Caspase-mediated cleavage of HuR in the cytoplasm contributes to pp32/PHAP-I regulation of apoptosis

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The RNA-binding protein HuR affects cell fate by regulating the stability and/or the translation of messenger RNAs that encode cell stress response proteins. In this study, we delineate a novel regulatory mechanism by which HuR contributes to stress-induced cell death. Upon lethal stress, HuR translocates into the cytoplasm by a mechanism involving its association with the apoptosome activator pp32/PHAP-I. Depleting the expression of pp32/PHAP-I by RNA interference reduces both HuR cytoplasmic accumulation and the efficiency of caspase activation. In the cytoplasm, HuR undergoes caspase-mediated cleavage at aspartate 226. This cleavage activity is significantly reduced in the absence of pp32/PHAP-I. Substituting aspartate 226 with an alanine creates a noncleavable isoform of HuR that, when overexpressed, maintains its association with pp32/PHAP-I and delays the apoptotic response. Thus, we propose a model in which HuR association with pp32/PHAP-I and its caspase-mediated cleavage constitutes a regulatory step that contributes to an amplified apoptotic response.

Introduction

In response to different assaults, cells initially activate survival pathways that prevent protein damage and facilitate recovery. If the stress is unsustainable, cell death is induced (Beere, 2004). Apoptosis, a major form of cell death, eliminates damaged cells when triggered by external and physiological stimuli. The main effectors of apoptosis are a set of proteases called caspases (cysteinyl and aspartate-specific proteases; Beere, 2004). Initiator caspases (2, 8, 9, and 10) are activated by upstream events and stimulate the activity of executioner caspases (3, 6, and 7), which promote apoptosis through the cleavage of protein substrates (Green, 2005). Although a study has suggested that caspase-3 and -7 show a high degree of homology and have redundant activities (Kuribayashi et al., 2006), recent in vivo experiments demonstrated that both are independently required for mitochondria-mediated apoptosis (Lakhani et al., 2006). The activation of caspase-9 occurs by a conformational change within a macromolecular complex that is assembled by a scaffolding molecule, Apaf-1, in response to a specific ligand, cytochrome *c* (cyt *c*; Zou et al., 1997). Cyt *c* induces the heptamerization of Apaf-1 followed by the recruitment of procaspase-9. This complex is known as the apoptosome and promotes the activation of procaspase-9, which processes caspase-3 and -7 to induce protein catabolism and apoptosis.

It is well established that the expression of several pro- and antiapoptotic proteins is in part regulated posttranscriptionally (Dimri et al., 1995; Lowe and Lin, 2000; Ikeguchi et al., 2002; Wang et al., 2003). Indeed, in response to some stresses, elevated protein levels of $p21^{Waf1}$, p53, and bcl-2 are observed, which cannot entirely be explained by an increase in their rate of transcription (Roninson, 2002; Yamasaki, 2003). Recent observations implicate the RNA-binding protein HuR in stress-induced apoptosis. For example, UV light treatment induces the stability of $p21^{Waf1}$ mRNA as well as the translation of p53 and the antiapoptotic factor prothymosin α (proTα) by stimulating the association of HuR with these messages (Wang et al., 2000;

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Abbreviations used in this paper: AMC, 7-amino-4-methylcoumarin; CP, cleavage product; cyt *c*, cytochrome *c*; HNS, HuR nucleocytoplasmic shuttling; HS, heat shock; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; RRM, RNA recognition motif; STS, staurosporine; wt, wild type.

The online version of this article contains supplemental material.

The Journal of Cell Biology, Vol. 180, No. 1, January 14, 2008 113–127
http://www.jcb.org/cgi/doi/10.1083/jcb.200709030

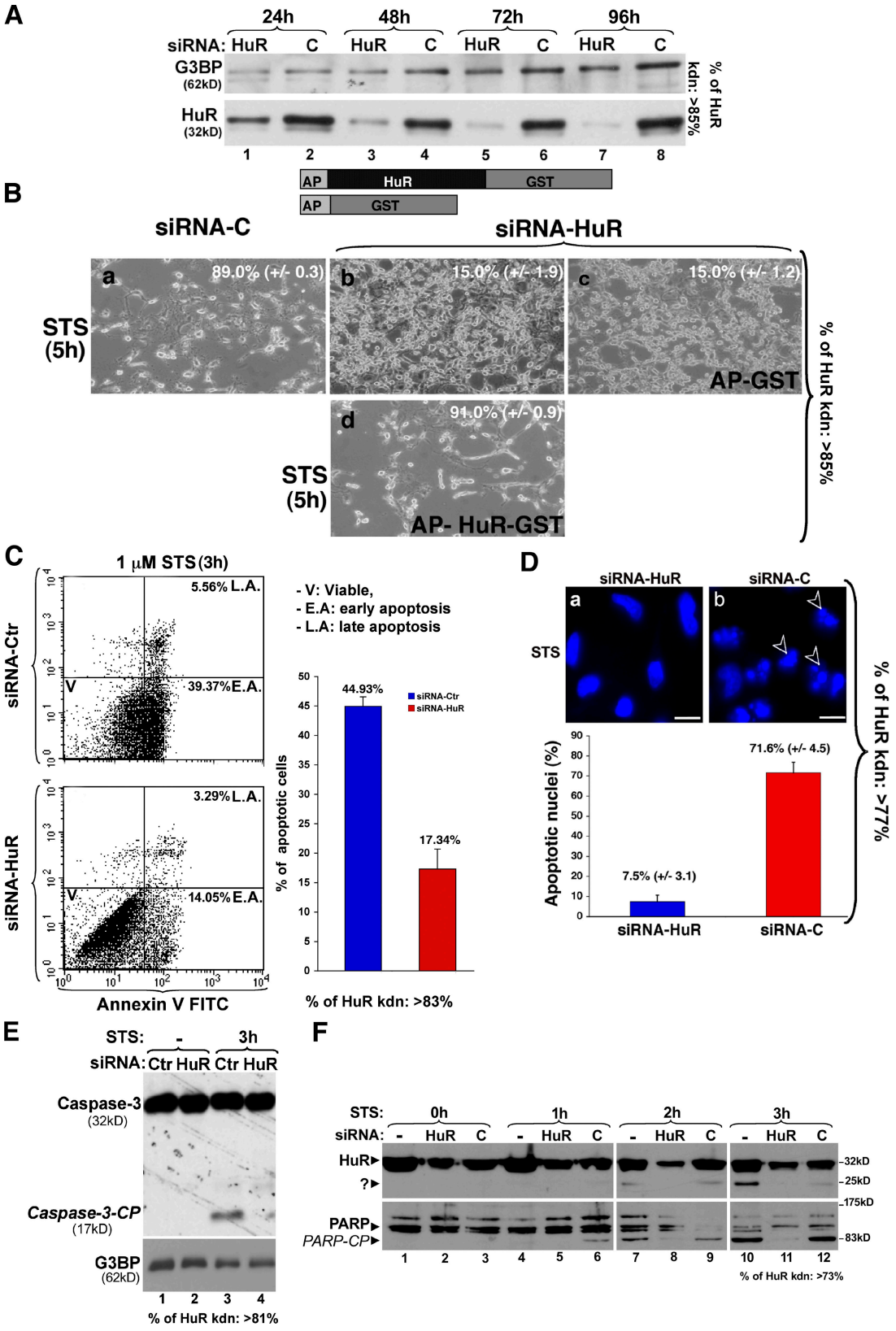

Figure 1. **HuR protein promotes cell death upon lethal stress.** (A) Knockdown of HuR expression by RNAi in HeLa cells. HeLa cells were transfected with RNAi duplexes against HuR mRNA (siRNA-HuR) or control (siRNA-C) and harvested every 24 h for 96 h. Western blot analyses were performed using 3 μg of total cell extract. The blot was probed with antibodies against HuR and G3BP (loading control). (B) AP–HuR-GST rescues the normal levels of cell death upon STS treatment. (top) Schematic representation of the constructs used to rescue siRNA-treated cells. HuR-GST and GST alone were conjugated to AP (antennapedia) peptide. (bottom) HeLa cells depleted (panels b–d) or not depleted (panel a) of HuR were incubated for 5 h with 1 μM STS. 16 h before stress, the cells were also incubated with 50 nM AP–HuR-GST (panel d) or AP-GST (panel c). The percentage of dead cells was quantified by subtracting the

Mazan-Mamczarz et al., 2003; Lal et al., 2005). HuR is a well-characterized, ubiquitously expressed posttranscriptional regulator belonging to the embryonic lethal abnormal vision phenotype/Hu family (in flies; Robinow et al., 1988). HuR has three highly conserved motifs belonging to the RNA recognition motif (RRM) superfamily and a hinge region between RRMs 2 and 3 named the HuR nucleocytoplasmic shuttling (HNS) domain (Brennan and Steitz, 2001). It has been shown that the HNS domain regulates the localization of HuR by mediating its association with adaptor proteins for nuclear export such as pp32/PHAP-I and APRIL (Brennan et al., 2000; Fan et al., 2003b; Jiang et al., 2003) and with import factors transportin-1, -2, and importin α (Rebane et al., 2004; Wang et al., 2004; van der Giessen and Gallouzi, 2007). Under normal conditions, transportin-2 regulates the nuclear import of HuR, whereas pp32/PHAP-I and APRIL are involved in its export (Gallouzi and Steitz, 2001; Rebane et al., 2004). Recently, it has been demonstrated that pp32/PHAP-I and SETα are implicated in both caspase-dependent and -independent cell death. SETα appears to have an antiapoptotic role in blocking caspase-independent cell death (Fan et al., 2003a), whereas pp32/PHAP-I acts as a proapoptotic factor by stimulating the activity of the apoptosome complex (Jiang et al., 2003). SETα and pp32/PHAP-I contain highly acidic domains through which they associate with the HNS-RRM3 motifs of the HuR protein (Brennan et al., 2000). This observation and the fact that, like HuR, pp32/PHAP-I shuttles between the nucleus and the cytoplasm (Brennan et al., 2000) raise the possibility that HuR also regulates pro- or antiapoptotic signaling pathways through protein–protein interactions. Although different stresses trigger the accumulation of HuR in the cytoplasm via a mechanism involving both pp32/PHAP-I and APRIL (Gallouzi et al., 2001; Higashino et al., 2005), the functional relevance of HuR association with its protein ligands in deciding cell fate is still elusive.

It has been observed that despite their ability to induce cell death, stresses like UV, proteasome inhibition, and amino acid starvation do not significantly affect the expression of HuR. Rather, they induce its partial cytoplasmic accumulation (Wang et al., 2000; Mazan-Mamczarz et al., 2003; Lal et al., 2005; Mazroui et al., 2007). Therefore, the role of HuR in cell death may be the result of changes in its cellular localization, which is mediated by protein partners. In this study, we address this, showing that HuR enhances caspase-mediated apoptosis by a novel regulatory mechanism by presenting evidence that links HuR cleavage via a specific caspase to amplification of the apoptotic response.

