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The eIF4G homolog DAP5/p97 supports the translation of select mRNAs during endoplasmic reticulum stress

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ABSTRACT

DAP5/p97 is a member of the eIF4G family of translation initiation factors that has been suggested to play an important role in the translation of select messenger RNA molecules. We have shown previously that the caspase-cleaved form of DAP5/p97, termed p86, is required for the induction of the endoplasmic reticulum (ER)-stress-responsive internal ribosome entry site (IRES) of the caspase inhibitor HIAP2. We show here that expression of DAP5/p97 is enhanced during ER stress by selective recruitment of DAP5/p97 mRNA into polysomes via the DAP5/p97 IRES. Importantly, enhanced translation mediated by the DAP5/p97 IRES is dependent on DAP5/p97 itself, thus providing a positive feedback loop. In addition, we show that activation of DAP5/p97 and HIAP2 IRES during ER stress requires DAP5/p97. Significantly, the induction of DAP5/p97 during ER stress is caspase-independent, whereas the induction of HIAP2 requires proteolytic processing of DAP5/p97. Thus, DAP5/p97 is a translational activator that selectively modulates translation of specific mRNAs during conditions of cellular stress in both a caspase-dependent and caspase-independent manner.

INTRODUCTION

Selective translation of messenger RNAs (mRNAs) has emerged as an important mechanism that regulates gene expression, particularly in response to various physiological and pathophysiological conditions that require rapid changes in gene expression profiles. mRNAs that employ selective translation utilize various regulatory elements, most often located within their 5′ untranslated regions (UTRs) that allow preferential translation. For example, the 5′ UTR of the transcription factor ATF4 contains two short upstream open reading frames that render translation of the ATF4 reading frame inefficient (1,2). However, translation of ATF4 is specifically increased under conditions of increased eIF2α phosphorylation, such as during endoplasmic reticulum (ER) stress and the unfolded protein response, although the rate of global protein synthesis is reduced (3). Another stress-induced mode of translation initiation takes advantage of internal ribosome entry site (IRES) elements located within 5′ UTRs that permit cap-independent translation (4). IRES were originally discovered in picornaviruses, where they initiate translation of naturally uncapped viral RNAs (5,6). Interestingly, cellular IRES are found largely in mRNAs that encode proteins with important roles in differentiation, cell growth and proliferation and the regulation of apoptosis, suggesting that the selective modulation of IRES-mediated translation is critical for the regulation of cell death and survival (4,7).

The precise molecular mechanism of cellular IRES-mediated translation is not fully understood. Several studies have shown that most, if not all, cellular IRES require various auxiliary proteins termed ITAFs (IRES *trans*-acting factors) for efficient IRES-mediated translation (8,9). While the requirement for ITAFs is not the same for all cellular IRES, it is generally believed that these factors may function as RNA chaperones that aid in the remodeling of proper IRES conformation and therefore allow access of the ribosome (4). Alternatively, ITAFs may also directly recruit the ribosome to an IRES through interaction with ribosomal subunits. The requirement for canonical initiation factors in IRES-mediated translation is even less understood. Several studies have suggested that members of the eIF4G family may be required for cellular IRES-mediated translation, in particular during conditions of cellular stress and compromised global protein synthesis. It was shown that caspase-cleaved fragments of two family members, eIF4GI and DAP5/p97, can specifically enhance translation mediated by several cellular IRES, including

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those of Apaf-1, c-myc, HIAP2, XIAP and DAP5/p97 mRNAs (10–13). Furthermore, using a HeLa cell-free translation system, the full-length DAP5/p97 was shown to support translation mediated by the c-myc, HIAP2 and XIAP IRES elements in eIF4G-depleted extracts (14). More recently, DAP5/p97 was suggested to control cell proliferation by regulating the translation of cell cycle proteins such as $p27^{Kip1}$ (15).

In this study, we have investigated the role of DAP5/p97 in modulating selective translation of IRES-containing mRNAs during ER stress. We show that DAP5/p97 is necessary for specific activation of at least two cellular IRES elements during pharmacologically induced ER stress. We find that expression of DAP5/p97 and an inhibitor of apoptosis protein HIAP2 are enhanced during ER stress; their mRNAs are selectively recruited to the polysomes via IRES elements located in their respective 5′ UTRs. We further find that this process is dependent on DAP5/p97, as reducing the levels of endogenous DAP5/p97 by RNA interference abrogated the IRES-mediated translation of both DAP5/p97 and HIAP2 during ER stress. Moreover, we find that proteolytic cleavage of DAP5/p97 to the DAP5/p86 isoform is not required for the translational induction of DAP5/p97 during ER stress. Thus, a positive feedback loop exists in which ER stress results in elevated levels of DAP5/p97 that, in turn, activate translation of specific mRNAs such as HIAP2 and DAP5/p97 itself under conditions of reduced cap-dependent translation.

MATERIALS AND METHODS

Cell culture and reagents

Human embryonic kidney (293T), or human cervical carcinoma (HeLa) cells were maintained in standard conditions in Dulbecco's modified Eagle's medium (DMEM; Wisent Inc.) supplemented with heat-inactivated 10% fetal calf serum (FCS), 2 mM L-glutamine and 1% antibiotics (100 U/ml penicillin–streptomycin). Transient transfections were performed using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's protocol. Briefly, cells were seeded at a density of 6×10^5 cells/well in 6-well plates and were transfected 24 h later in serum-free OPTI-MEM medium (Invitrogen, Carlsbad, CA) with 2 μg of DNA per well. siRNA transfections were performed using RNAifect according to the protocol provided by the manufacturer (Qiagen, Chatsworth, CA). Briefly, cells were seeded at a density of 5×10^5 cells/well in 6-well plates and were transfected 24 h later in serum-free DMEM with a 20 nM final concentration of DAP5/p97 siRNA or a non-silencing control siRNA (Qiagen). The target sequences of the DAP5/p97 siRNA oligonucleotides are as follows: #1- aatgtgggtgtagagtctaaa;#2-aagcactagacgaaggaa; #3- aaccagagtcagggactctta; #4- aaggaccgcatgttggagatt. Cells were collected for analysis 24, 48, or 72 h post-transfection. For the ER stress experiments, cells were treated with tunicamycin at indicated doses or DMSO for 24 h and then collected for analysis as described below. Caspase inhibition during ER stress was achieved by pre-incubating cells with 100 μM Z-VAD-FMK (Calbiochem, San Diego, CA) for 6 h, followed by co-incubation with 8.5 μM tunicamycin and 100 μM Z-VAD-FMK for an additional 24 h. The bicistronic vectors pβGal/(-162)/CAT (containing the XIAP IRES and hence referred to as pβGal/XIAP/CAT in the manuscript), pβGal/p97/CAT (containing the eukaryotic initiation factor DAP5/p97 IRES) and pβGal/HIAP2/CAT (containing the HIAP2 IRES) were described previously (10,11,15,16). The expression vectors for FLAG-DAP5/p97 and FLAG-p86 were described previously (11).

