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Antiapoptotic function of RNA-binding protein HuR effected through prothymosin α

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We report the antiapoptotic effect of RNA-binding protein HuR, a critical regulator of the post-transcriptional fate of target transcripts. Among the most prominent mRNAs complexing with HuR is that encoding prothymosin α (ProTα), an inhibitor of the apoptosome. In HeLa cells, treatment with the apoptotic stimulus ultraviolet light (UVC) triggered the mobilization of ProTα mRNA to the cytoplasm and onto heavier polysomes, where its association with HuR increased dramatically. Analysis of a chimeric ProTα mRNA directly implicated HuR in regulating ProTα production: ProTα translation and cytoplasmic concentration increased in HuR-overexpressing cells and declined in cells in which HuR levels were lowered by RNA interference. Importantly, the antiapoptotic influence engendered by HuR was vitally dependent on ProTα expression, since use of oligomers that blocked ProTα translation abrogated the protective effect of HuR. Together, our data support a regulatory scheme whereby HuR binds the ProTα mRNA, elevates its cytoplasmic abundance and translation, and thereby elicits an antiapoptotic program.
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Introduction

In response to stressful environmental conditions, mammalian cells elicit a series of adaptive changes collectively termed the *stress response*. Central to the stress response is the implementation of changes in gene expression patterns, which critically influence the cellular outcome. In turn, such gene regulatory events will dictate whether the stressed cell will engage in events such as growth arrest, proliferation, repair of damaged macromolecules, differentiation, or apoptotic death.

Post-transcriptional gene regulatory processes such as RNA splicing and maturation, as well as mRNA transport, stability, and translation, are gaining increasing recognition as key mechanisms controlling gene expression during the stress response (Sheikh and Fornace, 1999; Mitchell and Tollervey, 2000; Kaufman, 2002; Kedersha and Anderson, 2002). Such control mechanisms typically involve the association of transcripts with specific RNA-binding proteins (RBPs) that affect their subcellular localization, stability, and translation rate (Keene, 2001). A growing number of these ribonucleoprotein (RNP) associations have been found to be dependent on the presence of particular RNA sequences rich in adenines and uracils (also known as AU-rich elements or AREs), present in the 5′ or 3′ untranslated regions (UTRs) of the mRNA (Zhang *et al*, 2002; Bevilacqua *et al*, 2003). ARE-dependent RNPs have been described for many transcripts encoding proteins that directly influence cell survival upon exposure to damaging stimuli, such as p53, p27, bcl-2, and p21 (Wang *et al*, 2000a; Kullmann *et al*, 2002; Lapucci *et al*, 2002; Galbán *et al*, 2003; Mazan-Mamczarz *et al*, 2003). The ubiquitous member of the Hu/ELAV family of RBPs (which also comprises the primarily neuronal proteins HuB, HuC, and HuD), HuR binds target ARE-bearing mRNA subsets through its RNA-recognition motifs (RRMs), and has been proposed to participate in their export to the cytoplasm, where it increases their stability, modulates their translation, or performs both functions. Through its post-transcriptional influence on target mRNAs such as those encoding c-fos, c-myc, cyclooxygenase-2, tumor necrosis factor-α, GM-CSF, β-catenin, eotaxin, p27, cyclin A, cyclin B1, cyclin D1, p21, p27, p53, HuR has been proposed to play major roles in cell proliferation, tumorigenesis, the immune response, and the stress response (Brennan and Steitz, 2001; Dixon *et al*, 2001; Esnault and Malter, 2003; Gorospe, 2003).

The prothymosin α (ProTα) mRNA was recently identified as one of the major putative targets of HuR (Lal *et al*, 2004). The encoded ProTα is a small, highly acidic protein with a wide tissue distribution and a high degree of conservation among mammals (reviewed in Hannappel and Huff, 2003). ProTα was isolated from thymus extracts three decades ago, but progress in elucidating ProTα expression and function has been slow and surrounded by controversies regarding its subcellular localization, structural properties, post-translational modifications, related family members, immunomodulatory effects, and mechanisms controlling its expression (Pineiro *et al*, 2000). Nonetheless, there is a general agreement that ProTα promotes cell proliferation, is closely associated with neoplastic growth, and is capable of preventing cell death; consequently, ProTα is considered to be an oncoprotein (Eilers *et al*, 1991; Sburlati *et al*, 1991; Dosil *et al*, 1993; Smith *et al*, 1993; Wu *et al*, 1997; Rodriguez *et al*, 1998; Magdalena *et al*, 2000; Pineiro *et al*, 2000; Orre *et al*, 2001). An important breakthrough in elucidating ProTα function was the discovery that ProTα inhibited the formation of the apoptosome, a cytosolic macromolecular complex (700–1400-kDa) that assembles in cells committed to apoptotic death. In response to apoptogenic stimuli, cytochrome *c* is

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released from the mitochondria and binds apoptotic protease activating factor (Apaf)-1 monomers; Apaf-1 then oligomerizes to form the apoptosome, a heptameric structure that recruits and activates caspase-9, which in turn activates effector caspases (caspase-3, -6, -7), culminating in apoptotic cell death (Li *et al*, 1997; Rodriguez and Lazebnik, 1999; Kaufmann and Hengartner, 2001). ProTα was found to hinder the assembly of the apoptosome complex and thereby prevented the activation of caspase-9 and the ensuing apoptotic cascade of events (Jiang *et al*, 2003).

In this investigation, we set out to formally examine the association of HuR with target ProTα mRNA, to study HuR's influence on ProTα expression, and to assess the consequences of this interaction on apoptosis. Our results support a role for HuR in enhancing both the abundance of cytoplasmic ProTα mRNA and the translation of ProTα in response to irradiation with short-wavelength ultraviolet light (UVC), an apoptotic stimulus. We present evidence highlighting a functional interdependence between the prosurvival effects of HuR and those of ProTα following stressful stimulation, and propose that ProTα is a major effector of the antiapoptotic effects of HuR.

Results

Antiapoptotic effects of HuR in unstimulated and UVC-irradiated cells

In previous studies aimed at modulating HuR expression in cancer cells (Wang *et al*, 2000a, b; Lal *et al*, 2004), we consistently noted increased cell death in populations expressing reduced HuR levels (not shown). Here, we systematically investigated the effects of HuR on cell survival in response to UVC, a proapoptotic stimulus that damages DNA and other macromolecules. HuR abundance in the cytoplasm of HeLa cells increased rapidly (by 2 h) following UVC irradiation, remained elevated for at least 12 h, and decreased thereafter (Figure 1A), in keeping with earlier findings in other cell types (Wang *et al*, 2000a); UVC irradiation also triggered the appearance of cleaved poly(ADP-ribose) polymerase (PARP), a well-established marker of apoptosis. RNAi-based interventions to lower HuR expression in untreated (Untr.) HeLa cells (HuR siRNA group, Figure 1B and D) caused the appearance of nuclei with condensed and fragmented chromatin, distinct hallmarks of apoptosis, while no such nuclei were seen in the control population (Ctrl. siRNA). Following UVC irradiation, apoptotic nuclei were strikingly more abundant in cells expressing reduced HuR levels (Figure 1C). The changes in surviving cells as well as in the condensed/fragmented nuclei in each transfection and treatment group (Figure 1C) further revealed that HuR prevented cell death both in unstressed and in UVC-treated cells. The apoptotic features of populations expressing lower HuR levels were also observed when employing three other sequences targeting different regions of the HuR mRNA (not shown). Western blot analysis further revealed the different apoptotic response of these populations: in Ctrl. siRNA cells, PARP cleavage was only visible after UVC treatment, while in HuR siRNA cells, PARP cleavage was readily visible in unirradiated cells and increased markedly after UVC irradiation. Additional characterization of the apoptotic response by monitoring cleaved caspase-9 and cleaved caspase-3 (two additional apoptotic markers, Figure 1D) further indicated that knockdown of HuR triggered apoptosis in unirradiated cells and potentiated the toxicity of UVC irradiation.

