MKP-1 mRNA Stabilization and Translational Control by RNA-Binding Proteins HuR and NF90[†]

Yuki Kuwano, Hyeon Ho Kim, Kotb Abdelmohsen, Rudolf Pullmann, Jr., Jennifer L. Martindale, Xiaoling Yang, and Myriam Gorospe*

Laboratory of Cellular and Molecular Biology, National Institute on Aging-Intramural Research Program, National Institutes of Health, Baltimore, Maryland 21228

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The mitogen-activated protein (MAP) kinase phosphatase 1 (MKP-1) plays a major role in dephosphorylating and thereby inactivating the MAP kinases extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38. Here, we examine the posttranscriptional events underlying the robust MKP-1 induction by oxidants in HeLa cells. H₂O₂ treatment potently stabilized the MKP-1 mRNA and increased the association of MKP-1 mRNA with the translation machinery. Four RNA-binding proteins (RNA-BPs) that influence mRNA turnover and/or translation (HuR, NF90, TIAR, and TIA-1) were found to bind to biotinylated transcripts spanning the MKP-1 AU-rich 3' untranslated region. By using ribonucleoprotein immunoprecipitation analysis, we showed that H₂O₂ treatment increased the association of MKP-1 mRNA with HuR and NF90 and decreased its association with the translational repressors TIAR and TIA-1. HuR or NF90 silencing significantly diminished the H₂O₂-stimulated MKP-1 mRNA stability; HuR silencing also markedly decreased MKP-1 translation. In turn, lowering MKP-1 expression in HuR-silenced cultures resulted in substantially elevated phosphorylation of JNK and p38 after H₂O₂ treatment. Collectively, MKP-1 upregulation by oxidative stress is potently influenced by increased mRNA stability and translation, mediated at least in part by the RNA-BPs HuR and NF90.

In mammalian cells, proliferative and stress-causing stimuli often trigger shared signaling pathways. Prominent among these pathways are those that culminate in the activation of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 (7, 12, 43). MAPK activation, involving phosphorylation on tyrosine and threonine residues, leads to the phosphorylation of downstream effector proteins that carry out the necessary changes to respond to the stimulus (16). Through these phosphorylation events, MAPKs can alter the patterns of expressed genes, the rate of cell division, and the capacity of the cell to survive and respond to the particular challenge (38, 42). The magnitude and duration of MAPK activity are tightly controlled in order to prevent potentially harmful consequences of extended MAPK function. At the cellular level, the deleterious consequences of extended MAPK activity include excessive production of MAPK-regulated genes, uncontrolled proliferation, and unscheduled cell death. At the levels of tissue and organs, it can lead to complications such as autoimmune disease, septic shock, hypertension, and cancer (11, 17).

MAPK inactivation is performed by a family of MAPK phosphatases (22). The archetypal member of this family, MAPK phosphatase 1 (MKP-1 [also termed CL100, VH1, 3CH134, and DUSP1]) selectively inactivates all three MAPK families by dephosphorylating them at catalytic tyrosine and threonine

residues, but it acts preferentially upon p38 and JNK (13). Overexpression of MKP-1 in macrophages blocks the lipopolysaccharide (LPS)-triggered production of proinflammatory cytokines by inhibiting p38 (61); conversely, MKP-1^{-/-} mice have an exacerbated response to LPS, expressing elevated cytokine levels in serum and exhibiting prolonged activation of p38 and JNK in macrophages (54). These and many other studies illustrate the fact that MKP-1 provides negative feedback to MAPK activation in vivo. MKP-1 is highly induced by stress agents such as inflammatory stimuli, UV light, hypoxia, heat shock, and oxidants. MKP-1 expression is controlled at multiple levels. The chromatin on the MKP-1 locus was altered following treatment with arsenite (28), and several transcription factors (including Sp1, Sp3, and AP-1) were shown to influence the MKP-1 gene transcription in response to growth factors and stress stimuli (30, 44). However, little is known about the posttranscriptional regulation of MKP-1 expression, despite the fact that MKP-1 is an early response gene and, thus, is likely to be encoded by a short-lived mRNA (8, 50).

The transient, stimulus-driven stabilization of early response transcripts is controlled by sequence-specific RNA-binding proteins (RNA-BPs) that influence mRNA metabolism (21, 36). RNA-BPs that enhance mRNA decay include AU-binding factor 1 (AUF1 [also termed heterogeneous nuclear ribonucleoprotein D {hnRNP D}]), tristetraprolin (TTP), butyrate response factor 1 (BRF1), and KH domain-containing RNA-BP (KSRP) (6, 9, 31, 49, 60). RNA-BPs that inhibit mRNA decay include the embryonic lethal abnormal vision (Hu/elav) family of RNA-BPs, consisting of the ubiquitous HuR (HuA) protein and the primarily neuronal proteins HuB (Hel-N1), HuC, and HuD (3, 14, 37). The member of this family that is studied most extensively, HuR, is predominantly nuclear, but

* Corresponding author. Mailing address: Box 12, LCMB, NIA-IRP, NIH, 5600 Nathan Shock Dr., Baltimore, MD 21224. Phone: (410) 558-8443. Fax: (410) 558-8386. E-mail: myriam-gorospe@nih.gov.