Results

RNAi-mediated HuR depletion delays staurosporine-induced cell death

To investigate the role of HuR in stress response, we depleted its expression in HeLa cells using RNAi. Subsequently, these cells were treated with lethal doses of several stresses such as heat shock (HS; Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200709030/DC1; Gallouzi et al., 2000), staurosporine (STS; a potent apoptotic inducer; Fig. 1; Desagher et al., 1999), or proteasome inhibitor (MG132; Fig. S3; Mazroui et al., 2007). The siRNA duplex against HuR message (siRNA-HuR; van der Giessen et al., 2003) but not the control siRNA (siRNA-C) efficiently depleted HuR protein levels (>85%; Fig. 1 A). The percentage of death induced by 1 μM STS for 5 h was significantly lower (15%) in siRNA-HuR–treated cells compared with cells treated with siRNA-C (89%; Fig. 1 B). To test whether the decrease of cell death in HuR-depleted cells was associated with a reduction in apoptosis, we quantified the percentage of annexin V/propidium iodide (PI)–positive cells using flow cytometry (Martin et al., 1995). Annexin V binds to externalized phosphatidylserines at the surface of apoptotic cells. As shown in Fig. 1 C, the depletion of HuR resulted in a significant decrease in the number of annexin V–positive cells (from 44.9 to 17.3%; Fig. 1 C, compare siRNA-Ctr and siRNA-HuR dot plots and bar graph). The reduced levels of STS-mediated apoptosis were confirmed by the significant reduction in the number of condensed and fragmented nuclei in STS-treated HeLa cells depleted of HuR (7.5%) compared with the mock-depleted cells (71.6%; Fig. 1 D). Similar results were obtained with other stresses such as HS (Fig. S1) or MG132 (Fig. S3). Upon prolonged treatments with either 1 μM STS (>10 h), HS (>3 h at 45°C), or 10 μM MG132 (>36 h), HuR-depleted cells undergo death (unpublished data); thus, we concluded that depleting HuR significantly delays but does not prevent apoptosis.

To ensure that the observed delay in apoptosis was a direct consequence of the depletion of HuR, we performed rescue experiments using the cell-permeable peptide AP conjugated to either HuR-GST or to GST alone (Fig. S1 B; Gallouzi and Steitz, 2001). HuR knockdown cells were efficiently rescued from STS-induced cell death (>90%) with the addition of 50 nM AP–HuR-GST but not AP-GST (Fig. 1 B, b–d). In these experiments, 50 nM AP-conjugated proteins was not toxic to cells growing under normal conditions (Fig. S2 A, available at

number of adherent cells that remained upon STS treatment from the total number of cells before stress. Representative phase-contrast fields are shown. For each panel, five randomly chosen fields of 300 cells were counted before and after STS treatment. Values are ±SEM from five independent experiments. (C) HeLa cells treated with siRNA as described in A in the presence of 1 μM STS for 3 h were analyzed by staining with annexin V–FITC and PI and by flow cytometry. The percentage of apoptotic cells (right boxes) upon STS treatment was determined for HuR (siRNA-HuR) or control siRNA (siRNA-Ctr)-treated HeLa cells. The values were normalized to control untreated cells. (D, top) HeLa cells depleted of HuR were treated with 1 μM STS for 3 h. Cells were fixed, and nuclei were stained with DAPI to view the presence of condensed and fragmented nuclei. (bottom) The graph represents the number of apoptotic cells after treatment as described in E. For each bar, five randomly chosen fields of 300 nuclei were counted before and after STS treatment. The arrowheads point to apoptotic cells. (C and D) Values are the means ± SEM (error bars) from three independent experiments. (E) The cleavage of caspase-3 upon STS treatment is significantly reduced in the absence of HuR. siRNA-HuR– and C–treated cells 3 d after transfection were treated with 1 μM STS for 3 h. 25 μg of total cell extract was used for Western blotting, and the membrane was probed for caspase-3 and G3BP. The molecular mass of the caspase-3 CP (caspase-3–CP) is ∼17 kD, whereas the precursor is 35 kD. (F) STS-induced PARP cleavage depends on HuR protein. HeLa cells depleted or not depleted of HuR expression were treated with 1 μM STS for the indicated times. Western blot analyses were performed using 25 μg of total cell extract. The blot was probed with antibodies against HuR and PARP. Representative results of three independent experiments are shown. The percentage of HuR knockdown (kdn) for each panel is indicated. Bars, 20 μm.

http://www.jcb.org/cgi/content/full/jcb.200709030/DC1). Furthermore, as previously shown in HL-60 (Xu et al., 2005) and in C2C12 cells (van der Giessen et al., 2003), we observed that the HeLa cells used in our experiments efficiently took up the same amount of both AP–HuR-GST and AP-GST (>85%; Fig. S2, B and C).

To define the levels at which HuR affects apoptosis, we examined the processing of caspase-3 by Western blot analysis in cells expressing or not expressing HuR. Caspase-3 is one of the main executioner caspases that is processed by the apoptosome complex to generate a cleavage product (CP; caspase-3–CP) that represents the active form of the enzyme (Zou et al., 1997). HeLa cells treated with siRNA-HuR or siRNA-C were incubated for 3 h with 1 μM STS. Total extracts were then prepared and used for Western blot analysis. We observed that in HuR-depleted cells, the cleavage of caspase-3 was significantly reduced compared with the control (Fig. 1 E, lanes 3 and 4). We also examined the processing of a well-characterized caspase-3 target, poly(ADP-ribose) polymerase (PARP; a DNA repair enzyme; Janicke et al., 1998). We observed that PARP cleavage was impaired in HeLa cells lacking HuR and treated with 1 μM STS for various periods of time (Fig. 1 F, bottom; lanes 7–12). Our Western blot analysis using the anti-HuR antibody detected the appearance of a 24-kD fragment of HuR only in control cells 2 h after STS treatment and not in HuR-depleted cells (Fig. 1 F, top; lanes 7–12). This suggested that HuR itself could be a target for cleavage during caspase-mediated apoptosis. Thus, we investigated the link between the effect of HuR on apoptosis and the appearance of this 24-kD fragment.

Both caspase-7 and -3 are required for the cleavage of HuR at the $MGVD_{226}$ site located in the HNS domain

To observe this 24-kD fragment of HuR by Western blot analysis, at least 25 μg of cell extract was required. Because this quantity is 10-fold higher than normally used to detect full-length HuR, it is likely that only a small portion of the protein is cleaved to generate this stress-induced fragment. To define whether the appearance of this fragment correlates with caspase activity, we prepared total extracts from HeLa cells treated or not treated with 1 μM STS for 3 h in the absence or presence of the pancaspase inhibitor zVAD-FMK (Chipuk and Green, 2005). We observed that adding zVAD to STS-treated HeLa cells led to a significant reduction (>∼75%) in the intensity of the 24-kD band (HuR-CP; Fig. 2 A). Thus, we concluded that this fragment could be a caspase-generated CP of HuR. To determine which caspase was responsible for this cleavage activity, we first performed an in vitro cleavage assay using in vitro synthesized [^{35}S]methionine-labeled HuR and a set of recombinant human caspases. Despite the fact that both caspase-7 and -3 cleaved the ^{35}S-labeled PARP (Fig. 2 C), only caspase-7 cleaved HuR (Fig. 2 B, compare lane 6 with lanes 1–5 and 7–9). Even when the concentrations of other caspases were increased twofold, no HuR cleavage was seen (unpublished data). Caspase-3 and -7 have been shown in many cell systems to have a functional redundancy or to collaborate to cleave their target proteins (Lakhani et al., 2006). To investigate whether these two caspases cooperate to cause HuR cleavage in vivo, we depleted their expression in HeLa cells using specific siRNA duplexes. These cells were then treated with 1 μM STS for various periods of time and harvested to prepare total cell extracts. Western blot analysis using the anti-HuR or anti–caspase-3 or -7 antibodies demonstrated that the depletion of caspase-3 or -7 significantly reduced STS-mediated HuR cleavage (Fig. 2 D, lanes 5, 6, 8, and 9). Additionally, we observed that in siRNA–caspase-7–treated cells, the processing of caspase-3 was significantly reduced (Fig. 2 D, lanes 6 and 9). Likewise, the cleavage of caspase-7 was reduced in caspase-3 knockdown cells. These results suggested that in vivo, both caspase-7 and -3 are needed for the cleavage of HuR.

The epitope recognized by the monoclonal anti-HuR antibody (3A2) is located in RRM1 of HuR (Gallouzi et al., 2000). Therefore, our experiment showing that 3A2 also specifically recognized the 24-kD fragment (Figs. 1 F and 2 A) is an indication that the C-terminal region of HuR is likely to harbor the caspase-dependent cleavage site. To test this possibility, we prepared several deletion mutants of the HuR protein that were N-terminally tagged with GFP (Fig. 3 A). HeLa cells overexpressing equal amounts of these mutants (Fig. 3 B, lanes 1–4) were treated with 1 μM STS for 3 h (Fig. 3 B, lanes 5–8) and harvested to prepare total cell extracts. Western blot analyses using antibodies against GFP (Fig. 3 B) showed that STS treatment generated a CP with GFP-HuRΔRRM1-RRM2 (Fig. 3 B, lane 6) and GFP-wt-HuR (Fig. 3 B, lane 8) but not with the other mutants. Furthermore, as with endogenous HuR (Figs. 1 F and 2 A), the CPs are ∼9 kD smaller than the precursor proteins (Fig. 3 B, lanes 6 and 8). Because the calculated molecular mass of the HNS-RRM3 domain is 13.6 kD, these results support the existence of a caspase cleavage site within or close to the HNS motif of HuR (Fig. 3, A and B).

Caspases recognize a four–amino acid sequence in their targets and cleave at an aspartic acid residue (D) located at the P1 position with respect to the scissile bond. There are three potential caspase cleavage sites in the HNS-RRM3 motif: $MGVD_{226}$ and $QD_{254}AD_{256}$ (Fig. 3 A). The D residues in each of these sites were individually mutated to alanine (A), and the resulting constructs were transfected into HeLa cells (Fig. 3 C, lanes 1–5). The following day, apoptosis was induced by 1 μM STS for 3 h, and the appearance of HuR-CP was monitored by immunoblotting. Only the HuR $MGVD_{226}A$ ($HuRD_{226}A$) mutant was completely resistant to cleavage (Fig. 3 C, lane 8). In contrast, cleavage of the HuR mutants $HuRD_{254}A$ and $HuRD_{256}A$ (Fig. 3 C, lanes 9 and 10) were unaffected or only partially inhibited, respectively, suggesting that $MGVD_{226}$ constitutes the principal caspase-7/-3 cleavage site in HuR. Notably, overexpressing HuR appeared to cause some cleavage of wild-type (wt) GFP-HuR (Fig. 3, B [lane 4] and C [lane 1]). This cleavage is most likely the result of background apoptosis caused by transfection with HuR. In the absence of STS, the deletion mutants HuR-ΔHNS-RRM3 and HuR-ΔRRM1–RRM2 (which approximate the two products generated by the cleavage of HuR) also induced cell death by 17% and 28%, respectively (Fig. 3 D). These data established a direct correlation between HuR cleavage and caspase-mediated apoptosis.