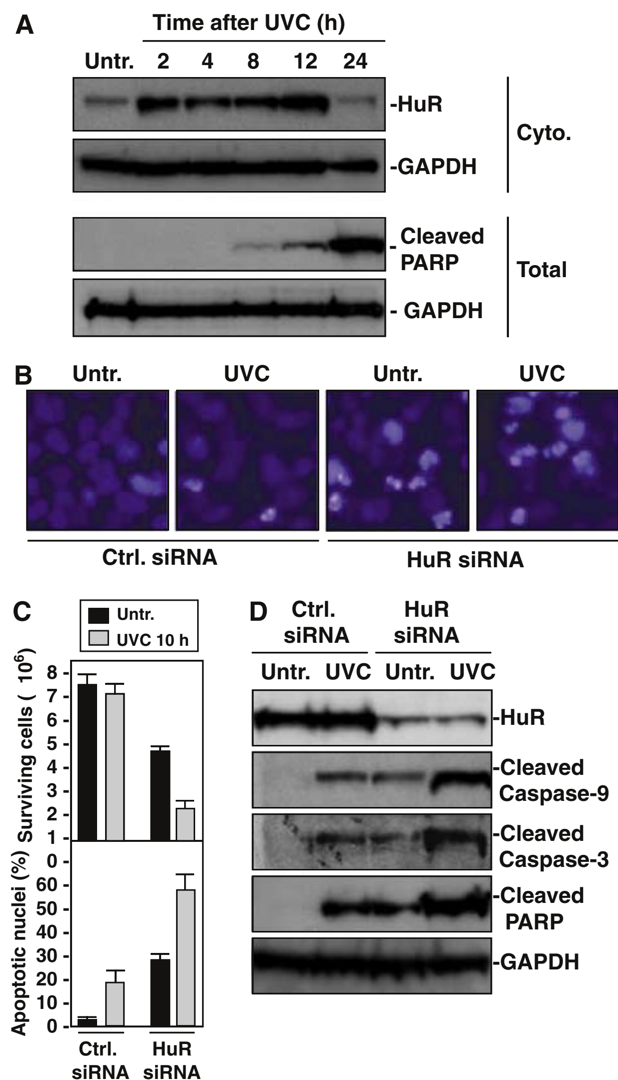

Figure 1 Downregulation of HuR expression in HeLa cells decreases cell survival. (**A**) At the times indicated after irradiation of HeLa cells with 30 J/m^2 UVC, cytoplasmic (Cyto., 10 μg) and whole-cell (Total, 20 μg) lysates were prepared and the abundance of HuR, cleaved PARP (a marker of apoptosis), and GAPDH (loading control) was assessed. (**B**) At 48 h after transfection with 20 nM of either a control siRNA (Ctrl. siRNA) or an siRNA targeting HuR (HuR siRNA), cells were either left untreated (Untr.) or were irradiated with UVC (UVC); cell viability 8 h later was assessed by Hoechst staining to monitor the presence of condensed and fragmented nuclei. Shown are representative fields from three independent experiments. (**C**) Graphs quantifying apoptotic cell numbers in cultures treated as explained in the legend of panel B; shown are the means±standard error of the means (s.e.m.) from three independent experiments. (**D**) Western blot analysis to monitor the abundance of HuR (with 10% remaining after siRNA-mediated knockdown), cleaved caspase-9, cleaved caspase-3, cleaved PARP, and GAPDH in whole-cell lysates prepared from cells that were treated as explained in the legend of panel B.

Conversely, ectopic overexpression of HuR by transfection (pHuR-T) reduced the number of apoptotic nuclei detected in control, vector-transfected (Vector) cells by 24 h after UVC irradiation, and largely prevented the cleavage of caspase-9, caspase-3, and PARP (Figure 2A–C). Further evidence of the

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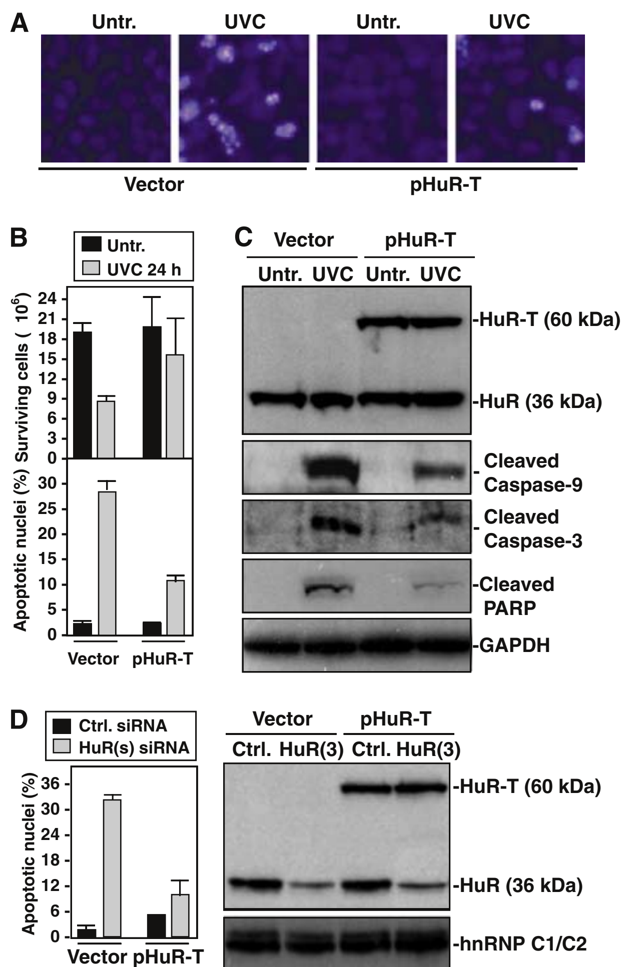

Figure 2 HuR overexpression in HeLa cells promotes cell survival. (**A**) At 12 h after UVC irradiation (30 J/m^2) of HeLa populations that had been transfected with either a control plasmid (Vector) or a plasmid overexpressing HuR (HuR-T), cell viability was assessed by Hoechst staining to monitor the presence of condensed and fragmented nuclei; representative fields are shown. (**B**) Graphs quantifying apoptotic cell numbers in cultures treated as described in panel A; shown are the means±s.e.m. from three independent experiments. (**C**) Western blot analysis to monitor the abundance of ectopically expressed HuR (HuR-T), endogenous HuR (HuR), cleaved caspase-9, cleaved caspase-3, cleaved PARP, and GAPDH in whole-cell lysates (20 μg) prepared from cells that were treated as described in panel A. (**D**) HuR downregulation-triggered apoptosis (elicited through an siRNA targetting the HuR 3′UTR, HuR(3) siRNA) was rescued by HuR-T overexpression. Left, quantification of apoptotic nuclei, shown as the means±s.e.m. from three independent experiments; right, Western blot analysis of the levels of HuR and HuR-T in each population (HuR(3) siRNA reduced endogenous HuR levels to 20% of the levels seen in control (Ctrl. siRNA) cells).

protective influence of HuR was obtained from 'rescue' experiments. When endogenous HuR expression levels were specifically knocked down by using an siRNA directed to the HuR 3′UTR (HuR(3)), cell survival was strongly reduced (Figure 2D), as anticipated (Figure 1); ectopic overexpression of HuR-T, using a vector that lacked the HuR 3′UTR and hence was insensitive to the effects of HuR(3) siRNA, significantly lowered the apoptotic phenotype (Figure 2D). Together, these results uncover an antiapoptotic function for HuR in both unstimulated and stress-treated cells.