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FIG. 1. H_2O_2 treatment elevates MKP-1 mRNA and protein levels and enhances MKP-1 mRNA stability and translational status. (A) HeLa cells were either left untreated or treated with 1 mM H_2O_2 and collected at the times indicated (h, left) or treated with the concentrations (mM) of H_2O_2 shown and then collected 4 h later (right). Total RNA was then extracted and used to measure the levels of the MKP-1 and housekeeping GAPDH mRNAs by RT, followed by conventional PCR. (B) Cells were treated, and RNA was collected as described in the legend to panel A; after RT, real-time qPCR was used to measure the levels of MKP-1 mRNA. After it was normalized to the levels of GAPDH mRNA, the relative abundance of MKP-1 mRNA is shown as a function of H_2O_2 treatment time (h, left) and dose (mM, right). Data are shown as the means plus the standard errors of the means (SEM) from three independent experiments. (C) To estimate mRNA half-life, HeLa cells were either left untreated (Untr.) or treated with H_2O_2 for 1 h (H_2O_2) and then treated with actinomycin D (2 μ g/ml) for the times shown. MKP-1 and GAPDH mRNA levels were measured by RT-qPCR, normalized to 18S rRNA levels, and plotted on a logarithmic scale to calculate the time required for each mRNA to reach one-half of its initial abundance (50%, dashed line). RT-qPCR results represent the mean values $\pm$ SEM from three independent experiments. (D) Whole-cell protein extracts were prepared from HeLa cells treated with 1 mM H_2O_2 for the times indicated (left) and for 8 h with the concentrations shown (right); the levels of MKP-1 and loading control β -actin were assessed by Western blotting analysis. (E) Representative polysome profiles obtained from HeLa cells that were either left untreated or treated with H_2O_2 (1 mM, 2.5 h) and fractionated through sucrose gradients. (F) The levels of MKP-1 mRNA (left) and housekeeping GAPDH mRNA (right) in each gradient fraction were measured by RT-qPCR and plotted as a percentage of the total MKP-1 or GAPDH mRNA levels in that sample. The translational activity associated with each fraction is indicated as untranslated (NB, not bound to polysomes; NT, not translated), moderately translated (LMW, low-molecular-weight polysomes), and actively translated (HMW, high-molecular-weight polysomes). Data represent the average of three independent experiments showing similar results.

its influence upon the expression of target mRNAs is linked to its cytoplasmic export (4, 15, 20). Oxidative stress triggered by exposure to H_2O_2 , arsenite, or UV light potentially induces HuR translocation to the cytoplasm, where it increases the half-life of many mRNAs encoding stress-regulated proteins (*c-fos*, p21, the cyclins A2, B1, D1, inducible nitric oxide synthase, tumor necrosis factor alpha [TNF- α], cyclooxygenase 2 [COX-2], and interleukin-3 [IL-3] [25, 35, 40, 47, 51, 52]). NF90 also stabilizes target mRNAs, as shown for IL-2, following T-cell activation (48). In addition to stabilizing target mRNAs, both HuR and NF90 have also been shown to modulate translation. HuR promotes the translation of target transcripts including p53, prothymosin α (ProT α), CAT-1, and cytochrome *c* mRNAs (3, 19, 26, 33), but it can also suppress the translation of other targets, including the mRNAs that encode p27, IGF-IR, Wnt5a, and several immune regulators (18, 23, 27, 34). NF90 was reported to suppress the translation of β -glucosidase (58, 59), but a broader role in translational repression has not been explored in detail.

The present study was prompted by the discovery that MKP-1 mRNA might be a target of HuR, given the presence of U-rich HuR motif hits (10, 32) in its 3' untranslated region (UTR). While we were testing this possibility in HeLa cells responding to H_2O_2 treatment, the MKP-1 3' UTR showed specific associations with several other RNA-BPs, but only HuR and NF90 formed ribonucleoprotein (RNP) complexes with MKP-1 mRNA in an H_2O_2 -inducible manner. Our results further revealed that both HuR and NF90 potentially stabilized the MKP-1 mRNA in response to H_2O_2 treatment and that HuR promoted MKP-1 translation.

MATERIALS AND METHODS

Cell culture, transfections, RNA interference, and plasmids. Human cervical carcinoma HeLa cells were maintained in Dulbecco's modified essential medium (Gibco-BRL) supplemented with 10% fetal bovine serum. Cells were treated with H_2O_2 in complete medium for the indicated times. For RNA interference analysis, Oligofectamine (Invitrogen) was used to transfect cells with small interfering RNAs (siRNAs) targeting HuR or NF90 or with a control siRNA. siRNAs (20 nM each) targeting the HuR consisted of a mixture of AATCTTAAGTTTCGTAAGTTA (HuR U1), TTCGTAAGTTATTCCTTTAA (HuR U3), and AAGTGCAAAGGGTTTGGCTTT (HuR H4); siRNAs specifically targeting the HuR 3' UTR [HuR(3')] were a mixture of HuR U1, HuR U3, and TTCCTTAAGATATATATTA (HuR U2). The siRNA targeting the NF90 coding region (CR) was GCCCACCTTGTCTTTTAT, and the control siRNA was TTCTCCGAACGTGT. The 3' UTR of MKP-1 was cloned into the 3' UTR of the enhanced green fluorescent protein (EGFP) gene in the pTRE-d2EGFP vector (BD Biosciences). Plasmid vectors for overexpressing HuR (pHuR-TAP) or NF90 (pcDNA3.1-NF90) were reported previously (26, 46). Plasmids were transfected using Lipofectamine 2000 (Invitrogen). All transfected cells were used 48 h later for analysis.

Western blotting analysis. Whole-cell, cytoplasmic, and nuclear extracts were prepared as described previously (25). Proteins were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes. MKP-1 was detected by using a polyclonal antibody against MKP-1 (Santa Cruz Biotechnology). Monoclonal antibodies recognizing HuR and α -tubulin, as well as polyclonal antibodies recognizing hnRNP C1/C2, TIA-1, and TIAR, were from Santa Cruz Biotechnology; a monoclonal antibody against NF90 was from BD Biosciences; a β -actin antibody was from Abcam. To detect MAP kinases, antibodies recognizing phosphorylated ERK1 (p-ERK1), p-p38, and p-JNK1/2, as well as total ERK1, p38, and JNK1/2, were from Cell Signaling. After samples were incubated with secondary antibodies, signals were detected by enhanced chemiluminescence (Amersham Biosciences).

RNP IP assays. For immunoprecipitation (IP) of endogenous RNA-protein complexes from whole-cell (1.5-mg) and cytoplasmic (450- μ g) extracts, reactions were carried out for 2 h at 4°C with protein A-Sepharose beads (Sigma) that had been precoated with 30 μ g of either mouse immunoglobulin G1 (IgG1; BD Biosciences), goat IgG (Santa Cruz Biotechnology), or antibodies recognizing HuR, TIA-1, TIAR, AUF1, or NF90. Beads were washed with NT2 buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM $MgCl_2$, and 0.05% Nonidet P-40) and then incubated with 20 U of RNase-free DNase I (15 min, 30°C) and further incubated in 100 μ l NT2 buffer containing 0.1% SDS and 0.5 mg/ml proteinase K (30 min, 55°C).