Cell viability

293T cells were transiently transfected in 6-well plates with either DAP5/p97 siRNA or non-targeting (scr) siRNA as described above. For rescue experiments, cells were additionally transfected with expression vectors encoding either GFP or DAP5/p97 cDNAs. Twenty-four hours post-transfection, the cells were harvested and cell viability was determined using a cell viability analyzer (Vi-Cell; Beckman Coulter, Fullerton, CA) for each treatment. All data are shown as an average ± SD of three independent experiments performed in triplicate. Western blots were performed on parallel samples to determine the levels of DAP5/p97 and GAPDH expression.

β-Galactosidase and CAT analysis

Transiently transfected cells were washed twice in 1 ml PBS and harvested in 300 μl CAT ELISA kit lysis buffer according to the protocol provided by the manufacturer (Roche Molecular Biochemicals, Indianapolis, IN). β-Galactosidase (β-Gal) enzymatic activity was determined by spectrophotometric assay using *o*-nitrophenyl-β-D-Galactopyranoside as previously described (17). CAT levels were determined using the CAT ELISA kit according to the protocol provided by the manufacturer (Roche Molecular Biochemicals). The relative IRES activity was determined as a ratio of CAT/β-Gal. All data are shown as an average ± SD of three independent experiments performed in triplicate.

Western blot analysis

Cells were harvested in ice-cold PBS and cell extracts were prepared in RIPA buffer [1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM sodium phosphate (pH 7.2), 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride] containing 10 μg/ml each of aprotinin, pepstatin A and leupeptin (all from Sigma). The lysates were then centrifuged at 14 000*g* for 10 min and supernatants were collected. Protein concentration in the supernatants was determined by protein assay kit (Bradford Assay, Bio-Rad, Richmond, CA). Equal amounts of protein samples were separated by 10% SDS–PAGE, transferred to PVDF membrane and analyzed by western blotting. The antibodies used were as follows: mouse monoclonal anti-GAPDH (Advanced ImmunoChemical Inc., Long Beach, CA), mouse monoclonal anti-HIAP2 (R&D), mouse monoclonal anti-GRP78/BiP (Transduction Laboratories, San Jose, CA),

rabbit polyclonal anti-cleaved PARP (Cell Signaling Technologies, Danvers, MA). Rabbit polyclonal antibody to DAP5/p97 was raised against the synthetic peptide EFLGKTPGQNAQKWIPAR (amino acids 37–53) and purified (Open Biosystems, Huntsville, AL). All antibodies were used at the manufacturer's suggested dilutions and conditions followed by secondary antibody (horseradish peroxidase-conjugated sheep anti-mouse or anti-rabbit IgG; Amersham Biosciences, Piscataway, NJ). Antibody complexes were detected using the ECL Plus and ECL western blotting detection systems (Amersham Biosciences). For the purposes of quantification of protein expression, parallel western blots were performed as described above but the secondary antibody used was Alexa Fluor 680 goat anti-mouse, anti-rat or anti-rabbit IgG (LI-Cor Inc, Lincoln, NE). Antibody complexes were then detected and quantified using the Odyssey Infrared Imaging system (LI-Cor Inc). All quantification data are shown as an average ± SD of three independent experiments.

Quantitative RT-PCR

Total RNA was isolated from tunicamycin-treated or control cells that were previously transfected with the pβGal/p97/CAT or pβGal/HIAP2/CAT reporter plasmids using the Absolutely RNA miniprep kit (Stratagene, La Jolla, CA) as directed by the manufacturer's instructions. For quantitative RT-PCR, reverse transcription was carried out using the First-Strand cDNA Synthesis kit (Amersham Biosciences, Piscataway, NJ, USA) with oligo $d(T)_{18}$ primers. The quantitative PCR was performed using the QuantiTect SYBR green PCR kit (Qiagen) and analyzed on an ABI Prism 7000 sequence detection system using the ABI Prism 7000 SDS Software. Quantitative PCRs were carried out to detect β-Gal (5′-ACTATCCCG ACCGCCTTACT-3′; 5′-CTGTAGCGGCTGATGTT GAA-3′) and CAT (5′-GCGTGTTACGGTGAAAAC CT-3′; 5′-GGGCGAAGAAGTTGTCCATA-3′) as described previously (18).

Analysis of polysome-associated mRNAs

Polysomes from treated and untreated cells were collected using sucrose-gradient centrifugation as described previously (19). RNA was isolated from individual fractions using the Absolutely RNA miniprep kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Indicated fractions (polysomes and 40S/60S/80S) were pooled and cDNA was generated for each pool using an oligo $d(T)_{18}$ primer and the Bulk 1st Strand Syntesis kit according to the protocol provided by the manufacturer (Amersham Biosciences). cDNA was then used as a template for quantitative PCR using the QuantiTect SYBR Green PCR kit (Qiagen) and analyzed on an ABI Prism 7000 detection system as described above. Quantitative PCR reaction were carried out to detect BiP (forward: TGCAGCAGGACATCAAGTTC; reverse: AGTTCCAGCGTCTTTGGTTG), DAP5/p97 (forward: CTCTTATCCCAGCTGCAAGG; reverse: CCCAGAG GTGGTGTTTGAGT), NF90 (forward: CCGTGATGT TCCCTGTTTCT; reverse: CGTGTGTTGGCACAAAC TTC), HIAP2 (forward: TGGCCTTTCATTCGTATCA AGA; reverse: TCTGGAGATGATCCATGGGTAGA), Actin (forward: CTGGAACGGTGAAGGTGACA; reverse: AAGGGACTTCCTGTAACAATGCA) and ATF4 (forward: CCTACGTTGCCATGATCCCT. reverse: CTTCTGGCGGTACCTAGTGG).