HuR binds the 3′UTR of the ProTα mRNA

Earlier studies had identified the ProTα mRNA, which bears a long, AU-rich 3′UTR (Figure 3, Schematic), as an HuR target transcript (Lal *et al*, 2004). In order to directly examine the formation of (HuR-ProTα mRNA) RNP complexes, binding assays were performed. First, cDNAs corresponding to either the 5′UTR, the coding region (CR), or the 3′UTR of the ProTα mRNA were prepared for use as templates for *in vitro* transcription and the corresponding biotinylated RNAs used in pulldown assays (Materials and methods). As shown, HuR prominently bound the 3′UTR transcript, but not the CR or 5′UTR transcripts; by contrast, the RBP hnRNP A1 was found to bind the 5′UTR transcript somewhat more strongly than the other two fragments (Figure 3A). Second, an immunoprecipitation (IP) assay was carried out under conditions that preserved endogenous RNP associations. Following RT–PCR analysis of the IP material employing ProTα-specific oligomers, a ProTα product was readily detected in the IP material obtained using an anti-HuR antibody, while only residual amplification of ProTα was observed in both the IgG1 control IP and in control RT–PCR reactions to amplify housekeeping genes GAPDH and SDHA (Figure 3B). These observations indicate that HuR can specifically bind the ProTα 3′UTR.

ProTα mRNA cytoplasmic abundance and association with HuR in the translating cell fraction increase after UVC

The levels of ProTα mRNA (as detected by Northern blotting, Figure 4A) and ProTα protein in whole-cell lysates (as detected by Coomassie staining due to unique ProTα characteristics that preclude its analysis by Western blotting (Pineiro *et al*, 2000; Sukhacheva *et al*, 2002), Figure 4B) remained unchanged following UVC irradiation. However, the cytoplasmic levels of ProTα mRNA increased approximately three-fold by 6 h after UVC irradiation, as assessed by either RT+real-time PCR analysis or by Northern blotting, while a concomitant three-fold reduction of ProTα mRNA in the nuclear compartment was measured (Figure 4C). The levels of positive control p21 mRNA increased in both compartments after UVC treatment, while the levels of negative control GAPDH mRNA remained unchanged (Figure 4C). These observations suggested that UVC increased ProTα mRNA levels in the cytoplasm and decreased them in the nucleus, possibly via a UVC-enhanced mRNA export. That HuR likely contributed to this increase in cytoplasmic ProTα mRNA was supported by the findings that cells overexpressing HuR (pHuR-T group) had overall higher levels of cytoplasmic ProTα mRNA and lower levels of nuclear ProTα mRNA than did control populations (Vector group, Figure 4D).

A closer analysis of the cytoplasmic components of HeLa cells, separated through sucrose gradients to discriminate the nontranslating and the translating fractions, revealed an increase in the ProTα mRNA in the translationally engaged, polysomal fractions (fractions 6–10) of UVC-irradiated cells, as monitored by both Northern blotting (Figure 5A, quantified in Supplementary data) and by RT+real-time PCR analysis (Figure 5B). The latter set of experiments also examined the abundance of UVC-regulated mRNAs encoding p21 or cyclin D1 (previously reported; Lal *et al*, 2004), as well as that of negative controls UBC, GAPDH, and SDHA mRNAs, whose levels and relative distribution on polysomes did not

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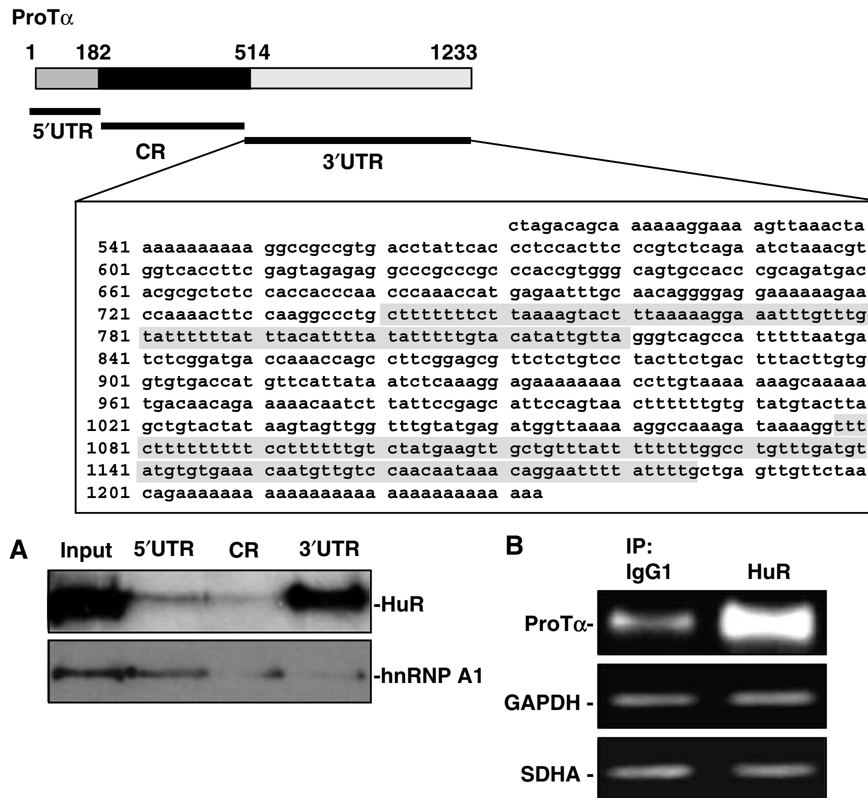

Figure 3 HuR binds endogenous and recombinant ProTα transcripts. Schematic of ProTα mRNA, depicting AREs (gray), as well as the transcripts (5′UTR, CR, and 3′UTR) used in biotin pulldown analysis. (**A**) Whole-cell lysates were prepared from HeLa cells and binding of equimolar amounts of the biotinylated transcripts (Supplementary data) to either HuR or hnRNP A1 was tested by biotin pulldown analysis; representative Western blots are shown. (**B**) The binding of endogenous HuR to endogenous target transcripts was detected by RT–PCR assay of material obtained by immunoprecipitation (IP) using either IgG1 or anti-HuR antibodies. Amplification of housekeeping transcripts encoding GAPDH and SDHA, bound at low levels with the IP material, showed equal loading of IP samples. PCR products are shown.

change significantly with UVC irradiation (Lal *et al*, 2004). The heightened presence of ProTα mRNA in the translating fractions further suggested that UVC might enhance the biosynthesis of ProTα protein.