The RNA isolated from the IP material was reverse transcribed using random hexamers and SSII reverse transcriptase (Invitrogen). Transcript abundance was then assayed by amplification of the cDNA, using gene-specific primer pairs and either conventional PCR (30 cycles) or real-time, quantitative PCR (qPCR) employing Sybr green PCR master mix (Applied Biosystems). PCR primers for the detection of ProT α , SIRT1, UBC, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were described previously (1).

Biotin pulldown analysis. For in vitro synthesis of biotinylated transcripts, cDNA from HeLa cells was used as a template for PCRs, whereby the T7 RNA polymerase promoter sequence (CCAAGCTTCAATACGACTCACTATAG GGAGA [T7]) was added to the 5' end of all fragments. Primers used for the amplification of sequences of GAPDH 3' UTR were described previously (41). The primers used for the preparation of biotinylated transcripts spanning the 5' UTR, CR, and 3' UTR of MKP-1 (GenBank accession no. NM_004417) were as follows: primers (T7)TCGCTGCGAAGGACATTTGGGCTGTG and GGCCG GCCTCAGCGCCCCAGCGT were used for preparing the MKP-1 5' UTR, and primers (T7)ATGGTCATGGAAGTGGGCACCC and TCAGCAGCTGG GAGAGGTCGTA were used to prepare the MKP-1 CR transcripts. To prepare partial MKP-1 3' UTR transcripts, the primers used were (T7)AAGGCCACG GGAGGTGAGGCTC and TCAGATGGACTTGATGTACCCA for 3' UTR-A, (T7)CAAAATGGGGCAGAAGAGAAAG and TGAACAAAC TGCTTAAGAT for 3' UTR-B, and (T7)GCAGTGTGGAAATACCAAGT and CAATAGAAATGCCATAATTTAT for 3' UTR-C. Biotinylated RNAs were synthesized using a MaxiScript T7 kit (Ambion). Whole-cell lysates (40 μ g for each sample) were incubated with 4 μ g of purified biotinylated transcripts for 1 h at room temperature. Complexes were isolated with paramagnetic streptavidin-conjugated Dynabeads (Dyna), and bound proteins in the pulldown material were assayed by Western blotting using antibodies recognizing AUF1, HuR, NF90, TIA-1, or TIAR, as described above.

Immunofluorescence. HeLa cells were washed with ice-cold phosphate-buffered saline (PBS) and fixed for 10 min using 2% formaldehyde in PBS. Cells were permeabilized using 0.1% Triton X-100 in PBS for 5 min, washed with ice-cold PBS, and blocked with 5% bovine serum albumin in PBS for 1 h at 25°C. After cell preparations were incubated with anti-HuR or anti-NF90 antibodies (in 5%

FIG. 2. RNA-BPs forming complexes with the MKP-1 mRNA. (A) Schematic of the MKP-1 biotinylated transcripts (CR, 3' UTR: full length [3' UTR], truncated 3' UTR fragments A, B, and C) used in pulldown assays. (B) Biotinylated MKP-1 fragments (CR, 3' UTR-A, -B, and -C) and a negative-control biotinylated GAPDH 3' UTR fragment were prepared, and their associations with HuR, AUF1, NF90, TIAR, or TIA-1 were tested by biotin pulldown assay using lysates from HeLa cells. The results shown are representative Western blotting signals using specific antibodies against each RNA-BP. (C to F) Binding of endogenous HuR, NF90, TIAR, or TIA-1 to endogenous mRNAs was detected by RT-qPCR assay of material obtained by IP from cytoplasmic fractions of untreated (–) or H_2O_2 -treated (2.5 h) (+) cells using IgG, anti-HuR, anti-NF90, anti-TIAR, or anti-TIA-1 antibodies. The levels of mRNAs encoding GAPDH, UBC (housekeeping controls), ProT α and SIRT1 (known HuR targets serving as positive controls), and MKP-1 present in the IP materials were detected by RT-qPCR analysis and shown as fold differences in abundance of the corresponding mRNAs in the RNA-BPs IP compared with those of the IgG IP. qPCR results represent the means $\pm$ standard errors of the means from four independent experiments. PCR products were visualized after electrophoresis in 1% agarose gels stained with ethidium bromide.

bovine serum albumin for 16 h at 4°C) and underwent additional washes with ice-cold PBS, they were incubated with a secondary antibody (Alexa 488), washed with PBS, and incubated with a solution of Topro-3 for 10 min. After preparations were washed thoroughly, they were embedded using ProLong Gold antifade reagent (Invitrogen); 24 h later, photos were taken using a fluorescence microscope (Zeiss Axiovert 35).

Analysis of translation: polysome gradients and nascent translation assays. For polysome analysis, untreated or H₂O₂-treated HeLa cells (5×10^5 cells) were incubated with 0.1 mg/ml cycloheximide for 10 min. Cytoplasmic extracts were prepared and fractionated through a linear sucrose gradient (10 to 50% [wt/vol]), as reported previously (25). Twelve fractions were collected using a fraction collector (Brandel) and monitored by optical density measurement (A_{254}). The RNA in each fraction was isolated with Trizol (Invitrogen). Following reverse transcription (RT)-qPCR analysis was performed using primer pairs for MKP-1 (CTGCCCTTGATCAACGTCTCA and ACCCTTCCTCCAGCATT CTT) or for GAPDH (41).

To assess the levels of nascent MKP-1 and GAPDH, *de novo* synthesis of MKP-1 and GAPDH was measured by incubating HeLa cells briefly (15 min) with L-[³⁵S]methionine and L-[³⁵S]cysteine (Easy Tag TMEXPRESS; NEN/Perkin Elmer, Boston, MA). Cells were lysed in RIPA buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.1% SDS, and 1 mM dithiothreitol), and the IP reactions were carried out in 1 ml TNN buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 5 mM EDTA, 0.5% NP-40) for 16 h at 4°C, using anti-MKP-1 (Santa Cruz Biotechnology), IgG1 (BD Pharmingen), or anti-GAPDH antibodies (Santa Cruz Biotechnology). After IP samples were washed extensively in TNN buffer, the samples were resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membrane filters, and visualized with a PhosphorImager (Molecular Dynamics). MKP-1 signals were quantified and presented as a percentage of the signals in control siRNA, untreated cells.