Analysis of global protein synthesis

HEK 293T cells were seeded at 2.5×10^5 cells per well in a 6-well plate. Twenty-four hours later, cells were transfected with 0.5 μg/well siRNA (scrambled control or DAP5/p97 siRNA) as described above. Twenty-four hours post-transfection, the media was replaced with methionine/cysteine-free minimal essential medium (Invitrogen) in which L-cysteine was added to a final concentration of 63 mg/l and the cells were incubated at 37°C for 15 min. Cells were metabolically labeled by incubating at 37°C for 20 min in labeling media supplemented with 100 μCi/ml [^{35}S]-Methionine (GE Healthsciences). Cells were then washed in ice-cold PBS and harvested for 20 min at 4°C in RIPA buffer containing 10 μg/ml each of aprotinin, pepstatin A and leupeptin. The lysates were then centrifuged at 14 000*g* for 10 min and supernatants were collected. Protein concentration in the supernatant was determined by protein assay kit (Bradford Assay, Bio-Rad) and equal amounts of protein samples (20 μg) were separated by 10% SDS–PAGE. The total proteins were visualized by Coomasie brilliant blue (R-250) staining and the ^{35}S incorporation was visualized by autoradiography.

Co-precipitation of mRNA:protein complexes

The co-precipitation of mRNA:protein complexes was performed essentially as described (20). Briefly, HEK293T cells were transfected with pCI, FLAG-DAP5/p97 or FLAG-DAP5/p86 and cytoplasmic lysates were harvested 24 h later by incubating 5×10^6 cells on ice for 5 min in 750 μl of cytoplasmic lysis buffer [20 mM Tris–Cl (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$, 0.3% IGEPAL CA-630, RNasin (Promega), 1 mM PMSF and protease inhibitors], then clarified by centrifugation at 10 000*g* for 10 min. Cytoplasmic extract of 375 μl was incubated with 50 μl of a 50% (v/v) suspension of anti-FLAG-coated agarose beads (Sigma) for 1 h at 4°C and then the beads were then washed five times in NT2 buffer [50 mM Tris–Cl (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$ and 0.05% NP-40]. Contaminating DNA was removed from the sample by incubating the beads in 100 μl of NT2 buffer supplemented with 2 μl DNase I (Invitrogen) for 40 min at 30°C. The beads were washed twice in NT2 buffer and bound proteins were digested by incubating the beads in 100 μl NT2 buffer containing 0.1% SDS and supplemented with 0.5 mg/ml Proteinase K for 10 min at 55°C. RNA was then extracted using phenol: chloroform and precipitated in the presence of Glycoblue (Ambion, Austin, TX). Precipitated mRNA was detected by RT-PCR and visualized on a 1.5% agarose gel by ethidium bromide staining.

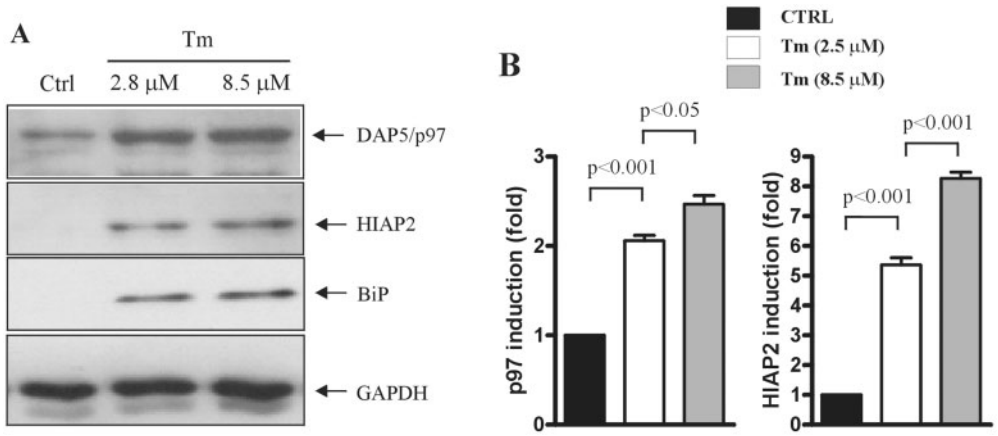

Figure 1. ER stress causes induction of eukaryotic initiation factor DAP5/p97 expression. (**A**) Western blot analysis of endogenous DAP5/p97 and HIAP2 proteins in 293T cell lysates treated with DMSO (Ctrl) or indicated doses of tunicamycin (Tm). The induction of ER stress that was confirmed by western blot analysis for the ER chaperone BiP and the membrane was probed with anti-GAPDH as a loading control. (**B**) The densitometric analysis of DAP5/p97 and HIAP2 protein expression (relative to GAPDH) from three independent experiments (average ± SD).

RESULTS

Endoplasmic reticulum stress enhances translation of DAP5/p97

Induction of ER stress by various triggers results in enhanced translation of the inhibitor of apoptosis (IAP) protein HIAP2 in various cell types (11,21). In addition, induction of ER stress leads to caspase-dependent cleavage of the DAP5/p97 protein, a member of the eIF4G family of initiation factors, to generate a p86 isoform that specifically enhances translation of HIAP2 via an IRES element located within the 5′ UTR of HIAP2 mRNA (11). While investigating the role of DAP5/p97 in ER stress, we have noticed that the levels of DAP5/p97 are also elevated following induction of ER stress by tunicamycin (an inhibitor of protein glycosylation) or thapsigargin (an inhibitor of ER calcium pump) (Figure 1 and data not shown). The induction of DAP5/p97 expression by tunicamycin was dose-dependent and paralleled the expression pattern of known ER stress-inducible genes GRP78/Bip and HIAP2 (Figure 1A). Examination of DAP5/p97 and HIAP2 mRNA levels from treated and untreated samples by quantitative RT-PCR revealed that the levels of mRNA did not change following drug treatment [Figure 2C and (11)]. These data indicate that the observed changes in DAP5/p97 protein levels are likely due to translational up-regulation.