Given the association of HuR with ProTα transcripts (Figure 3) and the UVC-triggered increase in HuR in actively translating polysomes (Figure 5A), direct evidence for the existence of (HuR-ProTα mRNA) complexes was sought in the various cell fractions following UVC irradiation. As shown in Figure 5C, IP followed by RT + real-time PCR analysis revealed a marked increase in the abundance of the (HuR-ProTα mRNA) complex, particularly in the polysomes (fractions 6–10). Taking into consideration the aforementioned reports describing a role for HuR in modulating the translation of target mRNAs, we hypothesized that ProTα translation could be regulated by HuR and set out to examine this possibility experimentally.

UVC increases ProTα translation in an HuR-dependent fashion

ProTα is predominantly a nuclear protein (Watts *et al*, 1989, 1990; Clinton *et al*, 1991) but its antiapoptotic effects are elicited in the cytoplasm (Jiang *et al*, 2003). While no UVC-triggered changes in global ProTα levels have been reported (Enkemann *et al*, 2000; Evstafieva *et al*, 2003; Figure 4B), it was possible that ProTα levels increased in the cytoplasm following irradiation. Accordingly, we investigated if the translation and cytoplasmic abundance of ProTα were altered following UVC irradiation. These studies were carried out by employing constructs that expressed chimeric EGFP-ProTα (pEGFP-ProTα, Figure 5D) for several reasons: first, to avoid using the endogenous ProTα promoter, which is activated by transcription factor c-myc (Eilers *et al*, 1991), itself encoded by an mRNA that is a target of HuR (Ma *et al*, 1996); second, to be able to transfer the protein onto filters for Western blot analysis (ProTα fails to bind to nitrocellulose membranes because it lacks sufficient hydrophobic amino acids); third, to be able to use anti-EGFP antibodies for analysis, thereby overcoming the limited usefulness of anti-ProTα antibodies available, which poorly detect it by Western blotting; and fourth, because the ProTα protein lacks any methionine or cysteine residues, needed for monitoring nascent translation (Materials and methods), an approach that allows the direct measurement of newly synthesized protein and thereby circumvents additional processes influencing protein levels (degradation, cleavage, etc.). Nascent translation of EGFP and EGFP-ProTα was assayed by performing a brief (20-min long) incubation in the presence of [^{35}S]-labeled amino acids followed by IP using either an anti-EGFP antibody or control IgG. As shown, UVC did not influence EGFP translation, as determined by monitoring the rate of nascent EGFP translation from construct pEGFP, but strongly elevated the translation of EGFP-ProTα (Figure 5E). Importantly, when assessing the levels of the chimeric ProTα protein following UVC

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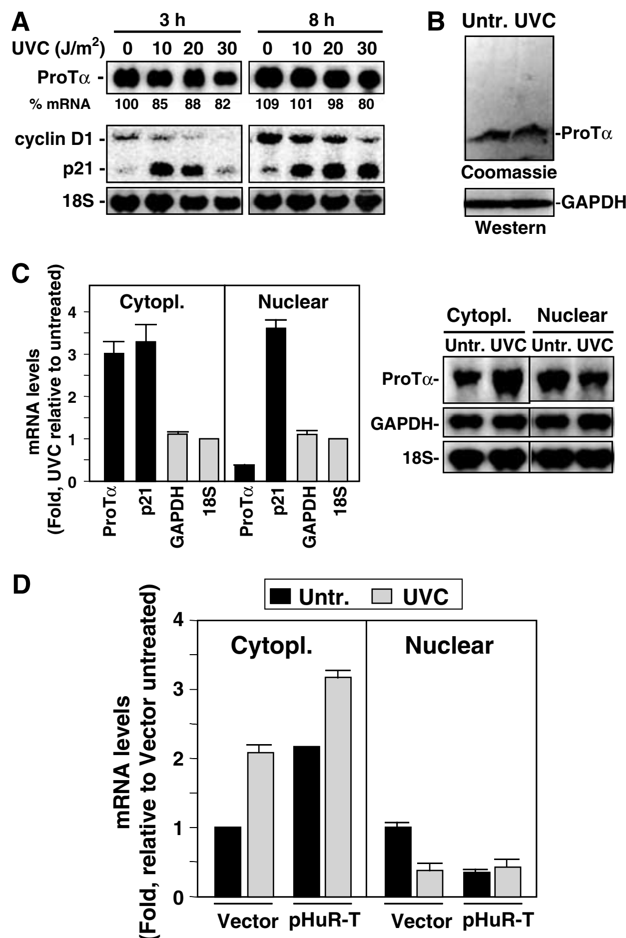

Figure 4 UVC- and HuR-dependent increase in cytoplasmic abundance of the ProTα mRNA. (**A**) The levels of mRNAs encoding ProTα, cyclin D1, and p21, as well as loading control 18S rRNA were assessed by Northern blotting following UVC irradiation of HeLa cells. % mRNA, percent ProTα mRNA signals relative to 18S signals. (**B**) The abundance of ProTα was monitored in whole-cell lysates (Materials and methods) that were prepared 8 h after 30 J/m^2 UVC irradiation, size-fractionated by SDS–PAGE, and visualized by staining the gels with Coomassie blue. (**C**) Changes in the levels of mRNAs encoding ProTα, p21, and GAPDH (as well as 18S rRNA) in the cytoplasmic (Cytopl.) or Nuclear fractions (Materials and methods) 6 h after UVC irradiation (30 J/m^2) were assessed by either RT + real-time PCR (left) or by Northern blotting (right). (**D**) Cells expressing normal HuR levels (Vector) or overexpressing HuR (pHuR-T, described in the legend of Figure 2) were either left untreated or irradiated with UVC. After 6 h, Cytopl. and Nuclear fractions were prepared and ProTα mRNA levels detected by RT + real-time PCR. Graphs depict the means ± s.e.m. from four independent experiments. Controls to monitor the adequate preparation of nuclear and cytoplasmic lysates are available (Supplementary data).

irradiation, a sizeable increase was detected in the cytoplasm (the cell fraction where ProTα inhibits the formation of the apoptosome) of UVC-treated cells (Figure 5F). These observations strongly support the notion that the translation of ProTα increased after UVC irradiation.

In order to investigate whether HuR was directly implicated in regulating ProTα translation by UVC, interventions to either elevate or knock down HuR levels were undertaken. Following HuR knockdown by siRNA, EGFP-ProTα expression was significantly reduced in the HuR siRNA populations, whereas EGFP expression was unchanged between the Ctrl. and HuR siRNA transfection groups (Figure 6A). That this reduction in EGFP-ProTα expression was due, at least in part, to a decrease in EGFP-ProTα translation in the HuR siRNA population was again determined through the assessment of nascent protein synthesis. After a pulse incubation with ^{35}S-amino acids followed by IP, EGFP-ProTα translation was found to be markedly reduced in HuR-knockdown populations, while EGFP translation was unaffected by the reduction in HuR levels (Figure 6B). Conversely, HuR overexpression increased the steady-state abundance of EGFP-ProTα (Figure 6C) and its translation rate (Figure 6D), but not those of control EGFP (Figure 6C and D). Northern blot analyses of EGFP mRNA and EGFP-ProTα mRNA revealed that the levels of the chimeric transcripts were unchanged among the populations expressing different ectopic HuR levels (not shown). These results underscore a role for HuR in regulating the translation of ProTα and hence its cytoplasmic abundance.