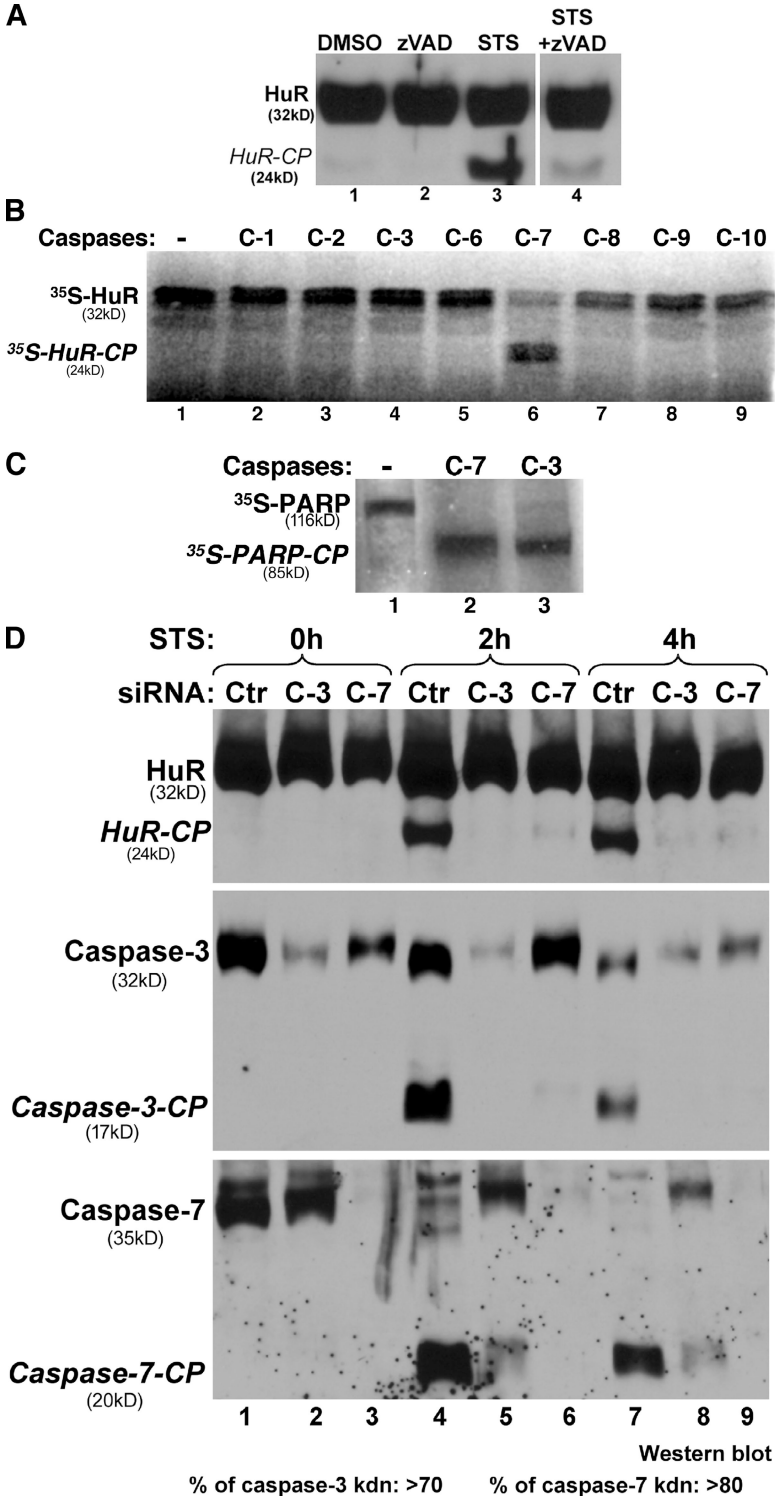

Figure 2. **Although only caspase-7 cleaves HuR in vitro, both caspase-3 and -7 are needed for the cleavage of HuR in HeLa cells.** (A) Active caspases are required for the appearance of the 24-kD fragment. Cells were incubated with DMSO (lane 1), zVAD (lane 2), STS (lane 3), or with both zVAD and STS (lane 4). Western blotting for HuR was performed as described in Fig. 1 A. (B and C) In vitro caspase cleavage assay. (B) [^{35}S]methionine-labeled HuR was incubated for 3 h at 37°C with 5 U of purified recombinant caspases (C-1–10). Samples were applied to SDS-polyacrylamide gels, and HuR cleavage was visualized by autoradiography. (C) [^{35}S]methionine-labeled PARP was incubated for 3 h at 37°C with 5 U of purified recombinant caspase-7 and -3. Samples were analyzed as described in B. (D) The effect of caspase-7 and -3 knockdown on HuR cleavage. HeLa cells were transfected with RNAi duplexes against caspase-7 (siRNA–C-7) or caspase-3 (siRNA–C-3) mRNA as well as control siRNA (siRNA-Ctr). 48 h later, they were treated with 1 μM STS for 3 h and were harvested to prepare total cell extracts. Western blot analyses were performed using antibodies against HuR, caspase-7, and caspase-3 proteins. The percentage of caspase-7 and -3 knockdown (% kdn) is indicated. (A–D) Representative results of three independent experiments for each panel are shown.

HuR accumulates in the cytoplasm in a pp32/PHAP-I–dependent manner in response to STS

The aforementioned experiments indicated that under apoptotic conditions, HuR is cleaved at the D_{226} residue located in its HNS domain. It has been shown that under stresses such as HS and UV, HuR accumulates partially in the cytoplasm (Gallouzi et al., 2000; Wang et al., 2000). Because the HNS region mediates the nucleocytoplasmic trafficking of HuR (Fan and Steitz, 1998), we tested the link between this cellular relocalization and the caspase-mediated cleavage of HuR. HeLa cells overexpressing equal amounts of GFP-HuR and -HuRD_{226}A (Fig. 3 C, lanes 1 and 3) were treated with 1 μM STS for 3 h and fixed. We observed that both proteins translocated into the cytoplasm upon STS treatment (Fig. 4 A), indicating that the D_{226}A mutation does not affect the cellular movement of HuR. We were

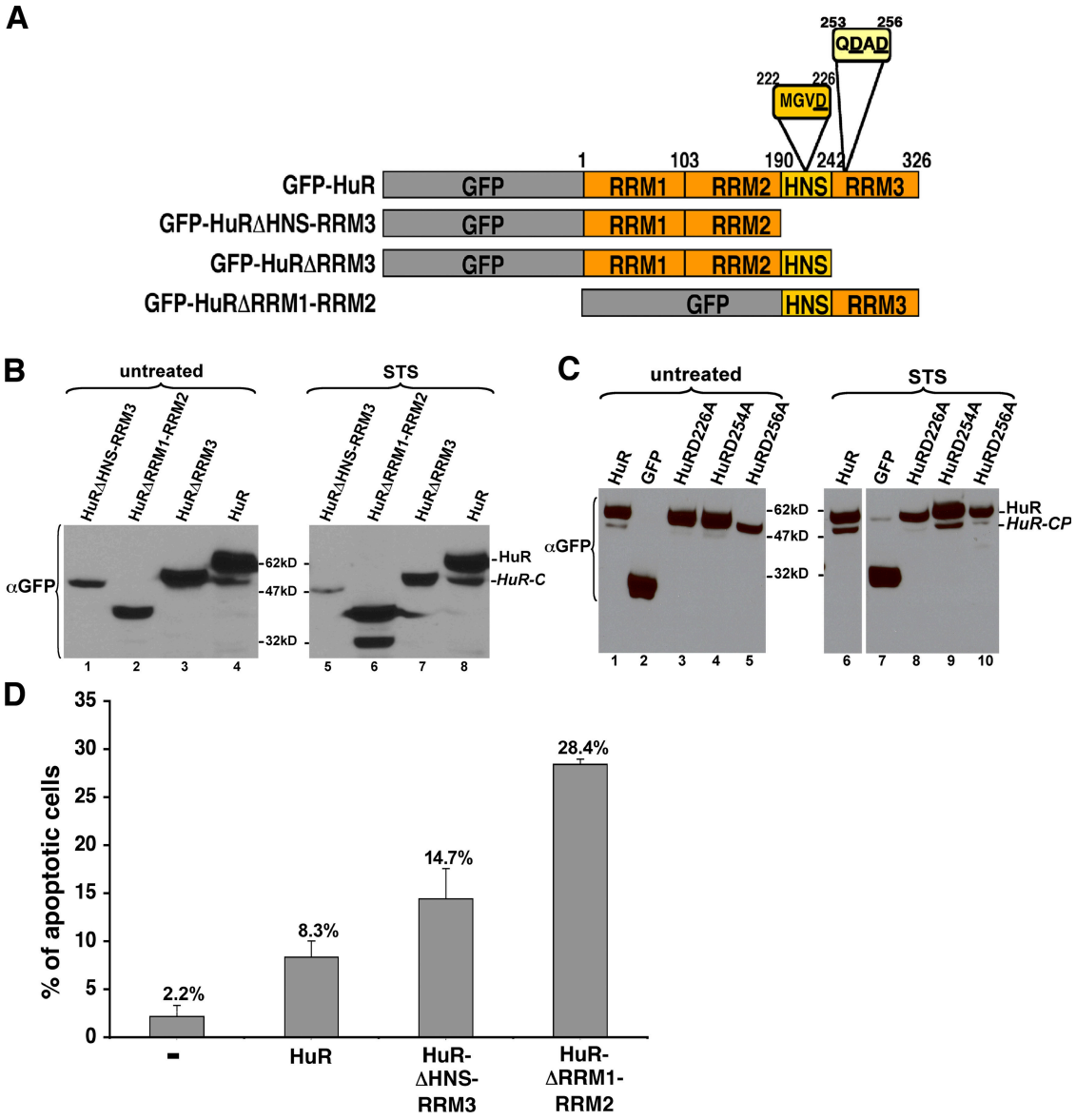

Figure 3. **Substituting the amino acid residue aspartate 226 with alanine protects HuR from caspase-mediated cleavage.** (A) Schematic diagram of the structure of full-length HuR fused to GFP as well as the deletion and point mutants. The positions of the Asp (D) mutated to Ala (A) are underlined. The functional domains of HuR are shown. Numbers refer to the amino acid sequences of the protein. (B) HuR is cleaved in its HNS domain. GFP-HuR and its truncated forms were expressed in HeLa cells. Subsequently, the cells were treated or not treated with 1 μM STS for 3 h. Immunoblot analysis was performed using anti-GFP antibodies. (C) The D_{226} residue is required for HuR cleavage. GFP, GFP-HuR, and its point mutation derivatives were produced in HeLa cells. Subsequently, the cells were treated with 1 μM STS for 3 h (right) or were left untreated (left). GFP and GFP-HuR proteins were detected by immunoblotting using anti-GFP antibodies. The white line indicates that intervening lanes have been spliced out. (D) Overexpression of the HuR CPs enhances cell death. HeLa cells grown under normal conditions were mock transfected (−) or transfected with GFP-HuR, GFP-HuR-ΔHNS-RRM3, or GFP-HuRΔRRM2–RRM3. 48 h after transfection, cells were fixed and DAPI stained. Percent death was calculated by counting the number of fragmented nuclei and expressing this number as a percentage of the total number of cells in a given field. Data are represented as means of three experiments ± SEM (error bars).