RNA decay assay after Tet-Off transcriptional inhibition. The plasmid pTRE-d2EGFP-MKP-1(3' UTR) was constructed by cloning a cDNA fragment spanning the MKP-1 3' UTR after the STOP codon of the EGFP CR in the pTRE-d2EGFP vector (BD Biosciences) following EcoRI and XbaI digestions. By 24 h after transfection of HeLa Tet-Off cells (Clontech) with control, HuR, or NF90 siRNAs, cells were transfected with pTRE-d2EGFP-MKP-1(3' UTR) or with the pTRE-d2EGFP control vector. Twenty-four hours later, doxycycline (Sigma) was added to a final concentration of 1 µg/ml, and the cells were harvested in Trizol reagent (Invitrogen). Total RNA was used for RT-qPCR to assess the expression of EGFP (using the primer pair CACATGAAGCAGCAGACTT and GGAT GTTGCCGTCCTCTTG), 18S rRNA, and GAPDH. At each time point, the abundances of EGFP or EGFP-MKP-1(3' UTR) and GAPDH mRNA transcripts were normalized to that of 18S rRNA and the half-lives calculated for each transfection group.

RESULTS

H₂O₂ treatment increases MKP-1 mRNA stability and translation. Treatment with H₂O₂ induced MKP-1 expression in HeLa cells in a time- and dose-dependent manner. MKP-1 mRNA levels were measured by RT of total RNA, followed by conventional PCR or real-time qPCR amplification. As shown in Fig. 1A and B, treatment with 1 mM H₂O₂ induced MKP-1 mRNA levels at 2 h, peaking by 8 h. MKP-1 protein levels followed this pattern of induction (Fig. 1D). The nature of the upper band appearing on MKP-1 Western blots was unclear; while it did not appear to be phospho-MKP-1, it could be an MKP-1-related protein (not shown).

MKP-1 expression in response to oxidative stress was already shown to increase through elevated transcription of the MKP-1 gene and through stabilization of the MKP-1 protein (5, 57). However, given the rapid and potent induction in expression of the MKP-1 mRNA and protein, we hypothesized that posttranscriptional events, including mRNA stabilization and translational upregulation, contributed to elevating MKP-1 levels. To determine if H₂O₂ treatment stabilized the MKP-1 mRNA, we studied the MKP-1 mRNA half-life by incubating cells with actinomycin D to block *de novo* gene transcription and then monitored the rate of MKP-1 mRNA decay by using RT-qPCR. As

FIG. 3. Effect of HuR or NF90 silencing on H₂O₂-increased MKP-1 mRNA stability. (A) After HeLa cells were treated with H₂O₂ for the times indicated, whole-cell (WCE), cytoplasmic (CE), and nuclear (NE) lysates were prepared, and HuR, NF90, α -tubulin, hnRNP C1/C2, and β -actin levels were tested by Western blotting analysis. (B) Immunofluorescence microscopy was used to assess NF90 (top) and HuR (bottom) distribution (green) in cells that were either left untreated (Unt.) or treated with H₂O₂ (1 mM, 2 h). Nuclei were visualized by using Topro-3 (red). Merged, overlay of NF90 and Topro-3 (top) or HuR and Topro-3 (bottom).

shown in Fig. 1C, the MKP-1 mRNA had a half-life of ~50 min in untreated cells, but it increased to >120 min after H₂O₂ treatment (Fig. 1C), indicating that treatment with the oxidant potentially stabilized the MKP-1 mRNA. To determine if the translation of MKP-1 was also influenced by H₂O₂ treatment, we monitored the fraction of MKP-1 mRNA that was associated with the translational machinery before and after it was stimulated with H₂O₂. Cytoplasmic extracts from either the untreated or the H₂O₂-treated cells were fractionated on sucrose gradients; the relative abundance of the MKP-1 mRNA in each fraction was then used to measure the degree of engagement of the MKP-1 mRNA with the translational apparatus. As shown in Fig. 1E,

FIG. 4. (A) Forty-eight hours after cells were transfected with control (Ctrl.) or HuR-directed siRNAs, HuR and loading control β -actin levels were determined by Western blotting analysis. (B) The stability of MKP-1 mRNA was measured in the cultures described in the legend to panel A following incubation with actinomycin D (2 μ g/ml) for the times indicated. The levels of MKP-1 mRNA (and GAPDH mRNA, not shown) were assayed by RT-qPCR analysis, normalized to 18S rRNA levels, and used to calculate mRNA half-life as described in the legend to Fig. 1C. (C) By 48 h after transfection of HeLa cells with either control or NF90-directed siRNAs, the expression levels of NF90 and β -actin were determined by Western blotting analysis. (D) In the cultures described in the legend to panel C, MKP-1 mRNA stability was calculated as described in the legend to Fig. 1C. The results represent the mean values $\pm$ standard errors of the means from three independent experiments.

general translation was reduced following H₂O₂ treatment, as the actively translating polysomal component (spanning fractions 8 to 11) declined markedly. By contrast, H₂O₂ triggered a dramatic shift in the relative distribution of MKP-1 mRNA toward heavier fractions of the gradient (Fig. 1F, left panel), consistent with H₂O₂ strongly promoting MKP-1 mRNA. Together, these findings indicate that H₂O₂ treatment both stabilized the MKP-1 mRNA and promoted its translation.