Interdependence of prosurvival effects of ProTα and HuR

Finally, the influence of ProTα and HuR on cell survival was investigated directly. Overexpression of ProTα (performed as described in Figure 6) was found to partly rescue the apoptosis triggered by HuR knockdown, both in unirradiated and in UVC-irradiated cells (Figure 7A). Similarly, in UVC-irradiated cells, ProTα overexpression potentiated the antiapoptotic phenotype of cells expressing higher HuR levels (Figure 7B). More significantly, a reduction of ProTα expression by using oligomers that did not change mRNA levels but suppressed ProTα translation (AS oligo, (Sburlati *et al*, 1991), as determined by Western blotting and ^{35}S incorporation (Figure 7C)) largely abrogated the protective influence of HuR overexpression (compare HuR-T groups between the S oligo and AS oligo populations) and further contributed to enhancing apoptosis (Figure 7D). Assessment of PARP cleavage (Figure 7D, right) served to confirm the relative extent of apoptosis in the S and AS oligo treatment groups. Together, these findings support an antiapoptotic role of HuR that is elicited through HuR-mediated increases in the levels of cytoplasmic ProTα mRNA and ProTα translation.

Discussion

This report describes the antiapoptotic influence of HuR and examines the mechanisms whereby HuR modulates the expression of ProTα, a protein that critically enhances cell survival (Jiang *et al*, 2003) and is encoded by an HuR target mRNA (Lal *et al*, 2004). Using UVC-irradiated HeLa cells as model system, HuR was found to strongly promote cell survival after UVC irradiation, and these effects were linked to HuR-mediated increases in cytoplasmic ProTα mRNA levels and in ProTα translation. Based on these observations, we investigate the existence of molecular and functional associations between the expression and prosurvival effects of HuR and ProTα during the stress response.

Post-transcriptional upregulation of HuR target transcripts

While a role for HuR in export of target mRNAs to the cytoplasm has been suggested (Keene, 1999; Gallouzi and Steitz, 2001), HuR has been extensively characterized as a

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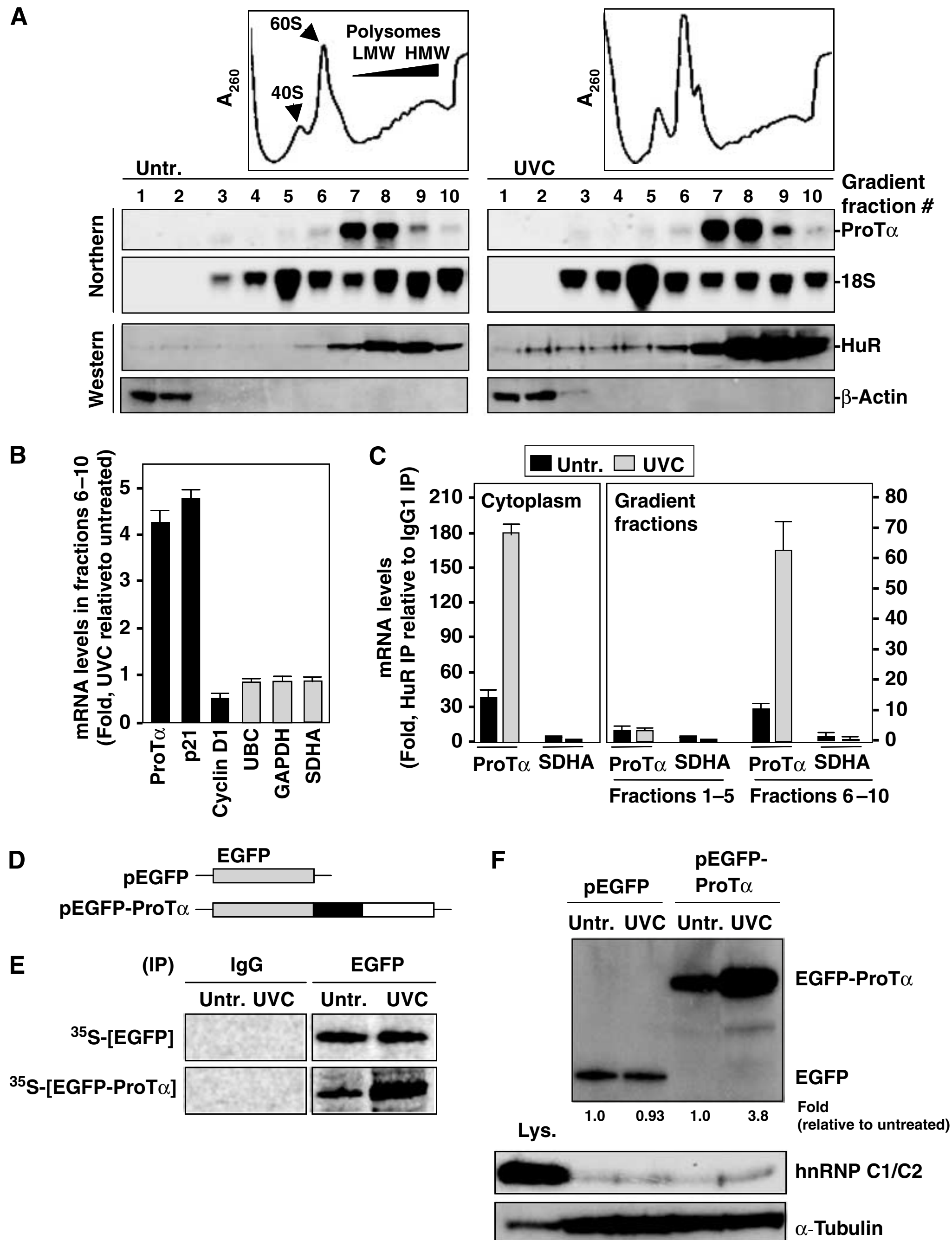

Figure 5 UVC-triggered increase in ProTα mRNA levels in polysomes, elevation of ProTα translation, and cytoplasmic accumulation of chimeric ProTα. (**A**) Representative polysome distribution profiles obtained after centrifugation of cytoplasmic lysates (prepared from either irradiated cells (6 h after receiving 30 J/m^2 UVC) or unirradiated cells) over sucrose gradients. From left to right, fractions lacked ribosomes or ribosome subunits (fractions 1, 2), contained ribosome subunits or single ribosomes (fractions 3–5), or spanned polysomes of increasing molecular weight (fractions 6–10). From each fraction, RNA was prepared for Northern blot analysis of ProTα mRNA and 18S rRNA, and protein was prepared for Western blot analysis of HuR and β-actin. (**B**) The polysome-associated levels of ProTα mRNA as well as those of control mRNAs encoding p21 and cyclin D1 (UVC-regulated) and those encoding UBC, GAPDH, and SDHA (not UVC-regulated) were quantified by RT + real-time PCR. (**C**) The abundance of HuR-bound mRNAs encoding either ProTα or GAPDH in the cytoplasmic fraction (left, 500 μg) or in pooled gradient fractions 1–5 and 6–10 (right) was investigated by HuR IP followed by RT + real-time PCR; data are shown as relative enrichment over the signals obtained using IgG1 IP in samples assayed by RT + real-time PCR. Graphs depict the means ± s.e.m. from four independent experiments. (**D**) Schematic of plasmids used in transfections to express either EGFP (pEGFP) or chimeric EGFP-ProTα (pEGFP-ProTα). Gray, EGFP cDNA; black, ProTα CR; white, ProTα 3'UTR. (**E**) Newly translated EGFP and EGFP-ProTα were assessed by incubating unirradiated cells (Untr.) or cells irradiated 6 h earlier (UVC) with L-[^{35}S]methionine and L-[^{35}S]cysteine for 20 min. Following IP using either IgG or anti-EGFP antibodies, samples were resolved by SDS–PAGE and transferred onto membranes for visualization of ^{35}S-radiolabeled signals. (**F**) EGFP and EGFP-ProTα expression levels in cytoplasmic lysates (20 μg) were determined by Western blotting using an anti-EGFP antibody. Assessment of hnRNP C1/C2 (a nuclear protein) and α-tubulin (a cytoplasmic protein) levels served to monitor the adequate preparation and loading of the cytoplasmic fractions. Data are representative from three independent experiments. Lys., whole-cell lysate (20 μg).