surprised to observe that HuR showed a diffused cytoplasmic staining upon STS treatment rather than being localized to stress granules (stress-induced cytoplasmic foci; Anderson and Kedersha, 2006), as was shown with other stresses (Gallouzi et al., 2000). To verify whether endogenous HuR behaves similarly to GFP-wt-HuR, HeLa cells were treated with 1 μM STS for various periods of time, and immunofluorescence experiments were performed using the anti-HuR antibody. As with GFP-HuR, STS treatment induced a rapid appearance of endogenous HuR into the cytoplasm (Fig. 4 B). The cytoplasmic translocation of HuR was detectable as early as 1 h after STS treatment, conditions under which cell shrinkage and caspase activities were not visible (Figs. 1 F [lanes 4 and 6] and 4 B [panel 6]). This observation was further supported by biochemical fractionation experiments. Nuclear and cytoplasmic fractions were obtained from HeLa cells treated or not treated with 1 μM STS for 3 h, and Western blot analysis showed that the levels of HuR increase in the cytoplasm upon STS treatment (Fig. 4 C, lanes 5 and 6). We also observed that the 24-kD fragment was detected only in the cytoplasmic fraction (Fig. 4 C, lanes 4 and 6). These results, together with the fact that zVAD did not prevent the STS-induced translocation of HuR in the cytoplasm (Fig. S4, available

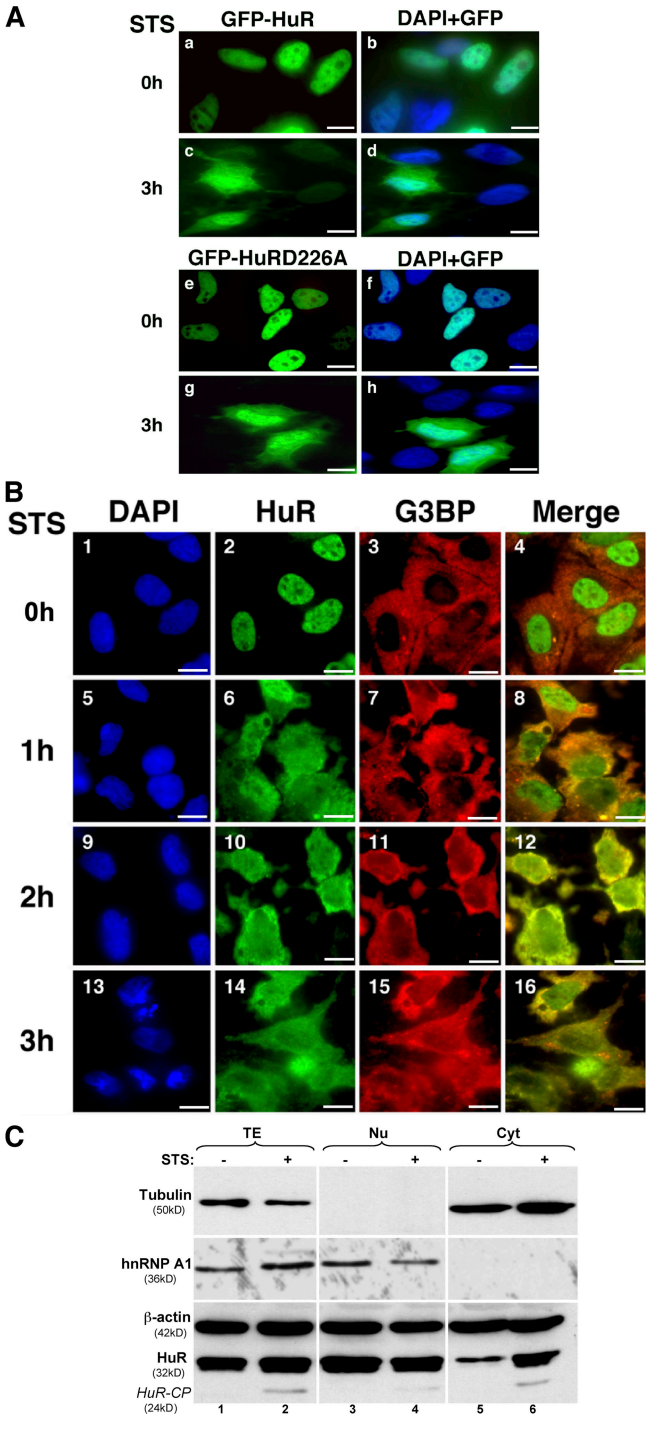

Figure 4. **The caspase-mediated cleavage of HuR is a cytoplasmic event.** (A) Like wt GFP-HuR, GFP-HuRD$_{226}$A accumulates in the cytoplasm upon STS treatment. HeLa cells were transfected with GFP-HuR (a–d) or GFP-HuRD$_{226}$A (e–h). 48 h after transfection, cells were treated with 1 μM STS as indicated. DAPI staining shows nuclei. Representative images of two independent experiments are shown for each time point. (B) STS induces the cytoplasmic accumulation of endogenous HuR. Cells were treated with STS for the indicated times and were fixed and processed for immunofluorescence using antibodies against HuR and the cytoplasmic protein G3BP. DAPI staining depicts nuclei. Representative images of four independent experiments are shown for each time point. (C) The HuR 24-kD CP is detected only in the cytoplasmic fraction. STS-treated cells were harvested at each indicated time point and lysed to prepare cellular fractions. Cytoplasmic and nuclear fractions were prepared and loaded on a 10% SDS-PAGE. Western blot analysis was performed to detect HuR. The anti-tubulin (cytoplasmic marker), -hnRNAP A1 (nuclear marker), and –β-actin (loading control) antibodies were used to assess equal protein loading among cellular fractions. Representative Western blots of three independent experiments are shown. TE, total cellular extract. Bars, 20 μm.

at http://www.jcb.org/cgi/content/full/jcb.200709030/DC1), suggested that during apoptosis, the cellular movement of HuR is independent of caspase activity.

Previous studies have suggested that HuR uses two pathways to translocate from the nucleus to the cytoplasm (Gallouzi and Steitz, 2001; Gallouzi et al., 2001; Higashino et al., 2005; Fries et al., 2006). One pathway involves the receptor CRM1 as well as HuR protein ligands pp32/PHAP-I and APRIL, whereas the other pathway involves an as yet unknown transport factor (Gallouzi and Steitz, 2001; Gallouzi et al., 2001; Fries et al., 2006). Under stress, however, HuR uses mainly the CRM1 route to go from the nucleus to the cytoplasm (Gallouzi et al., 2001; Higashino et al., 2005). To test whether this is also the case upon STS stress, we followed the cellular distribution of HuR and its ligands pp32/PHAP-I and APRIL. HeLa cells were treated or not treated with 1 μM STS for 3 h, fixed, and used for immunofluorescence experiments with antibodies against HuR, pp32/PHAP-I, or APRIL. We observed that both HuR and pp32/PHAP-I colocalized in the nucleus under normal conditions (Fig. 5 A, panels 1–3) as well as in the cytoplasm upon STS treatment (Fig. 5 A, panels 5–7). However, HuR and APRIL colocalized only in the nucleus before and after stress (Fig. 5 A, panels 9–11 and 13–15). These results were confirmed using cell fractionation and Western blot analysis (unpublished data). Because pp32/PHAP-I associates with the NHS motif of HuR and has been reported as an activator of the apoptosome (Jiang et al., 2003), we tested its role in both HuR cytoplasmic translocation and caspase activation. HeLa cells treated with siRNA-pp32/PHAP-I or siRNA-C were incubated or not incubated with 1 μM STS and were used for immunofluorescence experiments with antibodies against HuR and pp32/PHAP-I. Although knocking down pp32/PHAP-I by >50% did not affect the nuclear localization of HuR under normal conditions, the cytoplasmic translocation of HuR was significantly reduced upon STS treatment (Fig. 5 B, panels 5 and 13). Western blot analysis showed a significant reduction in the cleavage of both PARP and HuR in siRNA-pp32/PHAP-I–treated cells that were exposed to STS for 3 h (Fig. 5 C). Therefore, our observations indicated that in addition to being involved in the nucleocytoplasmic movement of HuR, pp32/PHAP-I also affects the caspase activation that is required for HuR cleavage.

HuR enhances apoptosome activity via its interaction with pp32/PHAP-I

Our knockdown and rescue experiments (Fig. 1) suggested that HuR plays a role in the cellular processes responsible for the activation of apoptosis. Because we have shown that pp32/PHAP-I interacts with HuR before and after stress (Gallouzi et al., 2001), it was possible that pp32/PHAP-I was involved in the proapoptotic function of HuR. To test this possibility, we reconstituted the apoptosome in a cell-free system and examined