To confirm that DAP5/p97 translation is indeed enhanced during ER stress, we examined the association of DAP5/p97 mRNA with polysomes in DMSO and tunicamycin-treated cells. Enhancement of DAP5/p97 expression at the translational level should result in recruitment of DAP5/p97 mRNA into the polysomal fraction. Polysomal mRNA was isolated from total cellular mRNA by sucrose gradient centrifugation. The typical optical density profile of the sucrose gradient recorded at 254 nm is shown in Figure 2A. The top fractions (fractions 2–5) represent free mRNAs and ribosomal complexes (40S, 60S and 80S) and were pooled together for future mRNA analysis. Conversely, the bottom fractions (fraction 6–10) represent mRNA molecules associated with polysomes. We observed that treatment of cells with tunicamycin resulted in a significant inhibition of proteins synthesis as demonstrated by a considerable decrease in the polysome peak and concomitant accumulation of free mRNAs and an increase in the monosome peak (Figure 2A). We performed control experiments to verify that tunicamycin-induced ER stress results in an inhibition of general protein synthesis (as evidenced by the distribution of actin mRNA) while supporting selective translation of ER stress-inducible genes (as evidenced by the distribution of BiP mRNA) (22). Indeed, examination of the distribution of actin mRNA showed that ~70% of total actin mRNA is associated with polysomes in DMSO-treated cells, whereas only 10% of total actin mRNA remains in polysomes during tunicamycin-induced ER stress (Figure 2B). In contrast, the mRNA of BiP, a known ER stress-inducible gene, remains associated with polysomes in tunicamycin-treated cells (Figure 2B).

Next, we analyzed the polysomal association of DAP5/p97 mRNA in DMSO and tunicamycin-treated cells. We observed that there was a significant increase in the amount of DAP5/p97 mRNA associated with polysomes in tunicamycin-treated cells (relative to actin mRNA) (Figure 2C). We did not observed any increase in the amount of total DAP5/p97 mRNA, indicating that enhanced transcription does not contribute to the observed increase in polysome-associated DAP5/p97 mRNA. As a control, we analyzed the expression of ATF4, which is known to increase in response to ER stress by both increased transcription and translation (23). As expected, we observed an increase in total ATF4 mRNA as well as polysomal-associated ATF4 mRNA (Figure 2C). Similar to DAP5/p97 mRNA, HIAP2 mRNA was also found to be mobilized into polysomes in ER-stressed cells without a concomitant increase in total HIAP2 mRNA levels (Figure 2C). In contrast, mRNA levels of NF90 (24), a transcription factor that

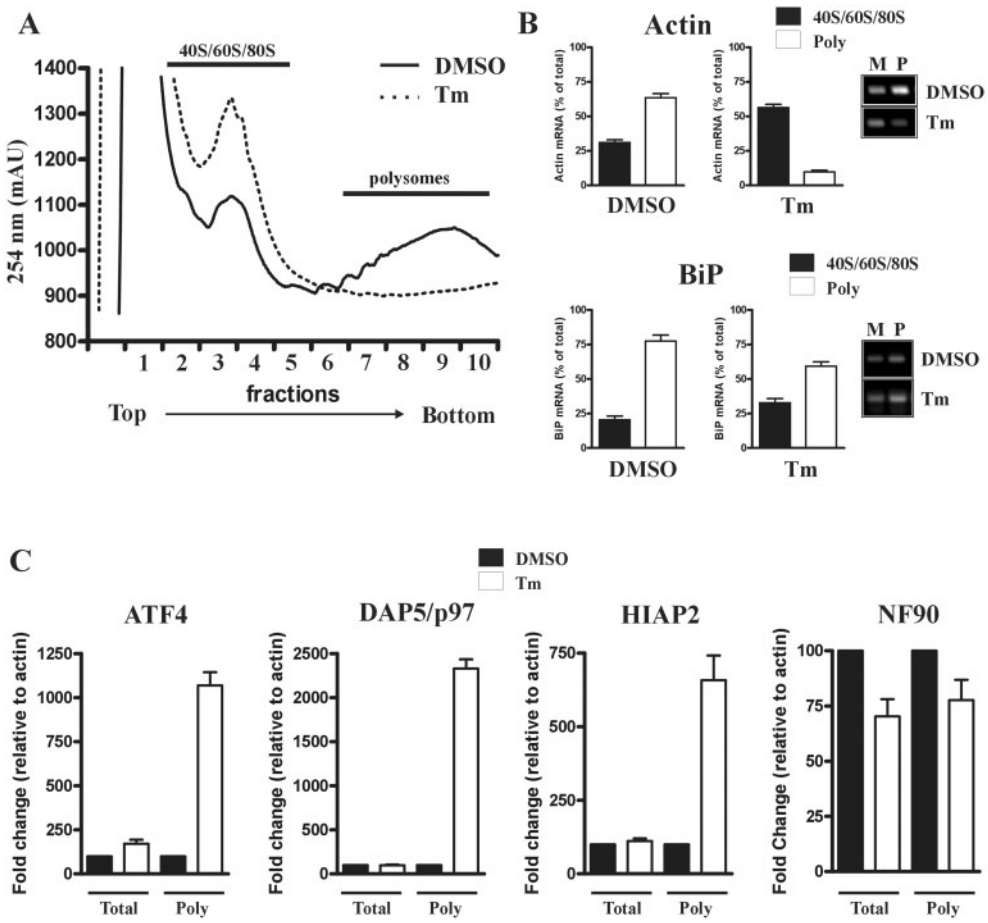

Figure 2. DAP5/p97 and HIAP2 mRNAs remain associated with polysomes during ER stress. (**A**) Polysome profiles from tunicamycin (Tm; 8.5 μM) or DMSO-treated cells were generated as described in Materials and Methods. (**B**) The polysomal distribution of actin and BiP mRNAs (shown as percentage of total RNA) was used to confirm the induction of ER stress and validate the qRT-PCR approach. Mean ± SEM of two independent experiments performed in triplicates is shown. The images of agarose gels on the right are representative of results from RT-PCR reactions that were run in parallel. (**C**) The levels of DAP5/p97 and HIAP2 mRNAs that remain associated with polysomes in Tm-treated cells (relative to actin) were determined as in (B). The polysomal association of ATF4, which is known to be translated during ER stress, was used as positive control; NF90, which is not known to be translated during ER stress, was used as negative control. Average ± SD of two independent experiments performed in triplicate. DMSO treated samples were set as 100.

is not known to be induced during ER stress and was chosen randomly as a control, decreased in both total and polysome fractions.