RNA-BPs binding to endogenous and recombinant MKP-1 mRNA. The MKP-1 mRNA has a long, AU-rich 3' UTR containing several predicted hits of a previously identified HuR motif (Fig. 2A) (32). We thus sought to determine if MKP-1 mRNA was the target of HuR or other RNA-BPs implicated in mRNA stabilization and/or translation (such as NF90, AUF1, TIA-1, and TIAR) and further determined if such RNP asso-

ciations changed in an H₂O₂-dependent manner. First, we assayed the existence of such RNPs by employing a biotin pull-down analysis. Biotinylated transcripts (as described in Materials and Methods) spanning either the CR or the 3' UTR (full-length or partial) of the MKP-1 mRNA were prepared and incubated with HeLa cell lysates. After the pulldown using streptavidin-coated beads, Western blotting analysis revealed that HuR, NF90, and TIAR associated specifically with various MKP-1 3' UTR transcripts but not with CR or GAPDH 3' UTR transcripts (Fig. 2B); NF90 showed a low-level association with the biotinylated GAPDH RNA, as previously reported (41). By contrast, neither TIA-1 nor AUF1 appeared to bind specifically to biotinylated MKP-1 transcripts (Fig. 2B). HuR (but not NF90) also associated with a biotinylated transcript spanning the MKP-1 5' UTR, but this interaction did not

have functional consequences, so it was not pursued further (see Fig. S1 in the supplemental material).

Second, RNP IP analysis was performed to study the association of endogenous HuR, NF90, TIAR, or TIA-1 with endogenous mRNAs, using cytoplasmic fractions of either untreated or H_2O_2 -treated (2.5 h) cells. When lysates from untreated HeLa cells were used, RNP IP assays revealed a ~12-fold enrichment in MKP-1 mRNA associated with HuR in anti-HuR antibody IP reactions relative to that of control IgG IP reactions (Fig. 2C). When using lysates from H_2O_2 -treated cells, the association of MKP-1 mRNA with HuR was >40-fold higher than that seen with control IgG IP reactions. As positive controls, the RNPs comprising HuR and the mRNAs of targets ProT α and SIRT1 were tested; these RNP associations increased and decreased following H_2O_2 treatment, as previously reported (1). As a negative control, the abundance of housekeeping GAPDH mRNA in HuR IP samples was comparable to that seen after IgG IP and remained unchanged by H_2O_2 treatment (Fig. 2C). Similarly, RNP IP assays using an anti-NF90 antibody indicated that (NF90-MKP-1 mRNA) RNP levels increased after H_2O_2 stimulation (Fig. 2D). The relative enrichment of these mRNAs in each RNP IP reaction was also tested by RT, followed by conventional PCR amplification and then visualized on agarose gels (Fig. 2C and D, right panels). By contrast, the MKP-1 mRNA dissociated from the translational repressors TIAR and TIA-1 in H_2O_2 -treated cells, as determined by RNP IP analysis. Biotin pulldown analysis recapitulated these results (see Fig. S2 in the supplemental material). Taken together, these observations indicate that HuR and NF90 associated more prominently with the MKP-1 mRNA after H_2O_2 treatment, while the translational repressors TIAR and TIA-1 dissociated from the MKP-1 mRNA in response to the oxidant (Fig. 2E and F).

After H_2O_2 treatment, HuR and NF90 increased in cytoplasmic abundance and stabilized MKP-1 mRNA. To investigate whether HuR and NF90 were involved directly in regulating MKP-1 expression, we first examined the levels and subcellular localization of HuR and NF90 in H_2O_2 -treated HeLa cells. H_2O_2 treatment did not change the abundance levels of HuR and NF90 in whole-cell extracts (Fig. 3A, WCE), but it did elevate the cytoplasmic presence of HuR and of NF90 (Fig. 3A, CE). These findings, which agreed with observations previously reported for colorectal carcinoma cells irradiated with UV light (48), were significant since regulated mRNA turnover and translation are primarily cytoplasmic events. Whether NF90 phosphorylation triggered its cytoplasmic increase, as reported for stimulated T cells (39), was not determined, but serine phosphorylation of NF90 did appear to be elevated following H_2O_2 treatment (see Fig. S3 in the supplemental material). Further evidence that HuR and NF90 translocated

to the cytoplasm after H_2O_2 treatment was obtained by immunofluorescence analysis (Fig. 3B).

Next, we determined if HuR and NF90 regulated MKP-1 mRNA stability by reducing their expression levels by using specific siRNAs in HeLa cells (Fig. 4A and C) and then measuring the MKP-1 mRNA half-life by RT-qPCR analysis. As shown, the half-life of MKP-1 in H_2O_2 -treated cells was potentially reduced from >180 min (Fig. 4B, control siRNA transfection group) to ~114 min (Fig. 4B, HuR siRNA transfection group). Similarly, NF90 silencing (Fig. 4C) also diminished the H_2O_2 -elicited stabilization of MKP-1 mRNA, from >180 min to ~164 min (Fig. 4D). These observations support the view that HuR and NF90 promote the stabilization of MKP-1 mRNA in response to H_2O_2 treatment, linked to their increased abundance in the cytoplasm after oxidant exposure.

HuR and NF90 increase MKP-1 mRNA stability via its 3' UTR. We hypothesized that the 3' UTR sequence of MKP-1 may specifically function as a mediator of mRNA turnover in response to H_2O_2 treatment. To test this possibility, we used a transcriptional pulse strategy based on the tetracycline (Tet) regulatory system (29, 56). HeLa Tet-Off cells that stably expressed a Tet-regulated repressor (tetracycline-controlled transactivator) were transiently transfected with a Tet-repressible EGFP reporter vector (pTRE-d2EGFP) containing the MKP-1 3' UTR [pTRE-d2EGFP-MKP-1(3' UTR)], as shown in the schematic of Fig. 5A. Forty-eight hours after the reporter plasmids were transfected, doxycycline was added to block tetracycline-controlled transactivator-driven transcription, and the rate of EGFP mRNA clearance was monitored by using RT-qPCR. In the absence of the MKP-1 3' UTR, the EGFP mRNA was very stable, with a half-life of >180 min (Fig. 5B, left); by contrast, insertion of the MKP-1 3' UTR lowered the half-life of the chimeric reporter [EGFP-MKP-1(3' UTR) mRNA] to ~50 min in untreated cells. It is important to note that this dramatic loss was completely prevented by pretreating cells with H_2O_2 (Fig. 5B, right), indicating that an instability sequence within the MKP-1 3' UTR treatment accelerated its decay in untreated cells and that treatment with the oxidant stabilized a transcript bearing this sequence.