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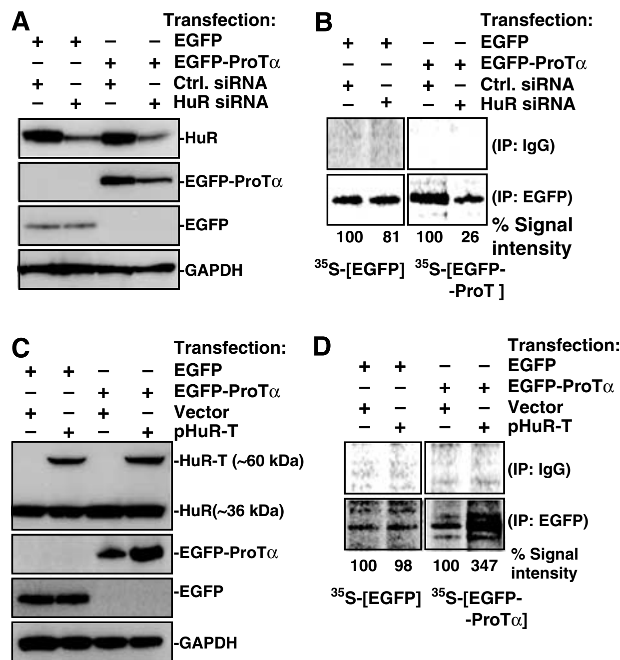

Figure 6 HuR promotes the translation and cytoplasmic accumulation of chimeric ProTα. HeLa cells expressing either normal (Ctrl. siRNA) or knocked-down HuR levels (HuR siRNA) were subsequently transfected with plasmids encoding EGFP or EGFP-ProTα; 24 h later, the expression levels of HuR, EGFP-ProTα, EGFP, and GAPDH were assessed by Western blotting (**A**) and the nascent translation rates of EGFP and EGFP-ProTα were examined as described for Figure 5E (**B**). HeLa cells expressing either higher (pHuR-T) or normal HuR levels (Vector) were transfected with plasmids encoding EGFP or EGFP-ProTα, and 24 h later the expression of HuR, HuR-T, EGFP-ProTα, EGFP, and GAPDH was assessed by Western blotting (**C**), and the nascent translation rates of EGFP and EGFP-ProTα were monitored as described in the legend of Figure 5E (**D**).

protein that stabilizes target mRNAs, as described for transcripts such as those encoding c-fos, VEGF, cyclooxygenase-2, p21, cyclin A, cyclin B1, matrix metalloprotease 9, GM-CSF, eotaxin, IL-2, c-myc, etc. (reviewed in Brennan and Steitz, 2001). However, HuR and other Hu/ELAV members have also been found to promote the translation of growing number of target transcripts. In addition to enhancing the ProTα translation, as described here, HuR was proposed to stimulate the translation of p53 and $p27^{Kip1}$ (Millard *et al*, 2000; Mazan-Mamczarz *et al*, 2003) (although one study instead reports the *repression* of $p27^{Kip1}$ translation by HuR; Kullmann *et al*, 2002), while HuB binds to and increases the translation of NF-M and Glut-1 mRNAs (Jain *et al*, 1997; Antic *et al*, 1999). The precise mechanisms mediating the enhanced translation of ProTα, p53, and $p27^{Kip1}$ by HuR are unclear, but may be linked to a mechanism of recruitment of these mRNAs to translationally active polysomes. In the present investigation, no differences in ProTα mRNA abundance (Figure 4A) or stability (Supplementary data) were observed, and only an HuR-mediated promotion of ProTα translation was apparent (Figure 6). To date, no studies addressing specific links between HuR-mediated stabilization and translation of target transcripts on a global level have been reported, but single-gene studies lend support to an emerging model whereby HuR binds to a given mRNA, likely assists in its nuclear export, protects it from degradation in the cytoplasm, and directs it to ribosomes, enhancing its translation (Keene 1999; Brennan and Steitz, 2001; Lal *et al*, 2004).

During the cellular response to genotoxic stresses, the presence of damaged DNA causes an inhibition of general transcription (reviewed in Svejstrup, 2002). Paradoxically, while the transcriptional machinery is inhibited, certain proteins participating in the DNA damage response, including those that control the cell division cycle, apoptosis, and DNA repair, must continue to be synthesized. How then does the cell modify its gene expression patterns to adequately sense the damage and elicit a proper response? Several studies support the notion that post-transcriptional events may provide leading mechanisms to control the expression of critical genes in response to DNA damage. For example, recent studies provide systematic demonstration that mRNA turnover accounted for at least one-half of the changes in mRNA steady-state levels following exposure to stresses (Fan *et al*, 2002; Kawai *et al*, 2004). Other post-transcriptional mechanisms (such as enhanced mRNA export, heightened translation, or increased protein stability) may likewise provide such regulation of specific DNA damage response proteins, thereby temporarily obviating the need for new transcription (Gorospe, 2003). In addition, post-transcriptional gene regulatory mechanisms would ensure that DNA damage affecting critical genes is not perpetuated by the production of defective proteins and would help preserve conditions of cellular homeostasis during a period of DNA repair. We propose that HuR is a key participant in the execution of such post-transcriptional regulation: it binds to mRNAs encoding proteins that regulate cell proliferation, repair, and apoptosis, likely functions in their nuclear export, helps preserve their cytoplasmic half-life, and enhances their translation. Accordingly, a broad post-transcriptional function for HuR will help ensure that key response proteins such as ProTα or p53 are in place through post-transcriptional mechanisms at a critical time of damage assessment and implementation of survival or apoptotic responses.