These observations are consistent with the notion that pharmacologically induced ER stress results in a significant increase in the translation of DAP5/p97 and HIAP2 due to the selective recruitment of their mRNAs into polysomes.

Translational induction of DAP5/p97 during ER stress is mediated by its IRES

The data described above confirm that the translation of DAP5/p97 and HIAP2 is selectively enhanced during ER stress. We have previously established the regulatory mechanism for the translational control of HIAP2 in response to ER stress (11). The 5′ UTR of HIAP2 encodes a short upstream open reading frame (uORF) that mediates repression of HIAP2 expression (25). Adjacent and overlapping with the uORF is an inducible IRES element that allows for selective translational induction of HIAP2 during pharmacologically induced ER stress (11) and also in cells treated with etoposide or sodium arsenite (26). The key step in the translational up-regulation of HIAP2 during ER stress is caspase-dependent cleavage of DAP5/p97, which generates the p86 fragment that specifically enhances IRES-mediated translation of HIAP2 (11). In addition, DAP5/p86 was shown to be involved in translation of other IRES elements such as those found in the Apaf-1 and DAP5/p97 mRNAs (10,12). We therefore wished to examine if translational induction of DAP5/p97 during ER stress is mediated by its IRES element. To assess DAP5/p97 IRES activity, we used the previously described bicistronic reporter plasmids containing the IRES elements of DAP5/p97, HIAP2 (positive control) and XIAP (negative control) (10,11,16). HEK293T cells were transiently transfected with bicistronic reporter constructs and the IRES activity was determined in control (DMSO treated) or tunicamycin-treated cells 24 h later. We observed dose-dependent induction of DAP5/p97 and HIAP2 IRES activity by tunicamycin treatment (Figure 3B). In contrast, the activity of the XIAP IRES

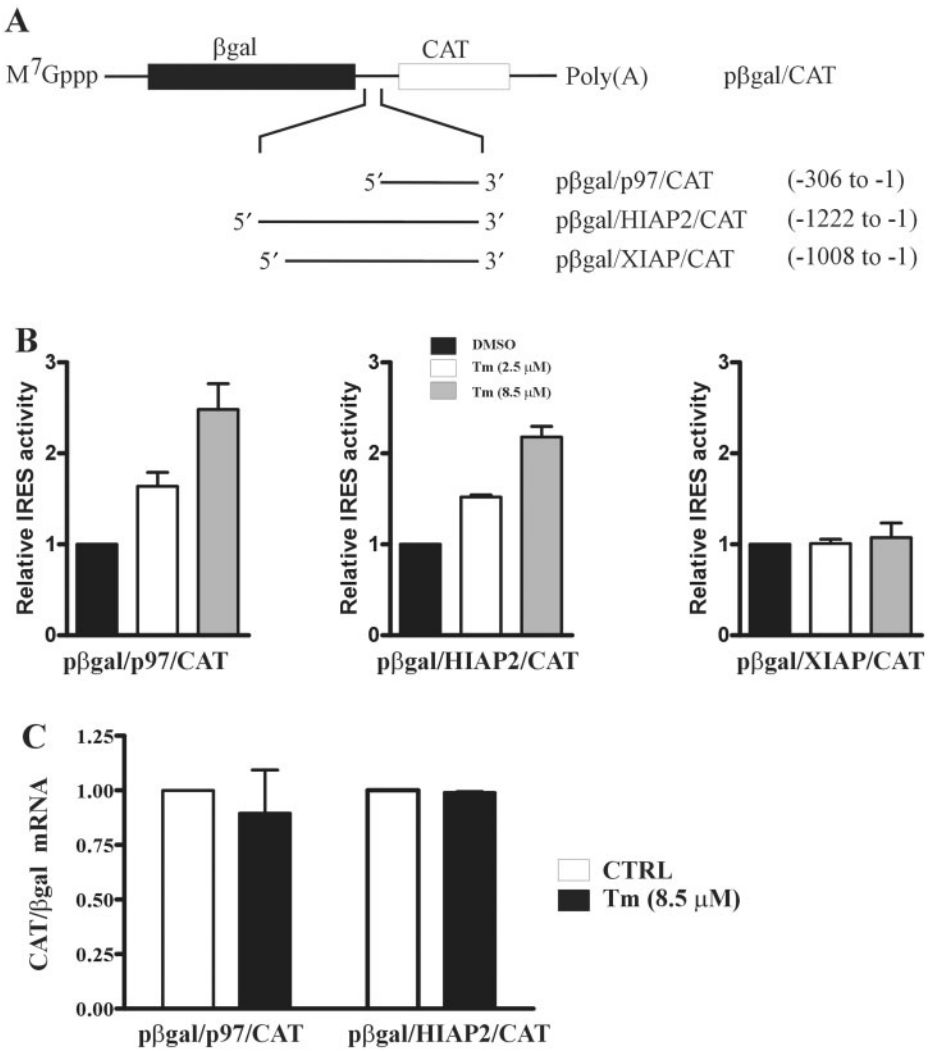

Figure 3. The activity of the DAP5/p97 IRES element is enhanced during ER stress. (**A**) Schematic diagram depicting the bicistronic reporter constructs harboring the IRES elements of DAP5/p97, HIAP2 and XIAP used in this study. (**B**) HEK293T cells were transiently transfected with the indicated bicistronic constructs and the effect of tunicamycin (Tm) treatment or DMSO treatment on IRES-mediated translation was determined by measuring the β-Gal and CAT levels as described in Materials and Methods. Relative IRES activity (CAT/β-Gal) was determined for three independent experiments performed in triplicate. Average ± SD. The activity of each construct in DMSO-treated cells was set as 1. (**C**) Quantitative RT-PCR analysis of β-Gal and CAT cistron RNA was used to test for cryptic promoter activity and spurious splicing of the DAP5/p97 IRES and HIAP2 IRES-containing bicistronic RNAs. Values are expressed as CAT relative to β-Gal ($2^{-[Ct(CAT) - Ct(\beta^{Gal})]}$). Average ± SD of three independent experiments performed in triplicate.