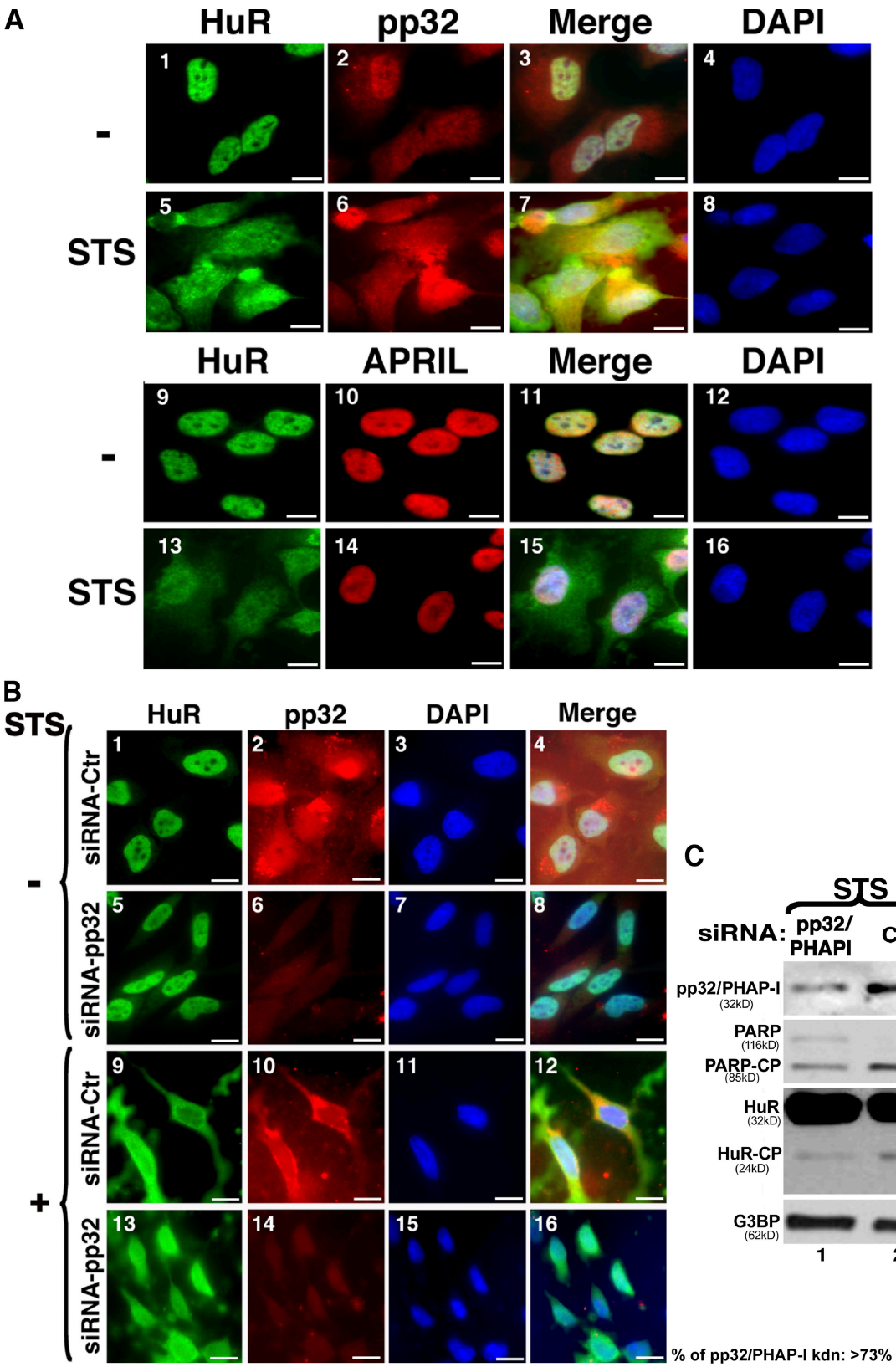

Figure 5. **Both the cytoplasmic accumulation of HuR and its caspase-7/-3–mediated cleavage depend on pp32/PHAP-I protein.** (A) STS treatment induces the cytoplasmic colocalization of HuR with pp32/PHAP-I but not with its ligand APRIL. Untreated cells or STS-treated HeLa cells were fixed and processed for immunofluorescence to detect HuR, pp32/PHAP-I, and APRIL. (B) The knockdown of pp32/PHAP-I prevents the cytoplasmic accumulation of HuR upon STS treatment. HeLa cells were transfected with RNAi duplexes against pp32/PHAP-I mRNA (siRNA-pp32) or control siRNA (siRNA-Ctr), and, 48 h later, they were treated with 1 μM STS for 3 h. Subsequently, these cells were fixed and used for immunofluorescence experiments with anti-HuR and -pp32/PHAP-I antibodies. Representative images of two independent experiments are shown for each time point. (C) siRNA-mediated depletion of pp32/PHAP-I results in the reduction of PARP and HuR cleavage. HeLa cells were transfected with RNAi duplexes as described in B. Western blot analyses using the

the effect of PHAP-I depletion on caspase-3 activity. To deplete pp32/PHAP-I, S100 extracts were incubated with GST-HuR or GST alone immobilized on a glutathione bead column and were used in an in vitro reconstitution assay of the apoptosome in the presence of cyt *c* and dATP (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200709030/DC1). In this assay we followed the release of a fluorophore (7-amino-4-methylcoumarin [AMC]) off the DEVD peptide (caspase-3 cleavage target site; Schafer and Kornbluth, 2006). Western blot analysis showed that the GST-HuR column depleted >80% of pp32/PHAP-I from the S100 extract compared with GST alone (Fig. 6 A, lanes 1 and 2). In contrast to the controls (Fig. 6 B, bars 2 and 3), the pp32/PHAP-I–depleted S100 extracts displayed weaker apoptosome activity in the presence of cyt *c*/dATP, as illustrated by reduced levels of hydrolysis of the caspase-3 substrate DEVD-AMC (Fig. 6 B, compare bar 4 with bars 2 and 3). This reduction is due specifically to the absence of pp32/PHAP-I because the addition of recombinant GST-pp32/PHAP-I to this extract (Fig. 6 B, bar 5) was able to reestablish the apoptosome activity to almost its normal level. The addition of GST-HuR with or without GST-pp32/PHAP-I to the pp32/PHAP-I–depleted S100 extract rescued the apoptosome activity to the same levels seen upon the addition of GST-pp32/PHAP-I alone (Fig. 6 B, compare bars 6 and 7 with bar 5). These observations suggested that the enhancement of apoptosome activity mediated by the pp32/PHAP-I–HuR complex reaches a threshold that cannot be potentiated.

Subsequently, we wished to test the effect of HuR CPs (HuR-CP1 and HuR-CP2) as well as the $HuRD_{226}A$ mutant on apoptosome activity in the presence or absence of pp32/PHAP-I. We were surprised to observe that the addition of HuR-CP1 or -CP2 further reduced the remaining apoptosome activity seen in the S100-Δpp32 extract (Fig. 6 B, compare bars 8 and 10 with bar 4). However, the apoptosome activity of these extracts increased upon the addition of GST-pp32/PHAP-I (Fig. 6 B, bars 9 and 11). The same result was obtained upon the addition of HuR-$D_{226}A$ (Fig. 6 B, compare bars 12 and 13 with bar 5). The fact that HuR-CP1 but not -CP2 associated with HuR in a pull-down assay (Fig. 7 A) and that HuR-CP2 but not -CP1 interacted with pp32/PHAP-I in an immunoprecipitation experiment (Fig. 7 B) indicated that the negative effect of these CPs on the apoptosome activity could be explained, in part, by their ability to interfere with HuR (CP1) or pp32/PHAP-I (CP2) functions.

The $D_{226}A$ mutation increases HuR association with pp32/PHAP-I and delays caspase-dependent apoptosis

Because the $D_{226}A$ mutation did not affect HuR cytoplasmic translocation under apoptotic conditions (Fig. 5 A) and prevented pp32/PHAP-I function in the apoptosome activation (Fig. 6 B, bar 13), we tested its effect on HuR-pp32/PHAP-I association. HeLa cells overexpressing equal amounts of GFP-wt-HuR or -$HuRD_{226}A$ proteins were grown under normal conditions and were used to prepare total cell extracts to perform an immunoprecipitation experiment with the anti-pp32/PHAP-I antibody. As expected, the overexpression of GFP-wt-HuR induced its own cleavage, and an association with pp32/PHAP-I was observed (Fig. 8 A, lanes 1 and 2). Furthermore, we also observed that the GFP-$HuRD_{226}A$ association with pp32/PHAP-I was fourfold higher compared with the interaction between pp32/PHAP-I and GFP-wt-HuR (Fig. 8 A, compare lane 2 with lane 4). This observation suggested that by increasing its association with pp32/PHAP-I, $HuRD_{226}A$ could interfere with the apoptotic process. To test this possibility, HeLa cells overexpressing GFP-wt-HuR, GFP-$HuRD_{254}A$, GFP-$HuRD_{256}A$, or GFP-$HuRD_{226}A$ were treated with a sublethal dose of STS (0.1 μM) for 3 h. We observed that although PARP cleavage was induced by the cleavable isoforms of HuR protein (wt, $D_{254}A$, and $D_{256}A$), it was significantly reduced by the noncleavable isoform GFP-$HuRD_{226}A$ (Fig. 8 B, compare lanes 3, 5, and 6 with lane 4). In addition, the $HuRD_{226}A$ mutant seemed to affect apoptosis by reducing the processing of caspase-3. HeLa cells expressing GFP-$HuRD_{226}A$, GFP-wt-HuR, or GFP alone were treated with STS for different times, and the apoptosome activity was assessed (Schafer and Kornbluth, 2006). At early stages of the apoptotic response (between 1 and 2 h of STS treatment), we observed only a basal level of caspase-3 activity (Fig. 8 C). However, at later stages (between 2 and 3 h and thereafter), we observed that caspase-3 activity was significantly reduced in the presence of GFP-$HuRD_{226}A$ (S100 + DA) compared with GFP-wt-HuR (S100 + HuR) or GFP (S100) alone (Fig. 8 C). These results suggested that $HuRD_{226}A$ significantly reduced the activation of the apoptosome (here between 2 and 3 h of STS treatment). These observations were further confirmed by Western blot analysis of caspase-3 cleavage in the same cells (Fig. 8 D). Thus, preventing HuR cleavage seems to negatively affect apoptosis. In addition, the significant increase in association between $HuRD_{226}A$ and pp32/PHAP-I was a good indication that the HuR–pp32/PHAP-I complex played an important role in the activation of apoptosis.

To determine whether the $HuRD_{226}A$ mutant might interfere with the proapoptotic function of endogenous HuR under these extreme conditions, we performed a rescue experiment as described in Fig. 1. HeLa cells depleted of HuR protein were incubated with AP–HuR-GST or AP–$HuRD_{226}A$-GST for 16 h before STS treatment. Western blot analysis using anti-PARP antibody showed that although AP–HuR-GST alone increased caspase-3 activity, the addition of AP–$HuRD_{226}A$-GST to these cells significantly reduced its activity and PARP cleavage (Fig. 8 E). This was also addressed in vivo by defining the percentage of cell death of HeLa cells incubated with 50 nM AP–HuR-GST or AP–$HuRD_{226}A$-GST and exposed to 1 μM STS for 3 h. The phase-contrast pictures showed that compared with AP–HuR-GST, AP–$HuRD_{226}A$-GST significantly reduced cell death (Fig. 8 F, compare panels c and g with panels d and h). These observations indicated that mutating the D_{226} to A not only protected HuR against cleavage but also delayed caspase-dependent apoptosis.

anti-pp32/PHAP-I, -HuR, and -PARP antibodies were performed using total cell extracts prepared from STS-treated cells. The anti-G3BP antibody was used as a loading control. (B and C) The percentage of pp32/PHAP-I knockdown (% kdn) is indicated. Bars, 20 μm.