To determine if HuR and NF90 participated in the oxidant-mediated stabilization, we tested the effect of knocking down HuR or NF90 on the reporter mRNA half-lives. Strikingly, silencing of HuR largely prevented the stabilizing effect of H_2O_2 , reducing the half-life of the chimeric EGFP-MKP-1(3' UTR) mRNA to ~78 min (Fig. 5C, right). Silencing NF90 also suppressed the stabilizing effect of H_2O_2 and lowered the EGFP-MKP-1(3' UTR) mRNA half-life to ~85 min (Fig. 5D, right). These results indicate that HuR and NF90 stabilize the MKP-1 mRNA in response to H_2O_2 via the MKP-1 3' UTR.

FIG. 5. HuR and NF90 increase MKP-1 mRNA stability via the MKP-1 3' UTR. (A) Schematic representation of the EGFP reporter plasmids containing the tetracycline-repressible promoter (*tetR*) used for the analysis. Twenty-four hr after control (B), HuR-directed (C), or NF90-directed (D) siRNAs were transfected into HeLa Tet-Off cells stably expressing a tetracycline-regulated repressor, pTRE-d2EGFP or pTRE-d2EGFP-MKP-1(3' UTR) plasmids were transfected; 24 h after transfection of the reporter plasmids, doxycycline (Dox) (2 μ g/ml) was added, and cells were collected at the indicated times to measure the amount of EGFP mRNA (left graphs) and GAPDH mRNAs (right graphs) by using RT-qPCR. Values were normalized to 18S rRNA levels in each sample and plotted using a semilogarithmic scale, and the times required for each mRNA to reach one-half of its initial abundance (50%, dashed line) were calculated. RT-qPCR results represent the means $\pm$ standard errors of the means from three independent experiments. Dashed lines, treated; solid lines, untreated (Untr.).

HuR enhances MKP-1 translation after H₂O₂ stimulation.

Since HuR and NF90 have also been implicated in controlling the translation of target mRNAs, we determined if these RNA-BPs might also influence MKP-1 translation. When untreated cultures were compared with H₂O₂-treated cultures, the distribution of MKP-1 mRNA along sucrose gradients shifted toward heavier polysome fractions in control fractions in control siRNA-transfected cells (Fig. 6A, left), as observed earlier with untransfected cells (Fig. 1F). In contrast, HuR silencing abrogated the shift in translation of MKP-1 mRNA after H₂O₂ treatment (Fig. 6B, left), supporting the notion that HuR contributed to enhancing MKP-1 association with actively translating polysomes following H₂O₂ treatment. NF90 silencing, on the other hand, had a modest influence on the translational engagement of MKP-1 mRNA after H₂O₂ treatment, as a large percentage of the total MKP-1 mRNA was still present in the actively translating fractions of the gradient (Fig. 6C, left, fractions 8 to 11). The polysomal association of a control housekeeping transcript (GAPDH mRNA) was reduced by H₂O₂ treatment but was not markedly different among the three silencing groups (Fig. 6A to C, right). According to these results, HuR contributes to enhancing MKP-1 translation after H₂O₂ treatment, while NF90 does not.

Additional evidence that HuR and NF90 influenced MKP-1 translation came from studies to assess de novo MKP-1 translation. After HuR or NF90 was silenced in HeLa cells, nascent translation of MKP-1 was measured by culturing cells in the presence of L-[³⁵S]methionine and L-[³⁵S]cysteine. This assay revealed that de novo translation of MKP-1 in H₂O₂-treated cells increased markedly, due in part to the higher MKP-1 mRNA levels. After NF90 silencing, the reduced MKP-1 mRNA stability did not cause a corresponding reduction in MKP-1 protein synthesis; instead, NF90 silencing elevated MKP-1 translation to the levels seen in the control group, supporting the view that NF90 might function as a repressor of MKP-1 translation. By contrast, HuR silencing caused a reduction in de novo MKP-1 translation (Fig. 6D).

Specificity of the influence of NF90 and HuR on MKP-1 expression. To further study the regulatory influence of these RNPs, we assayed endogenous MKP-1 expression levels after silencing HuR or NF90. Following H₂O₂ treatment, MKP-1 abundance was significantly reduced after HuR silencing (HuR siRNA) compared with that of control (control siRNA) cells (Fig. 7A and B). Next, we conducted “rescue” experiments to assess the specificity of this inhibitory effect. HuR expression was silenced in HeLa cells, using siRNAs that specifically targeted the 3' UTR of HuR [HuR(3') siRNA] while at the same time, HuR expression was restored using a plasmid to express a tandem

affinity purification (TAP)-tagged HuR (pHuR-TAP) construct, which lacked the HuR 3' UTR and hence was refractory to the effect of HuR(3') siRNA. As shown in Fig. 7C, MKP-1 protein levels were reduced in cells with silenced HuR, both in untreated and in H₂O₂-treated cells, but overexpression of HuR-TAP enhanced the H₂O₂-triggered production of MKP-1 (Fig. 7C). HuR residues S88, S100, and T118 were previously found to be phosphorylated by Chk2 (1). Accordingly, point mutations of these residues altered the association of HuR with MKP-1 mRNA (see Fig. S4 in the supplemental material). The HuR-TAP-mediated rescue of MKP-1 expression was also observed at the MKP-1 mRNA level, as determined by RT-qPCR analysis (Fig. 7D). Together, these results indicate that HuR specifically promotes MKP-1 expression after H₂O₂ treatment. Next, we determined if modulating the NF90 levels affected MKP-1 expression. The overexpression of NF90 by the use of an expression vector (pcDNA-NF90) unexpectedly reduced MKP-1 levels after H₂O₂ treatment, while MKP-1 mRNA levels increased modestly (Fig. 7E and F). Conversely, siRNA-mediated silencing of NF90 modestly increased MKP-1 protein levels while it moderately reduced MKP-1 mRNA levels (Fig. 7E and F). These results support the view that NF90 might function as a translational repressor of MKP-1 after H₂O₂ treatment.