remained unchanged in tunicamycin-treated cells, as reported previously (11). To eliminate the possibility that the observed IRES activity in tunicamycin-treated cells is due to the presence of cryptic promoters or spurious splicing events, we assessed the levels and integrity of the reporter mRNA by quantitative RT-PCR analysis as described (18). Total RNA was isolated from cells treated with either DMSO or tunicamycin that were previously transfected with the bicistronic plasmids. cDNA was produced by reverse transcription and was used as a template for quantitative PCR analysis using primers that amplify a portion of the β-Gal-coding region and a portion of the CAT-coding region. As shown in Figure 3C, the ratio of the CAT and β-Gal cistrons remained unchanged in cells treated with tunicamycin as compared to cells treated with DMSO. These data confirm that the integrity of the bicistronic RNA transcript produced from the reporter plasmids is not affected by tunicamycin. We therefore conclude that translational induction of p97 during tunicamycin-induced ER stress is mediated by the IRES elements in their respective mRNAs.

IRES-mediated translation of DAP5/p97 and HIAP2 is dependent on DAP5/p97

We have shown that the enhanced translation of DAP5/p97 and HIAP2 during tunicamycin-induced ER stress (as evidenced by the selective recruitment of DAP5/p97 and HIAP2 mRNAs to polysomes) is mediated by their respective IRES elements. DAP5/p97 is a translation initiation factor belonging to the eIF4G family that has been shown previously to stimulate the translation of several IRES elements (10–12,14). We therefore wished

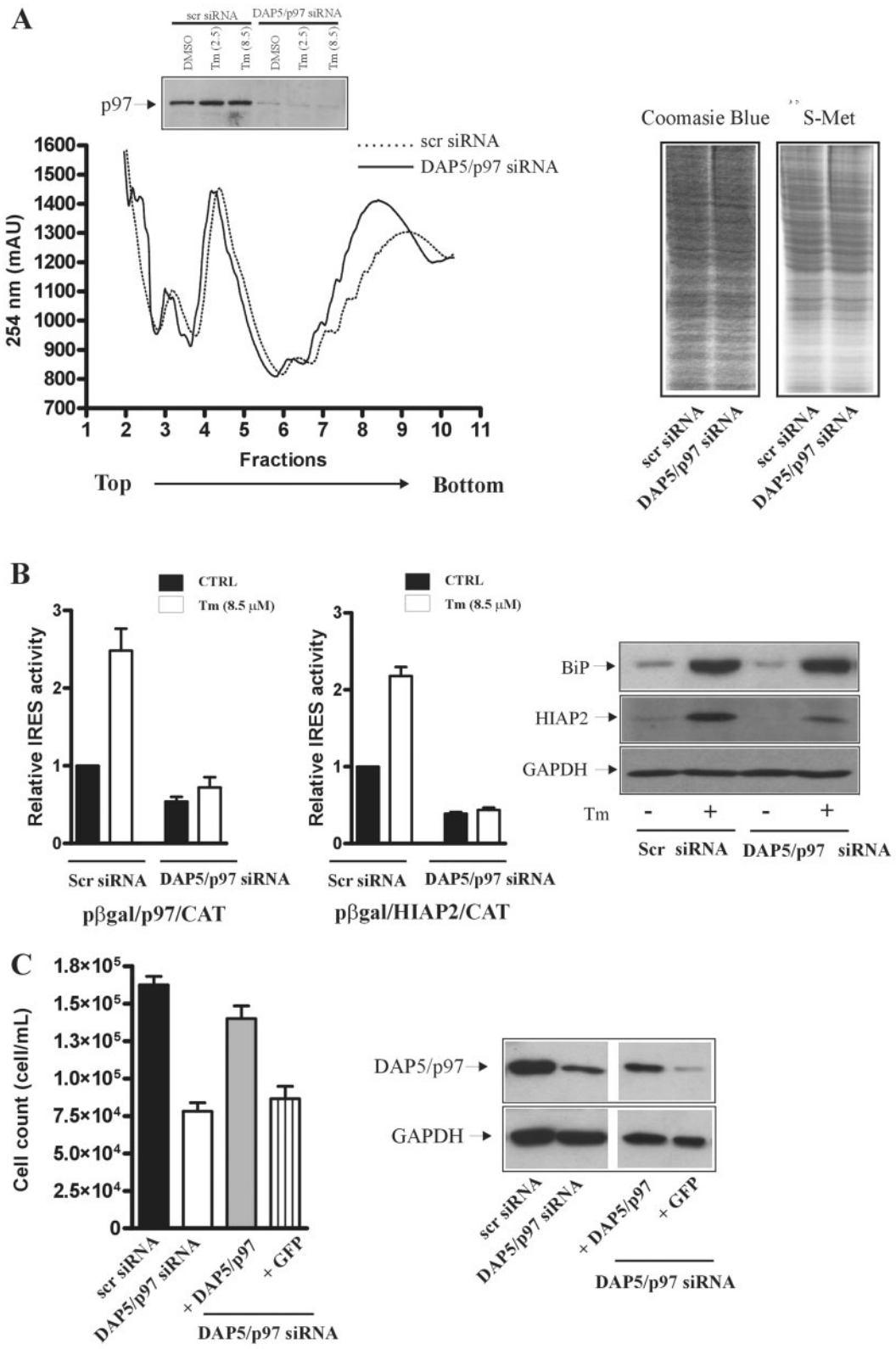

Figure 4. DAP5/p97 knockdown impairs IRES translation and reduces cell viability. (**A**) HEK293T cells were transiently transfected with the indicated siRNA molecules and the levels of DAP5/p97 protein were determined by western blot analysis 24 h later. Polysome profiles were generated from control (scr) or DAP5/p97 siRNA-transfected cells as described in Materials and Methods section. Metabolic labeling of control and DAP5/p97 siRNA-transfected cells was performed using ^{35}S-Met as described in Materials and Methods section and is shown on the right. (**B**) IRES activity of the DAP5/p97 and HIAP2 5′ UTRs was determined in control or DAP5/p97 siRNA-transfected cells treated with DMSO or 8.5 μM Tm using the bicistronic reporter plasmids described in Figure 3A. Average ± SD of three independent experiments performed in triplicate. Samples from

to examine the activity of the DAP5/p97 IRES and HIAP2 IRES in cells depleted of DAP5/p97 to determine if the activities of the DAP5/p97 IRES and HIAP2 IRES are dependent on the presence of DAP5/p97 in tunicamycin-treated cells. Cells were transfected with DAP5/p97 siRNA or non-silencing control siRNAs and the extent of DAP5/p97 knockdown was determined in DMSO and tunicamycin-treated cells. We observed that all four DAP5/p97 siRNAs, individually or in combination, significantly reduced expression of DAP5/p97 (Figure 4A and data not shown).