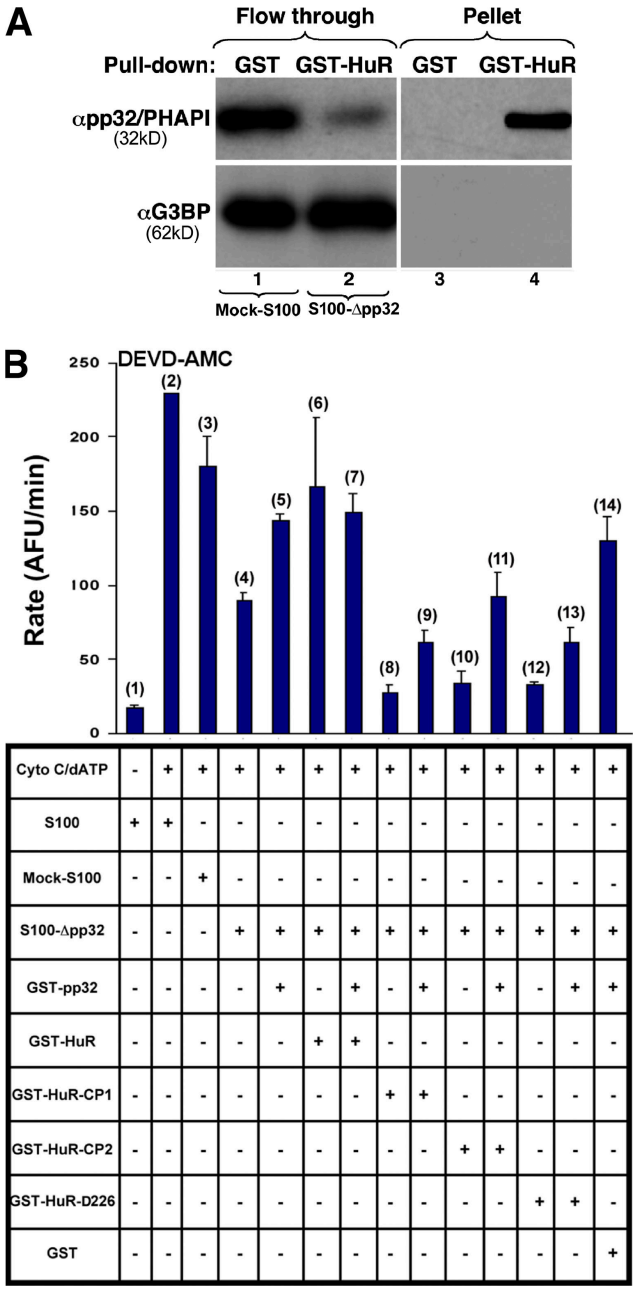

Figure 6. **Association between HuR and pp32/PHAP-I is part of the regulatory mechanism leading to pp32/PHAP-I–mediated apoptosome activation.** (A) Western blot analysis using the flow-through (lanes 1 and 2) or the pellets (lanes 3 and 4) prepared from S100 extracts incubated with GST-HuR or GST beads was performed using the anti-pp32/PHAP-I antibody. Detection of the cytoplasmic protein G3BP was used as a loading control. (B) S100 extracts were incubated with GST-HuR, GST, or buffer A alone for 16 h. The resulting flow-throughs, S100-depleted pp32 (S100-Δpp32), S100 mock depleted (Mock-S100), and S100 extracts were then incubated with 1 μg cyt *c*/1.5 mM dATP for 2 h at 30°C. For S100-Δpp32, the following proteins were added before incubation: GST-pp32 (bar 5), GST-HuR (bar 6), GST-pp32 and -HuR (bar 7), GST–HuR-CP1 (bar 8), GST-pp32 and –HuR-CP1 (bar 9), GST–HuR-CP2 (bar 10), GST-pp32 and -HCP2 (bar 11), GST-HuR-D_{226}A (bar 12), GST-pp32 and -HuR-D_{226}A (bar 13), and GST and -pp32 (bar 14). Apoptosome activation was monitored via cleavage of the fluorometric-conjugated caspase-3 substrate DEVD-AMC. Error bars represent SEM. AFU, arbitrary fluorescence unit.

Discussion

Our data demonstrate that HuR plays a key role in the enhancement of caspase-dependent apoptosis induced by extreme stress conditions. Although it is well established that HuR affects cell fate through its role as an RNA-binding protein (Brennan and Steitz, 2001; Abdelmohsen et al., 2007), evaluating the possibility that HuR also functions through an alternative pathway has been elusive. In this study, we identify a novel regulatory mechanism through which HuR participates in the apoptotic pathway. We show that HuR potentiates the apoptotic response by undergoing a caspase-mediated cleavage at a specific aspartate residue located in its HNS region. This cleavage activity occurs only after HuR enters the cytoplasmic compartment in a pp32/PHAP-I–dependent manner. HuR exerts its proapoptotic effects through its association with pp32/PHAP-I, a known enhancer of the apoptosome (Jiang et al., 2003; Hill et al., 2004). Thus, we propose a model whereby in response to a lethal stress, HuR accumulates in the cytoplasm, where it undergoes caspase-mediated cleavage. This cleavage appears to be important for pp32/PHAP-I–mediated enhancement of the caspase-dependent apoptosis (Fig. 9).

Previous studies had shown that HuR regulates the stability and/or the translation of mRNAs encoding for pro- ($p21^{Waf1}$, $p27^{Kip1}$, and p53) and antiapoptotic (proTα) proteins (Schiavone et al., 2000; Wang et al., 2000; Kullmann et al., 2002; Mazan-Mamczarz et al., 2003; Lal et al., 2005). However, no explanation has been given as to how HuR may be active in these two opposite processes. In response to different extracellular stimuli, HuR associates with mRNAs encoding for proteins that are involved in different mechanisms such as cell cycle, cell differentiation, and stress response (van der Giessen et al., 2003; Abdelmohsen et al., 2007). Thus, the cellular function of HuR could vary depending on the nature of the stimuli applied. Consistent with this idea, we noted that knocking down the expression of HuR in cells treated with a lethal dose of STS causes a significant delay in caspase-dependent apoptosis (Fig. 1). This observation implies that HuR promotes cell death under conditions in which the integrity of the cell is altered beyond repair. In contrast, it has been seen that upon mild stress, HuR promotes death resistance by affecting the translation of the mRNA encoding for proTα (Lal et al., 2005). Together, these and our findings argue that HuR can function during the two characteristic steps of the cell stress response. First, it participates in processes that activate prosurvival pathways to facilitate cell recovery (proTα). Then, if the stress becomes persistent, HuR promotes cell death.

To our surprise, while delineating the cellular mechanism by which HuR promotes apoptosis, we observed that HuR itself is cleaved in a caspase-dependent manner. We found that although only caspase-7 is able to cleave HuR in vitro (Fig. 2 B), in HeLa cells, this proteolytic activity involves both caspase-7 and -3 (Fig. 2). This caspase-mediated cleavage generates fragments of 24 kD (HuR-CP1) and 9 kD (HuR-CP2; Figs. 2 and 3). These data are consistent with the well-established notion that the cleavage cascade requiring a series of caspases represents a key process needed to generate active proapoptotic proteins.

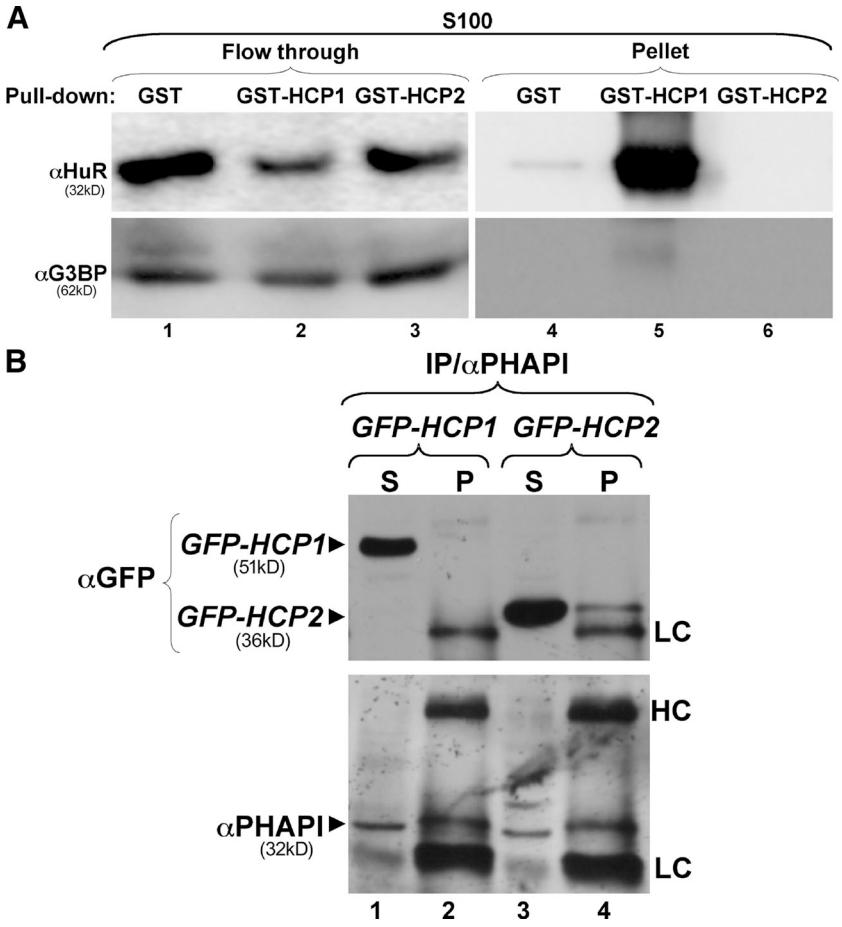

Figure 7. **HuR and pp32/PHAP-I differentially associate with HuR-CP1 and -CP2.** (A) HuR-CP1 but not HuR-CP2 associates with HuR in GST pull-down assays. S100 extracts were incubated with GST, GST–HuR-CP1 (HCP1), and–HuR-CP2 (HCP2) beads for 16 h. The resulting flow-through (lanes 1–3) and pellets (lanes 4–6) were analyzed by Western blotting using anti-HuR and -G3BP (loading control) antibodies. (B) HuR-CP2 but not HuR-CP1 associates with pp32/PHAP1 in vivo. HeLa cells were transfected with GFP-HCP1 (lanes 1 and 2) or GFP-HCP2 (lanes 3 and 4). 48 h after transfection, extracts were subjected to immunoprecipitation using anti-pp32/PHAP-I antibodies. The immunoprecipitates (P) and supernatants (S) were analyzed by Western blotting using anti-GFP and -pp32/PHAP-I antibodies. LC and HC indicate light chains and heavy chains of the anti-pp32/PHAP-I antibody, respectively. Arrowheads indicate the positions of GFP-HCP1, GFP-HCP2, and pp32/PHAP-I proteins.

Our results argue that cytoplasmic HuR becomes a member of this family of cell death effectors that are induced by caspase-mediated cleavage. HuR has features of a caspase target protein, such as a specific aspartate residue (D_{226}) cleavage site as well as the generation of active CPs (Figs. 3 and 8). Surprisingly, mutating this residue to alanine not only protected HuR protein from proteolysis (Fig. 3 C) but also generated a noncleavable isoform that is able to interfere with its role in apoptosis (Figs. 6 and 8). Together, these data suggest that as soon as a lethal stress is applied, HuR partially migrates to the cytoplasm and participates in activation of the apoptotic machinery to promote cell death (Figs. 3–5).