The influence of NF90 and HuR on the regulation of MKP-1 expression was further analyzed by testing the kinetics of HuR and NF90 binding to MKP-1 mRNA (Fig. 7G). Interestingly, after H₂O₂ treatment, the MKP-1 mRNA showed progressively greater association with HuR, while its association with NF90 was potentially reduced by 4 h of exposure. These results indicate that shortly after H₂O₂ treatment (Fig. 2C and D and 7G), both MKP-1 mRNA-stabilizing RNA-BPs were bound to the MKP-1 mRNA; NF90 dissociated thereafter (Fig. 7G), while HuR remained bound. In addition, we determined if the association of NF90 with MKP-1 mRNA was affected by HuR and vice versa. Silencing of HuR only modestly reduced the NF90-MKP-1 mRNA associations; likewise, silencing of NF90 only slightly reduced the HuR-MKP-1 mRNA associations, suggesting that the two RNA-BPs neither cooperate nor compete for binding to the MKP-1 mRNA and instead interact with the MKP-1 mRNA independently of one another (Fig. 7H). These findings further indicate that the changes in RNP composition contributed to the enhancement of MKP-1 mRNA stability and translation.

Effect of HuR and/or NF90 silencing on H₂O₂-induced activation of MAPKs. To test the consequences of these MKP-1 regulatory processes upon cellular events, we assayed the effects of H₂O₂ on MAPK phosphorylation. Forty-eight hours after HeLa cells were transfected with HuR or NF90 siRNAs, the cells were treated with H₂O₂ for the indicated times, whereupon

FIG. 6. Effect of HuR or NF90 silencing on H₂O₂-increased MKP-1 mRNA translation. (A to C) HeLa cells were transfected with control (Ctrl.) siRNA (A) or siRNAs directed to HuR (B) or NF90 (C). Forty-eight hours later, cells were either left untreated (Untr.) or treated with H₂O₂ for 2.5 h (H₂O₂), and cytoplasmic extracts were fractionated through sucrose gradients, and the presence of MKP-1 mRNA (left) and GAPDH mRNA (right) in each fraction was assessed by RT-qPCR, as explained in the legend to Fig. 1F. The data (triplicate measurements per point) are representative of three independent experiments showing similar results. (D) Following siRNA transfections as indicated, nascent MKP-1 production was monitored following a brief (15-min-long) incubation of HeLa cells with L-[³⁵S]methionine and L-[³⁵S]cysteine after either no treatment (–) or treatment with H₂O₂ (2.5 h) (+). Following IP using either anti-MKP-1, anti-GAPDH, or control IgG antibodies, the incorporation of radiolabeled amino acids into the newly synthesized MKP-1 and GAPDH proteins was assessed by SDS-PAGE. The radiolabeled MKP-1 signals were visualized and quantified using a PhosphorImager and shown as the percentage of signals relative to that of the control, untreated cells.

FIG. 8. Effect of HuR or NF90 silencing on H_2O_2 -triggered activation of MAPKs. Forty-eight hours after HeLa cells were transfected with HuR and/or NF90 siRNAs, cells were treated with H_2O_2 for the times indicated, whereupon whole-cell extracts were prepared (as described in Materials and Methods), and the activation of p38, JNK, and ERK was determined with anti-phospho-ERK1, anti-phospho-JNK, and anti-phospho-p38 MAPK antibodies. Western blots were probed with anti-ERK1, anti-JNK, or anti-p38 MAPK antibodies. MKP-1, HuR, NF90, and loading control β -actin were also measured by Western blotting analysis. Ctrl., control.

whole-cell extracts were prepared (as described in Materials and Methods), and the activation of MAPKs p38, JNK, and ERK was determined using antibodies that recognized p-ERK, p-JNK, and p-p38. Importantly, HuR-silenced cells showed stronger and more prolonged phosphorylation of p38, JNK, and ERK MAPKs,

along with a reduction in MKP-1 expression levels (Fig. 8). NF90 silencing had a modest influence on p38 and JNK phosphorylation but did not measurably affect p-ERK levels. These results implicate HuR in the upregulation of MKP-1 expression levels in response to H_2O_2 treatment, in turn reducing MAPK activity in response to the oxidant.

DISCUSSION

Our studies reveal that the oxidant-triggered induction of MKP-1 is potently influenced by two posttranscriptional processes, mRNA stabilization and increased translation. These findings are particularly significant given that only transcriptional mechanisms and chromatin-related changes were previously believed to control MKP-1 levels (24, 28, 57). However, the notion that MKP-1 was regulated only transcriptionally was difficult to reconcile with its long 3' UTR (Fig. 2A) and with its status as an immediate-early gene, two features characteristic of genes regulated posttranscriptionally. Here, the RNA-BPs HuR and NF90 were found to associate with the MKP-1 3' UTR in an H_2O_2 -dependent manner (Fig. 2). HuR both stabilized the MKP-1 mRNA and promoted its translation, two well-documented functions of HuRs (4, 15). While NF90 also stabilized the MKP-1 mRNA, it appeared to suppress MKP-1 translation; these effects of NF90 were in keeping with earlier reports showing that NF90 stabilized the IL-2 mRNA and suppressed the translation of β -glucosidase mRNA (48, 59). The opposed effects of HuR and NF90 upon MKP-1 translation were partly resolved by kinetic analysis of the RNPs. HuR binding to MKP-1 mRNA persisted for at least 8 h following H_2O_2 treatment, while NF90 binding was largely lost by 4 h after exposure to the oxidant (Fig. 7G). Emerging from these findings is a model whereby HuR and NF90 could bind to MKP-1 mRNA and contribute to its rapid and potent stabilization after H_2O_2 treatment; afterward, HuR remains bound and further enhances MKP-1 translation, whereas NF90 dissociates from the MKP-1 mRNA and, hence, avoids inhibiting its translation.