First, we investigated the effect of DAP5/97 down-regulation on global protein synthesis by metabolic labeling and by examining polysome profiles from DAP5/p97 siRNA and control siRNA-treated cells. We found that reduced expression of DAP5/p97 had no significant effect on global protein synthesis (Figure 4A). In contrast, the activities of the DAP5/p97 IRES and HIAP2 IRES were severely impaired in control as well as tunicamycin-treated cells transfected with p97 siRNA (Figure 4B). Furthermore, transfection of cells with DAP5/p97 siRNA, but not the control non-silencing siRNA, significantly reduced the levels of endogenous HIAP2 protein in response to ER stress (Figure 4B). Therefore, DAP5/p97 is necessary for IRES-mediated translational induction of HIAP2 and p97 in response to ER stress.

We have shown previously that DAP5/p97-mediated induction of HIAP2 expression increases the resistance of cells to ER stress (11). We therefore wished to determine if down-regulation of DAP5/p97 levels sensitizes cells to tunicamycin treatment. Surprisingly, we noted that transfection of cells with DAP5/p97 siRNA resulted in a significant loss of cell viability (Figure 4C), precluding us from assessing the tunicamycin sensitivity of the cells. The loss of viability was specific to DAP5/p97 down-regulation, as non-silencing siRNA did not affect cell survival. Furthermore, we were able to rescue cell viability of DAP5/p97 siRNA-transfected cells by elevating the levels of DAP5/p97 using an overexpression plasmid (Figure 4C). Taken together, our data suggest that loss of p97 is not compatible with cell survival, likely by impinging on the translation of specific cellular mRNAs.

Translational induction of DAP5/p97 during ER stress is caspase-independent

Caspase cleavage of DAP5/p97 to the p86 isoform has been shown to be important for the activation of several IRES elements (10–12). Moreover, the DAP5/p86 isoform potently induces HIAP2 IRES activity, and caspase activity is required for the induction of HIAP2 IRES activity during ER stress (11). A recent report has shown that DAP5/p97 can affect IRES activity independent of caspase cleavage, suggesting that DAP5/p97 may be able to affect IRES activity independently of proteolytic cleavage to the DAP5/p86 isoform (27). As we have found that DAP5/p97 is absolutely required for the induction of DAP5/p97 IRES activity during ER stress, we therefore wished to determine if caspase activity is required for the translational induction of DAP5/p97 during ER stress. Cells were pre-treated with the pan-caspase inhibitor Z-VAD-FMK or DMSO for 6 h and then incubated with 8.5 μM tunicamycin in the presence or absence of 100 μM Z-VAD-FMK for an additional 24 h. Protein extracts were harvested and the levels of BiP, p97, GAPDH and cleaved PARP were assayed by western blot analysis. Surprisingly, we found that translational induction of DAP5/p97 during ER stress occurs in both the presence and absence of caspase activity (Figure 5A, lanes 2 and 4). These results indicate that translational induction of DAP5/p97 during ER stress occurs in a caspase-independent manner.

To further confirm the function of full-length DAP5/p97 in DAP5/p97 IRES activation, we cotransfected HEK293T cells with a DAP5/p97-overexpressing plasmid and either the DAP5/p97 IRES or the HIAP2 IRES-containing bicistronic reporter plasmids. We found that while overexpression of DAP5/p97 had no effect on the activity of the HIAP2 IRES [Figure 5B and (11)] it resulted in significantly enhanced activity of the DAP5/p97 IRES (Figure 5B). Thus, full-length DAP5/p97 is sufficient to drive the translation mediated by its own IRES element.

We next sought to determine how full-length DAP5/p97 is able to modulate the activity of the DAP5/p97 IRES but not the HIAP2 IRES. We hypothesized that full-length DAP5/p97 may be able to bind to DAP5/p97 IRES-containing mRNA but not to HIAP2 IRES-containing mRNA, whereas DAP5/p86 may bind to both p97 and HIAP2 mRNAs. To test this hypothesis, we co-precipitated mRNAs associated with either full-length DAP5/p97 or the DAP5/p86 caspase-cleavage product of p97. HEK293T cells were transfected with plasmids expressing FLAG-DAP5/p97, FLAG- DAP5/p86 or the empty pCI vector. Cytoplasmic extracts were harvested 24 h later, mRNA: protein complexes were co-precipitated using anti-FLAG-coated agarose beads and the co-precipitated mRNA was isolated by phenol: chloroform extraction and amplified by RT-PCR. As expected we were able to co-precipitate both DAP5/p97 and HIAP2 mRNA with DAP5/p86 (Figure 5C, lane 12). Surprisingly, we found that both DAP5/p97 and HIAP2 mRNA were co-precipitated with full-length DAP5/p97 (Figure 5C, lane 8). We could only weakly co-precipitate GAPDH mRNA with full-length DAP5/97 and not at all with DAP5/p86 (Figure 5C, lanes 8 and 12), indicating some degree of specificity of DAP5/p97 and DAP5/p86 for mRNA ligands. These data indicate that both full-length DAP5/p97 and the DAP5/p86 isoform can bind to IRES-containing mRNAs and therefore suggest that factors other than the ability of these proteins to associate with their mRNA ligands (such as protein–protein interactions) control the effect of

DMSO-treated cells transfected with scrambled siRNA were set as 1. The levels of BiP, HIAP2 and GAPDH proteins were determined by western blot analysis and are shown on the right. (**C**) Cell viability of control (scr) or DAP5/p97 siRNA-transfected cells was determined 24 h post-transfection by counting viable cells using the Vi-Cell cell viability analyzer (Beckman Coulter). Average ± S.D of three independent experiments performed in triplicate. For rescue experiments, the indicated plasmids were transfected into the cells at the time of siRNA transfection. The extent of DAP5/p97 expression and/or knockdown was assessed by western blot analysis of parallel samples.