Antiapoptotic influence of RNA-binding protein HuR

Our findings also uncover ProTα as a critical downstream effector of the HuR-elicited survival program. Damaging stimuli such as UVC (Nijhawan *et al*, 2003) trigger apoptosis by causing the release of cytochrome *c* from the mitochondria to the cytosol, where it activates Apaf-1 and promotes it oligomerization into the apoptosome. The antiapoptotic function of ProTα is attributed to its ability to inhibit the function of the apoptosome (Jiang *et al*, 2003; Nicholson and Thornberry, 2003), thereby blocking the cleavage of caspase-9 and preventing the ensuing cascade of events. In the present investigation, HuR was found to associate with the ProTα mRNA and to enhance its translation and cytoplasmic abundance in response to UVC. ProTα-mediated survival was reduced when its translation and cytoplasmic accumulation were diminished in cells that either expressed reduced HuR (Figures 5–7) or had been treated with oligomers that blocked ProTα translation (Figure 7C and D; Sburlati *et al*, 1991). The ProTα-elicited protection might have been more robust if ProTα had been used instead of EGFP-ProTα, although the chimeric protein does appear to retain functional characteristics of the endogenous protein (Rubtsov *et al*, 1997; Sukhacheva *et al*, 2002; Karetsou *et al*, 2004). Moreover, the antiapoptotic effects of HuR relied on the enhanced

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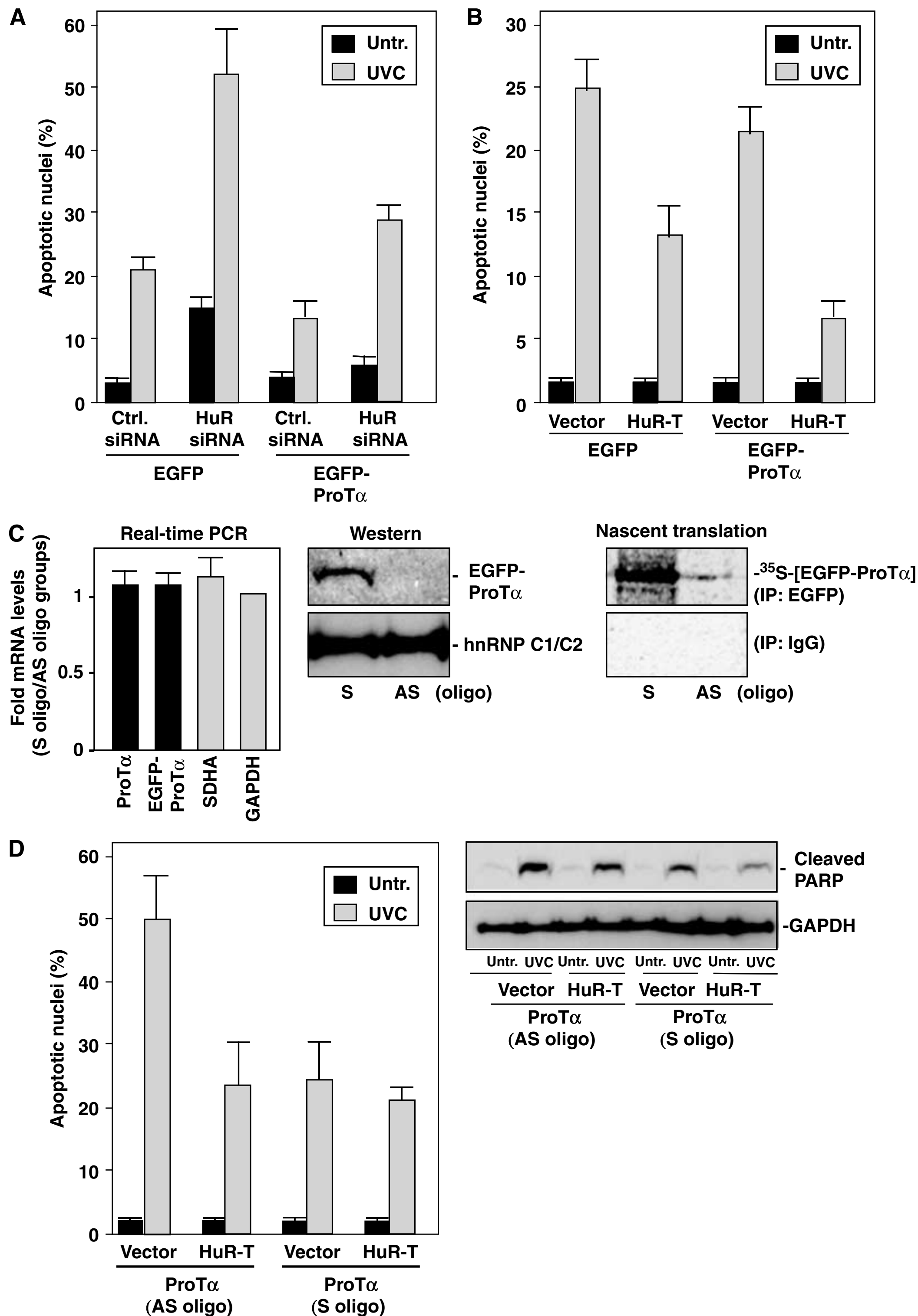

Figure 7 HuR- and ProTα-engendered protection against UVC are mutually dependent. (**A**) HeLa populations that expressed either normal or reduced HuR levels (Ctrl. siRNA and HuR siRNA, respectively, described in the legend of Figure 1) were transfected with plasmids that expressed either chimeric ProTα (EGFP-ProTα) or EGFP (as explained in the legend of Figure 6) and irradiated with UVC; apoptotic nuclei were scored 8 h later. (**B**) Cells that either overexpressed HuR or expressed normal HuR levels (HuR-T and Vector, respectively, described in the legend of Figure 2) were transfected with plasmids that expressed chimeric ProTα (EGFP-ProTα) or EGFP (EGFP) (Figure 6), and apoptotic nuclei were scored 12 h after UVC irradiation. (**C**) HeLa cells were incubated either with an oligomer that inhibited ProTα translation (AS oligo) or with a control oligomer (S oligo) (Sburlati *et al*, 1991), whereupon the levels of ProTα expression were assessed by monitoring mRNA levels (Real-time PCR), protein abundance (Western), and protein biosynthesis (Nascent translation). (**D**) The cell populations described in panel B were incubated with either AS oligo or with S oligo; 8 h after UVC irradiation, the percentage of apoptotic nuclei were quantified (left), and protein lysates were prepared to assess the degree of PARP cleavage by Western blotting (right). GAPDH signals revealed even loading and transfer of samples; Western data are representative of three independent experiments. Graphs represent the means±s.e.m. from three independent experiments.

translation of ProTα, since interventions to decrease ProTα production abrogated the prosurvival effects of HuR (Figure 7).

HuR and cancer

In order to become malignant, cancer cells must acquire a number of traits, including proliferation without growth signals, insensitivity to growth inhibitory signals, avoidance of replicative senescence, evasion of apoptosis, tissue invasion and metastasis, maintenance of angiogenesis, and evasion of antitumor immune response (Hanahan and Weinberg, 2000; Dunn *et al*, 2004). HuR levels are elevated in cancer (Audic and Hartley, 2004) and, interestingly, it has been proposed to regulate genes critical to the development of each of the aforementioned traits. It can help cells attain the ability to proliferate without external growth signals through its positive influence on the expression of growth factors such as EGF (Sheflin *et al*, 2004); it can assist cells in eluding growth inhibitory signals and avoiding replicative senescence by promoting the expression of proliferative and proto-oncogenic factors such as c-myc, c-fos, cyclin A, cyclin B, and cyclin D1 (Ma *et al*, 1996; Wang *et al*, 2000b; Wang *et al*, 2001); it can augment the cell's ability to invade and metastasize by elevating the expression of target mRNAs encoding matrix metalloproteases such as MMP-9 (Akool *et al*, 2003) and metastasis-associated protein 1 (MTA1, López de Silanes *et al*, 2004); it can promote angiogenesis through its ability to bind to the HIF-1 and VEGF mRNAs and enhance its expression (Levy *et al*, 1998, Sheflin *et al*, 2004); and finally, by regulating mRNAs that encode the immunosuppressive cytokine TGF-β and the T-cell inhibitor galectin-1 (Nabors *et al*, 2001; López de Silanes *et al*, 2004), HuR can help the tumor evade immune recognition, another common adaptive mechanism in malignancy.