Is HuR upregulation of MKP-1 levels in response to oxidative stress a component of HuR's antiapoptotic program? As shown in this report, HuR associated with MKP-1 mRNA more prominently after H_2O_2 treatment, thereby increasing

FIG. 7. Rescue experiments performed to assess the specific influences of NF90 and HuR on MKP-1 expression. (A and B) By 48 h after transfection of either control (Ctrl.) or HuR siRNAs into HeLa cells, the cells were stimulated with H_2O_2 , and lysates were collected over the first 2 h (A) or subsequently at 24 h (B) to measure the levels of MKP-1, HuR, and loading control β -actin by Western blotting analysis. (C) HuR "rescue" experiment. HeLa cells were cotransfected with siRNAs {[HuR(3')] siRNA, which targets the HuR 3' UTR, or Ctrl. siRNA} along with plasmid pTAP or pHuR-TAP; 48 h later, cells were treated with H_2O_2 , and whole-cell lysates were collected 3 h later to assess the levels of MKP-1, HuR, and loading control β -actin by Western blotting analysis. (D) Total RNA was extracted from cells treated as explained in the legend to panel C, and MKP-1 mRNA levels were analyzed by RT-qPCR; following normalization to 18S rRNA, the relative MKP-1 mRNA levels in each transfection group were calculated and are represented as the means $\pm$ standard errors of the means (SEM) from three independent experiments. (E) HeLa cells were transfected with empty (pcDNA) or NF90-expressing plasmids (pcDNA-NF90) or with NF90 or Ctrl. siRNAs. Forty-eight hours later, cells were treated with H_2O_2 , and whole-cell protein extracts were collected 4 h later for Western blotting analysis of MKP-1, NF90 and β -actin levels. (F) Total RNA was extracted from cells treated as explained in the legend to panel E, and MKP-1 mRNA levels were analyzed by RT-qPCR. Following normalization to 18S rRNA, the relative MKP-1 mRNA levels in each transfection group were calculated and are represented as the means $\pm$ SEM from three independent experiments. (G) The kinetics of association of endogenous HuR or NF90 with endogenous MKP-1 mRNA were assayed by RNP IP analysis using cytoplasmic fractions prepared from H_2O_2 -treated cells that were collected at the times indicated. The levels of MKP-1 mRNA were normalized first to the levels of 18S rRNA, and its enrichment was calculated relative to the levels of MKP-1 mRNA in IgG IPs (as described in the legend to Fig. 2C to F). The data (means $\pm$ SEM of four independent experiments) represent the percent differences in enrichment in the HuR IP and NF90 IP groups at various times after H_2O_2 treatment. (H) MKP-1 mRNA associated with HuR in control and NF90-silenced cells, as well as MKP-1 mRNA associated with NF90 in control and HuR-silenced cells. The data (means $\pm$ SEM from triplicate samples) are representative of five independent experiments.

MKP-1 mRNA stability and translation rate. Accordingly, elevation of HuR levels promoted MKP-1 expression, while silencing of HuR lowered MKP-1 abundance. The latter effect was studied further by examining MAPK activity following H₂O₂ treatment (Fig. 8). We observed a sustained activation of p38 and JNK that was linked to the reduction in MKP-1 levels. In light of the proapoptotic influences of the MAPKs p38 and JNK, the resistance to H₂O₂ and anisomycin treatments seen in embryonic fibroblasts derived from MKP-1-null mice (55, 62), and the overall prosurvival effect of HuR (2), we propose that the upregulation of MKP-1 by HuR is an important component of the HuR-elicited antiapoptotic program. However, while MKP-1 overexpression did elicit protection against H₂O₂ stress, MKP-1 silencing appeared to have no specific influence on cell survival (see Fig. S5 and S6 in the supplemental material). Therefore, whether MKP-1 is part of a growing number of HuR-induced antiapoptotic factors, such as ProTα, p21, Bcl-2, and Mcl-1, remains to be analyzed in depth. The proteins encoded by these HuR target mRNAs are postulated to function together in the prevention of cell death under unstimulated conditions, as well as in response to mild damage (2). The relative contribution of these antiapoptotic effectors to the overall cellular defense afforded by HuR likely depends on the type, timing, and magnitude of the damaging stimulus. Whether MKP-1 also participates in HuR-engendered cytoprotection awaits further study.

Posttranscriptional influence of NF90 upon MKP-1. As a DNA- and RNA-binding protein, NF90 has been ascribed a number of gene regulatory functions. Posttranscriptionally, NF90 was shown both to stabilize the IL-2 mRNA following T-cell activation and to suppress the translation of β-glucosidase (48, 59). In the present investigation, NF90 was found to have both types of effects upon the MKP-1 mRNA, stabilizing it but suppressing its translation. Given these opposing functions, NF90 had little net influence upon MKP-1 protein levels (Fig. 7E and 8). However, the kinetics of association of NF90 with the MKP-1 mRNA suggest that NF90 together with HuR could contribute to the initial stabilization of the MKP-1 mRNA. We hypothesize that NF90 later dissociates from the MKP-1 mRNA, thereby sparing MKP-1 from translational suppression, while HuR remains bound to the MKP-1 mRNA and enhances its translation. NF90 and HuR have affinity for shared, as well as unique, segments of the MKP-1 3' UTR. However, the dissociation of NF90 does not appear to increase the binding of HuR to the MKP-1 3' UTR. Neither does there seem to be cooperation in the binding of NF90 and HuR, since silencing NF90 did not affect the association of HuR with MKP-1 mRNA, and conversely, the silencing of HuR did not influence the association of NF90 with the MKP-1 mRNA (Fig. 7H). Efforts are under way in our laboratory to systematically identify NF90 target mRNAs and to determine if their stability and translation are regulated similarly to those of MKP-1 mRNA.

Posttranscriptional regulation of MKP-1 expression in tissue homeostasis. Overexpression of MKP-1 in macrophages decreases the LPS-induced production of inflammatory cytokines by inhibiting the p38 MAPK pathway (53). Consistent with the involvement of MKP-1 in these processes, macrophages derived from MKP-1 knockout mice exhibit prolonged H₂O₂-stimulated p38 and JNK activation resulting in enhanced

production of TNF-α and IL-6 compared with those derived from wild-type mice (45). HuR was recently shown to suppress the translation of inflammatory cytokines (18); according to the findings presented here, HuR may also prevent inflammation by elevating MKP-1 expression levels, as this is the major phosphatase to inhibit MAPK signaling in the innate immune response to inflammatory stimuli (reviewed in reference 53). Furthermore, since oxidative injury is a major source of damage in numerous other conditions, including atherosclerosis, cancer, and neurodegeneration, the influences of HuR and NF90 upon MKP-1 expression warrant further study in numerous pathophysiologic processes.

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