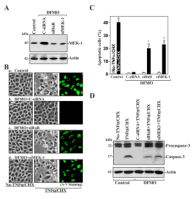

Fig. 7.

Effects of MEK-1 or HuR silencing on apoptosis in polyamine-deficient cells. (***A***) Cells were cultured with DFMO for 4 days and then transfected with either siRNA specifically targeting the MEK-1 mRNA coding region (siMEK-1), siHuR, or C-siRNA. The levels of MEK-1 protein were measured by Western blot analysis 48 h after transfection; equal loading was monitored by β-actin immunoblotting. (***B***) TNFα/CHX-induced apoptosis in cells treated as described in panel ***A***: ***a***) control cells; ***b***) DFMO-treated cells transfected with C-siRNA; ***c***) DFMO-treated cells transfected with siHuR; ***d***) DFMO-treated cells transfected with siMEK-1. Apoptosis was measured by morphological analysis (*middle*) and ApoAlert annexin-V (A-V) staining (*right*) 4 h after treatment with TNFα/CHX. Original magnification, ×150. (***C***) Percentage of apoptotic cells in cultures processed as described in panel ***B***. Values are the means ± SE from six samples. * $p < 0.05$ compared with cells that were not treated with TNFα/CHX. + $p < 0.05$ compared with DFMO-treated cells that were transfected with C-siRNA and then treated with TNFα/CHX for 4 h. (***D***) Representative immunoblots for procaspase-3 and caspase-3 in cells that were processed as described in panel ***B***. Whole-cell lysates were harvested 4 h after treatment with TNFα/CHX, and the levels of procaspase-3 and caspase-3 were examined by Western blot analysis. Three experiments were performed that showed similar results.

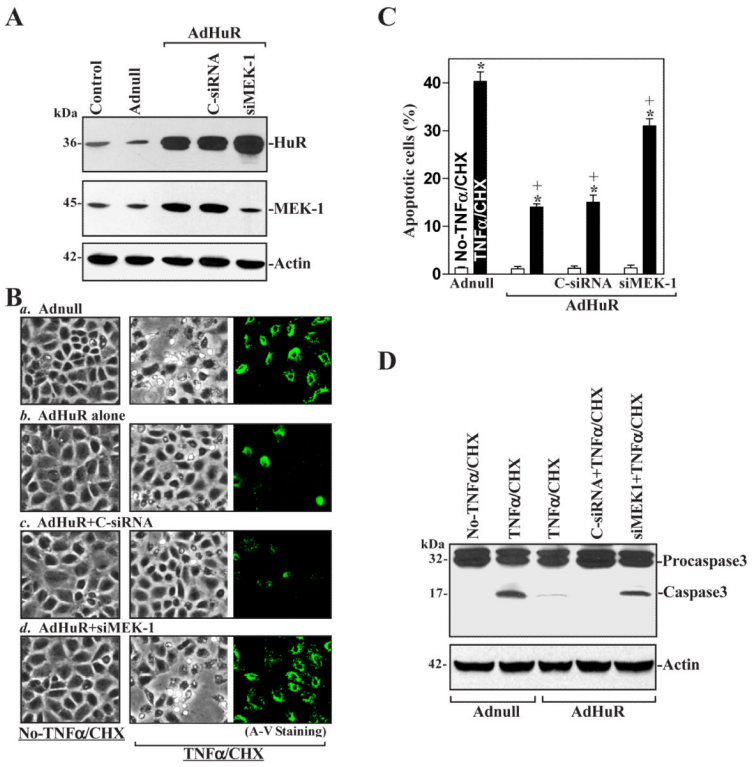

Fig. 8.

Effect of MEK-1 silencing on apoptotic sensitivity in cells overexpressing HuR. (***A***) Representative HuR and MEK-1 immunoblots. Cells were transfected with either siMEK-1 or C-siRNAfor 24 h and then infected with AdHuR or Adnull (100 pfu/cell). HuR and MEK-1 protein levels were examined by Western blot analysis 24 h after infection, and equal loading was monitored by β-actin immunoblotting. (***B***) TNFα/CHX-induced apoptosis in cells described ***A***: ***a***) cells infected with Adnull; ***b***) cells infected with AdHuR; ***c***) cells infected with AdHuR and transfected with C-siRNA; ***d***) cells infected with AdHuR and transfected with siMEK-1. Apoptosis was measured by morphological analysis (*middle*) and ApoAlert annexin-V (A-V) staining (*right*) 4 h after treatment with TNFα/CHX. Original magnification, ×150. (***C***) Percentage of apoptotic cells as described in ***B***. Values are means ± SE of data from six samples. * $p < 0.05$ compared with groups that were not treated with TNFα/CHX. + $p < 0.05$ compared with cells infected with Adnull and then treated with TNFα/CHX. (***D***) Representative immunoblots for procaspase-3 and caspase-3 in cells that were processed as described in ***B***. Whole-cell lysates were harvested 4 h after treatment with TNFα/CHX, and the levels of procaspase-3 and caspase-3 proteins were examined by Western blot analysis. Three experiments were performed that showed similar results.