Activation of the apoptosome is a cytoplasmic event that involves Apaf-1 oligomerization upon cyt *c* release from the mitochondria (Cain et al., 2002). It has been shown that HuR associates with the apoptosome activator pp32/PHAP-I through its HNS and RRM3 motifs (Brennan et al., 2000). Here, we observed that HuR diffuses and colocalizes in the cytoplasm with pp32/PHAP-I upon STS treatment (Fig. 5). Knocking down the expression of pp32/PHAP-I protein significantly reduces the cytoplasmic accumulation of HuR. This is consistent with previously published data showing that pp32/PHAP-I is part of the HuR complex that translocates to the cytoplasm under different stress conditions (Gallouzi et al., 2000, 2001). Likewise, we found that the percentage of total HuR that is cleaved in the cytoplasm (Fig. 3) corresponds to the same amount that associates with pp32/PHAP-I (Brennan et al., 2000). Thus, it is possible that by cleaving HuR within its HNS motif, caspase-7/-3 induce the release of an active part of HuR that participates in pp32/PHAP-I–mediated apoptosome activation. This is likely the case because overexpressing the noncleavable isoform of HuR, $HuRD_{226}A$, in HeLa cells delays the STS-induced cell death and stabilizes the pp32/PHAP-I–HuR complex in the cytoplasm (Fig. 8). Moreover, interfering with apoptosome activation by depleting the expression of pp32/PHAP-I using RNAi significantly reduces the cleavage of both HuR and PARP proteins (Fig. 5 C). These and our in vitro depletion/rescue experiments (Fig. 6) argue that HuR plays a critical role in activating the apoptosome complex via its association with pp32/PHAP-I. The results described herein shed light on a new player involved in the stimulatory effect of pp32/PHAP-I on the apoptosome. However, pp32/PHAP-I does not directly interact with any of the apoptosome components (Hill et al., 2004), and an apoptosome-activating mechanism remains unknown. It was demonstrated that the same C-terminal acidic domain of pp32/PHAP-I that is critical for its proapoptotic activity (Hill et al., 2004) is also involved in its association with HuR (Brennan et al., 2000). Our data suggest that the cleavage of HuR plays an important role in the proapoptotic function of pp32/PHAP-I.

Our data showing that RNAi-mediated depletion of pp32/PHAP-I leads to a significant reduction in PARP and HuR cleavage (Fig. 5 C) argue that an active apoptosome is required

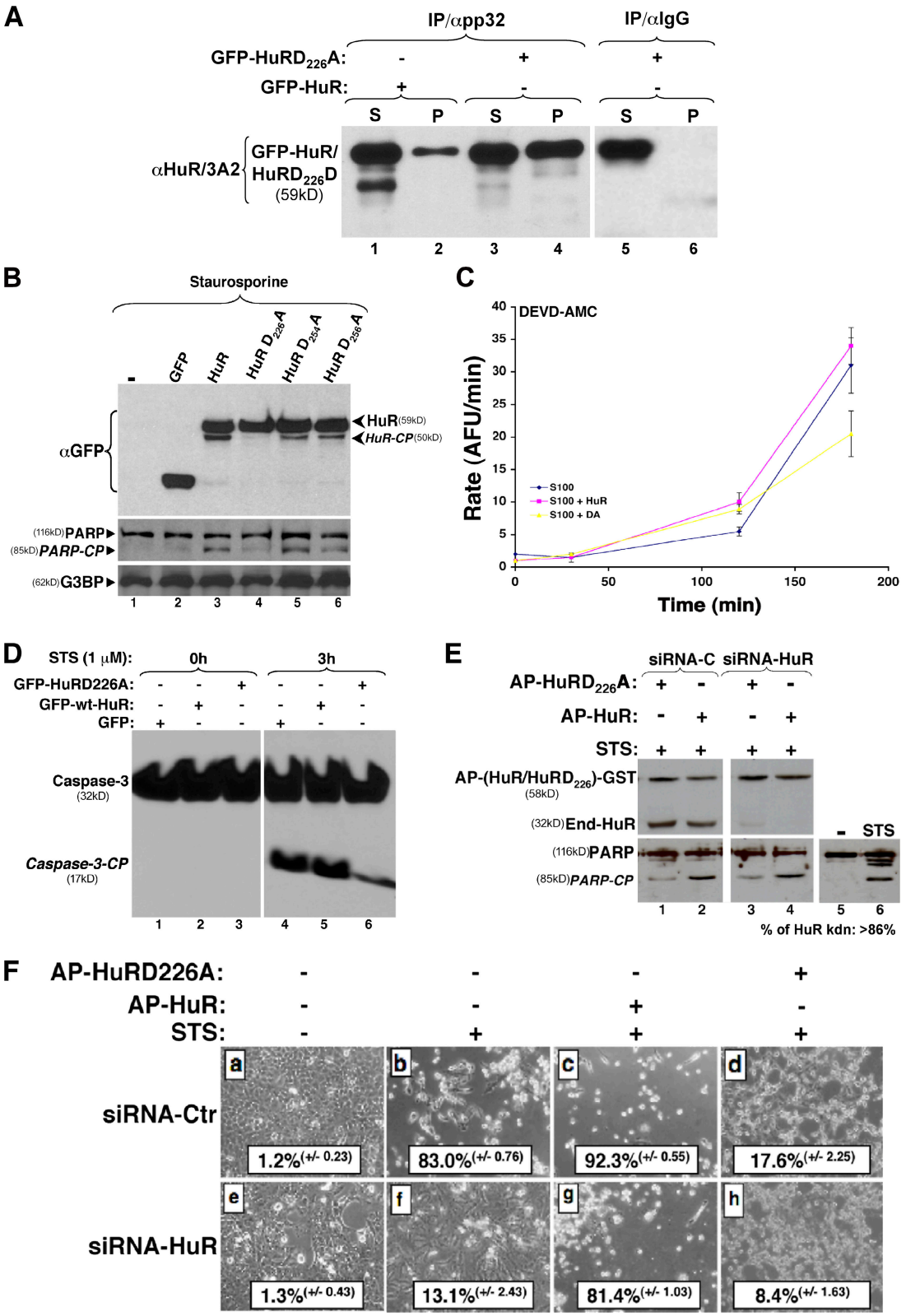

Figure 8. **HuRD$_{226}$A maintains a strong interaction with pp32/PHAP-I and delays apoptosis.** (A) HuRD$_{226}$A associates with pp32/PHAP-I with higher efficiency than wt-HuR. HeLa cells were transfected with GFP-HuR (lanes 1 and 2) or GFP-HuRD$_{226}$A (lanes 3 and 4). 48 h after transfection, extracts were subjected to immunoprecipitation using anti-pp32/PHAP-I (lanes 1–4) or IgG (lanes 5 and 6) antibodies. The immunoprecipitates (P) and supernatants (S) were analyzed by Western blotting using anti-HuR (3A2) antibody. (B) HuRD$_{226}$A reduces PARP cleavage. GFP, GFP-HuR, and its point mutation derivatives were produced in HeLa cells. The cells were then treated with 0.1 μM STS for 3 h. GFP and GFP-HuR proteins were detected by immunoblotting using anti-GFP, -PARP, and -G3BP (loading control) antibodies. (C and D) HeLa cells expressing GFP, GFP-HuR, or GFP-HuRD$_{226}$A were treated with 1 μM STS for the indicated times. 100 μg of total cytoplasmic extracts were incubated with 10 μM DEVD-AMC fluorogenic peptide, and the release of AMC was measured over 30 min. Error bars represent SEM. AFU, arbitrary fluorescence unit. (D) Western blot analysis of the cleavage of caspase-3 by immunoblotting using

for HuR proteolysis. These observations suggest two possibilities: (1) at early stages of the cell stress response, a basal apoptosome activity could be present in HeLa cells, resulting in caspase-7/-3 activation before the apoptosome reaches its full capacity; and (2) caspase-7/-3 could be activated in an apoptosome-independent manner. Our time-course experiments, in which we followed caspase-3 processing during cell response to STS treatment, favor the first possibility. We observed that at early stages (<100 min) of apoptotic response, when HuR localizes to the cytoplasm, the apoptosome harbors a basal level of activity (Fig. 8 C). However, at later stages (>100 min), we observed a significant increase in caspase-3 activation (Fig. 8, C and D), correlating with the appearance of HuR-CP1 (Fig. 1 F). These observations imply that the small amount of activated caspase-7/-3 at early stages of apoptosis could be sufficient to cleave HuR, generating the HuR-proapoptotic active forms. This form will then participate in the amplification loop that enhances the apoptosome activity, which feeds down on more HuR processing during apoptosis.

Although our data argue that HuR promotes apoptosis through a mechanism that is independent of its RNA-binding activity, we cannot rule out the possibility that the HuR CPs enhance apoptosis by also posttranscriptionally affecting the expression of some proapoptotic mRNAs. Indeed, recent observations indicated that the depletion of HuR results in a significant decrease in the expression of caspase-9 mRNA and protein (unpublished data). This suggested that HuR, through its RNA-binding activity, could also affect apoptosome formation. Thus, through their RNA-binding motifs, HuR-CP1 (RRM1–RRM2) or HuR-CP2 (RRM3; Fig. 3) could affect the stability and/or the translation of proapoptotic mRNAs. This suggests a model in which the caspase-mediated HuR cleavage represents the regulatory step during which HuR switches from an antiapoptotic function at early steps of the stress response to a proapoptotic role when cell death is unavoidable. This raises the possibility that in cancer cells, the cleavage of HuR could be altered, such as by a mutation to yield a noncleavable isoform and/or a defect in caspase-7/-3 activity, interfering with its RNA- and protein-mediated proapoptotic function. Therefore, investigating the effect of cell transformation on HuR functions as a proapoptotic factor will open the door to the possibility of defining posttranscriptional regulators as potential targets for cancer therapy.

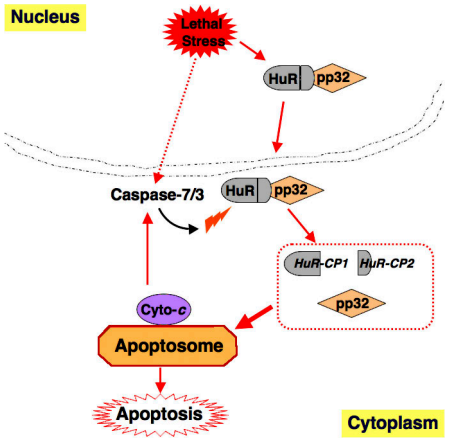

Figure 9. **Model of how HuR protein participates in the pp32/PHAP-I–mediated enhancement of apoptosome activity.** Upon induction of stress-induced apoptosis, HuR and its partner pp32/PHAP-I translocate together to the cytoplasm. In the cytoplasm, HuR is targeted for cleavage in a process involving both capase-7 and -3. This cleavage event contributes to the pp32/PHAP-I–mediated enhancement of apoptosome activity. How HuR and/or its CPs participate with pp32/PHAP-I in cell death by apoptosis is under investigation.