The finding that HuR regulates ProTα expression reported in the present study strongly supports the notion that HuR can actively enable cancer cells to evade apoptosis. Together with its ability to enhance the expression of genes critical to the other biological traits of malignancy, we hypothesize that HuR plays a central, multidirectional role in the path to cancer development. Substantiating this concept are reports indicating that HuR expression was universally elevated in cancers derived from a wide range of tissues examined (Blaxall *et al*, 2000; Erkinheimo *et al*, 2003; López de Silanes *et al*, 2003). Indeed, the HuR family of proteins was initially identified as specific tumor antigens present in individuals with paraneoplastic neurological disorder, providing the first indication that they could have a cancer-regulatory function (Dalmau *et al*, 1990; Szabo *et al*, 1991). Furthermore, the human HuR gene has been localized to chromosome 19p13.2, a locus that is associated with a number of translocations and oncogenic gains in human tumors (Larramendy *et al*, 1997; Ma and Furneaux 1997; Mao *et al*, 2002). Investigation of the tumorigenic potential of cells expressing varying levels of HuR using nude mice revealed that heightened HuR levels led to the development of larger and faster-growing tumors, while low HuR-expressing cells gave rise to significantly smaller and slow-developing tumors (López de Silanes *et al*, 2003). An assessment of whether such a pivotal function for HuR in colon carcinogenesis can be extended to cancer growth arising from other tissues is underway.

In summary, we propose that HuR exerts an antiapoptotic function through mechanisms that rely on binding the ProTα mRNA, elevating its cytoplasmic levels, and enhancing the translation of the encoded prosurvival protein. In light of the regulatory paradigm presented here, a reassessment of HuR's impact on the cell's response to immune, proliferative, differentiation, and stressful stimuli is warranted, as we seek a more complete understanding of the post-transcriptional events contributing to the maintenance of cellular homeostasis.

Materials and methods

Cell culture, treatment, RNA interference, and scoring of apoptotic nuclei

Human cervical carcinoma HeLa cells were cultured in Dulbecco's modified essential medium (Gibco-BRL) supplemented with 10% fetal bovine serum and antibiotics. Unless otherwise indicated, irradiated cells received 30 J/m^2 UVC. For HuR RNAi analysis, cells were transfected with 20 nM of siRNAs in medium containing 3% fetal bovine serum described under 'Supplementary data'. At 48 h after transfection, cells were treated with UVC, allowed to recover for the times indicated, and then either collected for analysis or stained with Hoechst 33342 (1 μg/ml, 30 min) to visualize nuclei. To score apoptotic nuclei, 500 cells were counted from duplicate plates; experiments were performed three times independently.

Plasmids and transient transfections

Cloning of the coding region and 3′UTR of ProTα downstream of the EGFP coding region in plasmid pEGFP-C1 (BD Biosciences), and cloning of the coding region of HuR upstream of TAP (pcDNA3-TAP, a kind gift from C-Y Chen, (Chen *et al*, 2001)) are described as 'Supplementary data'. Plasmids pEGFP-ProTα and pHuRT (8 μg each when used separately, 4 μg each when used jointly) were transfected using Lipofectamine 2000; 48 h after transfection cells received 30 J/m^2 UVC, and were analyzed at varying times afterwards.

Cell fractionation

For the preparation of cytosolic fractions, $\sim 5 \times 10^6$ cells were scraped in 400 μl of lysis buffer (10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 2.5 mM $MgCl_2$, and 40 μg/ml digitonin). The lysate was incubated in ice for 5 min and centrifuged (2000 *g*, 8 min), and the supernatant was designated as the soluble cytosolic fraction. Whole-cell lysates were prepared using RIPA buffer as described (Lal *et al*, 2004). To monitor ProTα mRNA levels in the cytoplasm and the nucleus, cytoplasmic extracts were made using digitonin (described above), and nuclear extracts were prepared by resuspending the resulting nuclear pellet in RIPA buffer and lysing by mild sonication. RNA was isolated from these fractions using the Trizol reagent (Invitrogen).

Linear sucrose gradient fractionation was performed as described (Lal *et al*, 2004; Supplementary data). For Western blot analysis, SDS–PAGE sample buffer was added to an aliquot of each fraction. For Northern blotting and RT–PCR, RNA was isolated from 500 μl of each fraction using 3 ml Trizol.

Detection of RNA and protein

RNA was isolated using the Trizol reagent and Northern blot analysis to detect mRNAs encoding p21 and cyclin D1, as well as to detect 18S rRNA was performed as previously described (Wang *et al*, 2000a), using excess oligomer probes in each case. For the detection of ProTα transcripts, a ProTα PCR product was labeled using random primers, [α-^{32}P]dATP, and Klenow enzyme.

For Western blot analysis, proteins were resolved by 12% SDS–PAGE and transferred onto PVDF membranes (Invitrogen). Commercial antibodies are described (Supplementary data); a monoclonal anti-hnRNP A1 antibody was a generous gift from Dr G Dreyfuss. Following incubation with appropriate secondary antibodies, signals were detected by enhanced chemiluminescence. Endogenous ProTα was isolated from whole-cell lysates by phenol:chlorofom (1:1) extraction followed by SDS–PAGE and Coomassie blue staining of the polyacrylamide gel (Evstafieva *et al*, 2003).

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The RNA isolated from either IP material or from pooled polysomal fractions was reverse-transcribed using random hexamers and SSII RT (Invitrogen), and the resulting cDNA amplified by PCR using gene-specific primer pairs for 25–30 cycles (oligomer sequences and PCR conditions described as Supplementary data).

Binding assays

IP of ribonucleoprotein complexes was previously described (Lal *et al*, 2004; Supplementary data). *In vitro* transcription and biotin pulldown assays were described previously (Lal *et al*, 2004) except that whole-cell lysates (40 μg) and equimolar transcript concentrations (16.8 pmol per reaction) were used here. Primers used to prepare templates for *in vitro* transcription are listed (Supplementary data).

Analysis of nascent protein

Newly translated EGFP or EGFP-ProTα proteins were measured by incubating 5×10^6 cells with 1 mCi L-[^{35}S]methionine and L-[^{35}S]cysteine (Easy Tag ™EXPRESS, NEN/Perkin Elmer) per 60-mm plate for 20 min, whereupon cells were lysed using TSD lysis buffer (50 mM Tris (pH 7.5), 1% SDS, and 5 mM DTT) and EGFP or EGFP-ProTα were immunoprecipitated using polyclonal anti-GFP antibody for 18 h at 4°C; IgG was used in control IP reactions. Beads were washed in TNN buffer (50 mM Tris (pH 7.5), 250 mM NaCl, 5 mM EDTA, 0.5% NP-40) and IP material was resolved by 12% SDS–PAGE, transferred onto PVDF membranes, and visualized using a PhosphorImager.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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