Materials and methods

Plasmid construction and protein purification

GFP-conjugated constructs were prepared using GST-HuR plasmid as a template (Brennan et al., 2000). All primers were generated with restriction sites in their 5′ end, BglII (forward), and EcoRI (reverse), and the products were cloned into pAcGFP1-C1 vector (BD Biosciences). GFP-wt-HuR was generated using the primers forward (5′-GGCAGATCTAATGGTTATGAAGACCACA-3′) and reverse (5′-GGCGAATTCTTATTTGTGGGACTTGTTGGTT-3′). We generated three deletion mutants using the following primers: GFP-HuR-ΔHNS-RRM3, forward (5′-GGCAGATCTAATGGTTATGAAGACCACA-3′) and reverse (5′-GGCGAATTCTTAGTACAGCTGCGAGAGGAG-3′); GFP-HuR-ΔRRM1–RRM2, forward (5′-GGCAGATCTTACCACTCGCCAGCGCGA-3′) and reverse (5′-GGCGAATTCTTATTTGTGGGACTTGTTGGTT-3′); and GFP-HuR-ΔRRM3, forward (5′-GGCAGATCTAATGGTTATGAAGACCACA-3′) and reverse (5′-GGCGAATTCTTAGGAGGAGGCGTTTCCTGG-3′). The point mutants GFP-HuR$_{226}$A, GFP-HuRD254A, and GFP-HuRD256A were generated by Norclone Biotech Laboratories. AP–HuR-GST and AP–HuR$_{226}$A-GST were produced and used as described previously (van der Giessen et al., 2003). GST-HCP1 and GST-HCP2 plasmids were constructed, and proteins were produced as follows: the first 226 amino acids of HuR (1–226; HCP1) or the last 100 amino acids (227–326; HCP2) were amplified by PCR using wt HuR as the template. A BglII site was created at the 3′ end, and an EcoRI site was generated at the 5′ end of the PCR product for both HCP1 and HCP2. GFP-HCP1 was generated using the primers forward (5′-GGCAGATCTAATGGTTATGAAGACCACA-3′) and reverse (5′-GGCGAATTCTTAATCGACGCCCATGGG-3′). We generated GFP-HCP2 using the primers forward (5′-GGCAGATCTCACATGAGCGGGCTCTCT-3′) and reverse (5′-GGCGAATTCTTAGTACAGCTGCGAGAGGAG-3′). Those PCR fragments were cloned within the BglII–EcoRI sites of pAcGFP1-C1 vector (BD Biosciences), and both fragments were inserted in frame of the GFP gene.

Cell culture, transfection, and stress treatments

HeLa CCL-2 cells (American Type Culture Collection) were grown and maintained in DME (Invitrogen) containing 10% FBS (Invitrogen), penicillin/streptomycin, and L-glutamine according to the manufacturer's directions (Sigma-Aldrich). DNA and siRNA-HuR duplexes were transfected into HeLa cells as described previously (van der Giessen et al., 2003) except that 60 nM of each siRNA-HuR duplex was transfected into cells. The knockdown of pp32/PHAP1, caspase-7, and caspase-3 expression was performed using a premade siRNA duplex (QIAGEN). The experiments were performed according the manufacturer's instructions.

For the rescue experiments, subconfluent HeLa cells depleted or not depleted of HuR protein were incubated in culture media with 50 nM AP-HuR or AP-HuR$_{226}$A. These cells were incubated with the AP-conjugated proteins for 16 h and were stressed with 1 μM STS (Sigma-Aldrich) as indicated. zVAD and MG132 were obtained from Sigma-Aldrich.

anticaspase-3 antibody. (E and F) The HuR$_{226}$A mutant interferes with the stimulatory function of HuR on apoptosis. (E) 50 nM AP–HuR-GST (lanes 2 and 4) or AP–HuR$_{226}$A-GST (lanes 1 and 3) was added to siRNA-HuR or -Ctr STS-treated HeLa cells. Total extracts from these cells were prepared and used for Western blot analyses to follow PARP cleavage. The blots were probed with antibodies against HuR and PARP. (F) Phase-contrast images were taken from the same cells described in E. The percentage of cell death was determined as described in Fig. 1 B. For each panel, five randomly chosen fields of 300 cells were counted before and after STS treatment. Values are the means ± SEM from three independent experiments. (E and F) The percentage of HuR knockdown (% kdn) for these two panels is indicated under panel E.

Preparation of cell extracts, immunoblotting, immunoprecipitation, GST pull-down, and immunofluorescence

Total cell extracts were prepared and Western blots were performed as described previously (Di Marco et al., 2005). The blots were probed with antibodies to HuR (3A2; Gallouzi et al., 2000), G3BP (Gallouzi et al., 1998), spectrin (Chemicon International), and tubulin (Sigma-Aldrich) as well as caspase-9, PARP, pp32/PHAP-I, GFP, and bax (all were purchased from Santa Cruz Biotechnology, Inc.). zVAD was added at 10 μM. Immunoprecipitation experiments were performed as described previously (Di Marco et al., 2005) except for a modification for pp32/PHAP-I antibody, which was incubated with protein G agarose (Millipore). HeLa S100 extract was prepared as described below in the Apoptosome activation assay section. GST pull-down was performed as described previously (Brennan et al., 2000) using HeLa S100 extracts. Essentially, 1 mg S100 proteins was incubated with 5 μg GST recombinant proteins for 16 h at 4°C. Supernatant flow-through was separated from protein-bound beads by centrifugation at 3,400 rpm for 5 min. Immunofluorescence was performed as previously described (Brennan et al., 2000; Mazroui et al., 2002).

Determination of the percentage of cell death

For the percentage of cell death, we counted the number of adherent HeLa cells expressing or not expressing HuR protein before and after STS treatment. The percentage of cell death was defined as described previously (Schafer and Kornbluth, 2006).

In vitro cleavage assays

[^{35}S]methionine-labeled HuR was obtained by coupled in vitro transcription/translation using the TNT reticulocyte lysate system (Promega). More precisely, 1 μg cDNA construct was incubated with T7 RNA polymerase, rabbit reticulocyte lysate, amino acid mixture minus methionine, and [^{35}S]methionine (GE Healthcare) for 90 min at 30°C in a final reaction volume of 25 μl.

Cleavage of 4 μl of the in vitro transcribed/translated radiolabeled HuR was performed by incubation at 37°C for 3 h in the presence of 5 U of purified recombinant caspases as indicated by the manufacturer's instructions (EMD). The cleavage reactions were terminated by the addition of 5 μl of 5× Laemmli sample buffer, after which samples were boiled for 5 min, and 18 μl was applied to 12% polyacrylamide gels. Gels were then fixed for 20 min in 10% acetic acid and 40% methanol, soaked in Enlightning (PerkinElmer) for another 20 min, dried for 90 min at 80°C, and exposed to film.

Apoptosome activation assay

Cells were collected by scrapping, and the cell pellet was washed with ice-cold PBS and resuspended in 5 vol of buffer A (20 mM Hepes, pH 7.5, 10 mM KCl, 2 mM EDTA, 250 mM sucrose, 1 mM DTT, 0.1 mM PMSF, and 1× protease inhibitor cocktail). All centrifugations were performed at 4°C. After 15 min of incubation on ice, cells were Dounce homogenized with a 15-ml pestle B (Wheaton). Lysates were then cleared by centrifugation for 5 min at 1,000 *g* to remove intact cells, cell debris, and nuclei. The soluble lysate was then centrifuged at 10,000 *g* for 10 min to prepare total cytosolic extract or was centrifuged at 100,000 *g* for 1 h to prepare S100 extract. The resulting supernatants were used for the apoptosome activation assay. For this assay, 100 μg S100 was incubated with 1 μg cyt *c*/1.5 mM dATP and 2.5 mM $MgCl_2$ in a total volume of 20 μl of buffer A for 1 h at 30°C. The activity of the apoptosome was monitored via caspase-3 activity, which was measured by fluorometry as the release of AMC from DEVD-AMC. The rate of cleavage of this fluorogenic substrate was measured over 30 min in 1-min intervals and expressed as arbitrary fluorescent units/min (excitation of 380 nm and emission of 460 nm).

Annexin V–FITC/PI assay

HeLa cells were treated for 48 h with siRNA-Ctr or -HuR and were incubated or not incubated with 1 μM STS for 3 h. Apoptotic and necrotic cells were identified by annexin V–FITC and PI staining, respectively, as well as by flow cytometry.

Microscopy and digital image

Images were acquired at room temperature with a microscope (Axiovert 200M; Carl Zeiss, Inc.) with a 63× oil objective (Carl Zeiss, Inc.), and a digital camera (Axiocam HR; Carl Zeiss, Inc.) was used for immunofluorescence photography. The original images were acquired using Axiovision 4.5 software (Carl Zeiss, Inc.). Digital images were manipulated and arranged using Photoshop 6.0 (Adobe). The luminosity and brightness were adjusted and applied to whole images to obtain the best visual reproduction, ensuring that linearity in the brightness scale was maintained. Images were included in figures using Photoshop 6.0.

Online supplemental material

Fig. S1 shows that HeLa cells depleted of HuR and exposed to HS (45° for 1 h) present a significant delay in triggering caspase-mediated apoptosis. Fig. S2 shows that the concentration (50 nM) of AP–HuR-GST and AP-GST used in the rescue experiments is not toxic to the cells. Fig. S3 shows that HeLa cells depleted of HuR and exposed to the proteasome inhibitor MG132 are resistant to death by apoptosis. Fig. S4 shows that the STS-induced cytoplasmic localization of HuR occurs in a caspase-independent manner. Fig. S5 shows a schematic representation of the different steps of the in vitro assay for the apoptosome activity performed in Fig. 6. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200709030/DC1.

We are grateful to Drs. R. Mackenzie, S. Richard, and V. Dormoy-Raclet for comments on the manuscript. We also thank S. Falfoul for his technical help.

This work was supported by a Canadian Institutes of Health Research (CIHR) Cancer Consortium Training Grant Fellowship Award to S. Di Marco, a National Cancer Institute of Canada (NCIC) Postdoctoral Terry Fox Fellowship and a CIHR Postdoctoral Fellowship to R. Mazroui, a Fonds de la Recherche en Sante Quebec Master's Fellowship to C. von Roretz, a National Institutes of Health/National Human Genome Research Institute grant (HG003679) to S.A. Tenenbaum, a CIHR operating grant (MOP-79410) to M. Saleh, and an NCIC operating grant (016247) to I.-E. Gallouzi. I.-E. Gallouzi is a recipient of a TierII Canada Research Chair.

Submitted: 6 September 2007
Accepted: 12 December 2007

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