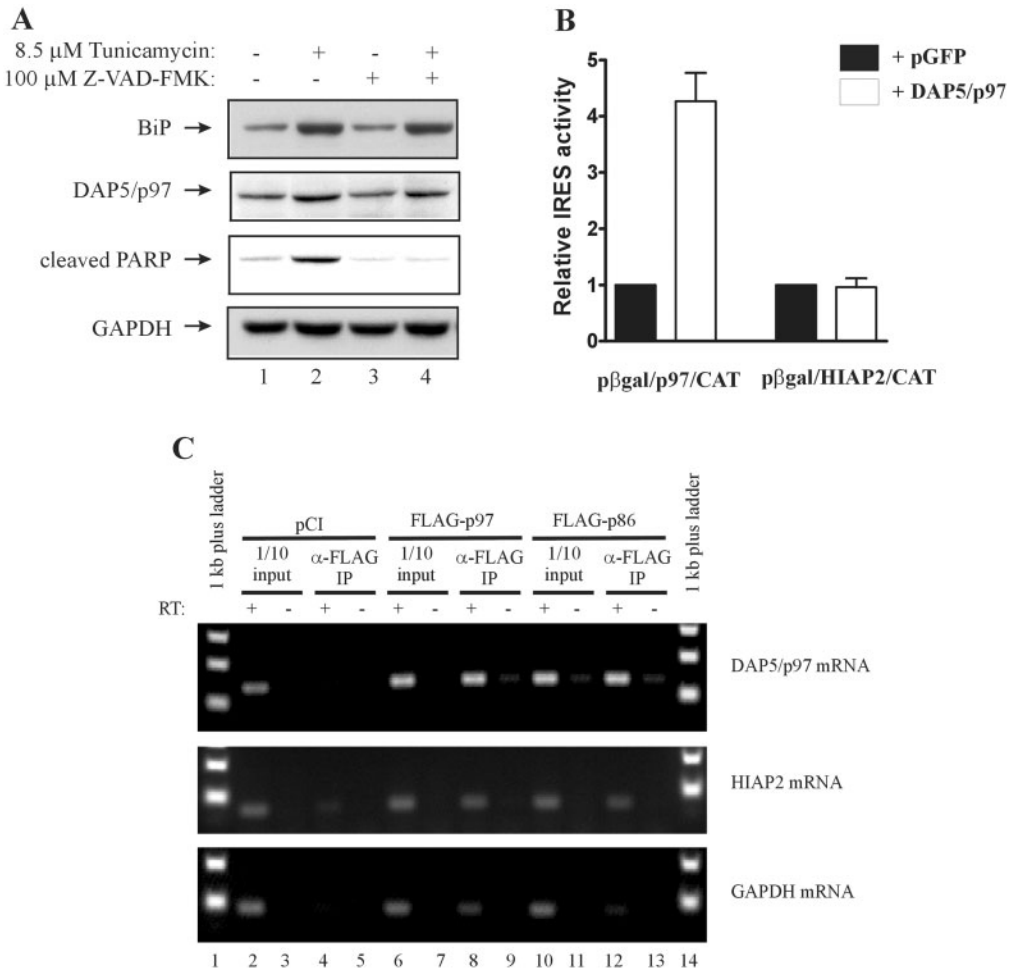

Figure 5. Translational induction of DAP5/p97 during ER stress is caspase-independent. (**A**) HEK293T cells were pre-treated with 100 μM Z-VAD-FMK or DMSO for 6 h and were then incubated with 8.5 μM tunicamycin or DMSO in the presence or absence of 100 μM Z-VAD-FMK for an additional 24 h. Protein extracts were harvested, separated by 10% SDS–PAGE, transferred to PVDF membrane and the levels of BiP, p97, GAPDH and cleaved PARP were determined by western blot analysis. (**B**) IRES activity of the DAP5/p97 and HIAP2 5′ UTRs was determined in DAP5/p97-overexpressing or control plasmid transfected cells using the bicistronic reporter plasmids described in Figure 3A. Average ± SD of three independent experiments performed in triplicate. Samples from the control plasmid transfected cells were set as 1. (**C**) Both DAP5/p97 and HIAP2 mRNA associate with full-length DAP5/p97. HEK293T cells were transfected with pCI, FLAG- DAP5/p97 or FLAG- DAP5/p86 and mRNA:protein complexes were co-precipitated using anti-FLAG coated agarose beads as described in Materials and Methods section. cDNA was produced from precipitated mRNA by reverse transcription (RT) using an oligo $d(T)_{18}$ primer, which was subsequently amplified by PCR using gene-specific oligonucleotide primers for DAP5/p97, HIAP2 and GAPDH. The resulting products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining.

full-length DAP5/p97, as well as the DAP5/p86 isoform, on IRES activity.

DISCUSSION

In this work, we demonstrate that a member of the eIF4G translation initiation factor family, DAP5/p97, is necessary for the specific activation of at least two cellular IRES elements during pharmacologically induced ER stress. We find that DAP5/p97 and HIAP2 protein levels are enhanced during ER stress; their mRNAs are selectively recruited to the polysomes via IRES elements located within their respective 5′ UTRs. We further find that this process is dependent on DAP5/p97, as reducing the levels of endogenous DAP5/p97 by RNA interference abrogated both the translation and IRES activity of DAP5/p97 and HIAP2 during ER stress. Thus, in a positive feedback loop the triggering of ER stress results in elevated levels of DAP5/p97 that, in turn, activate translation of specific mRNAs such as HIAP2 and DAP5/p97 itself under conditions of reduced cap-dependent translation. Moreover, while cleavage of p97 to the DAP5/p86 isoform is required for the induction of HIAP2 IRES activity during ER stress (11), the translational induction of DAP5/p97 during ER stress is mediated by DAP5/p97 itself in a caspase-independent manner. The pivotal role of DAP5/p97 is further strengthened by our observation that siRNA-mediated reduction of DAP5/p97 results in cell death.

DAP5/p97 was identified by several groups by virtue of its homology to eIF4G (28), as a target of the APOBEC-1 editing enzyme that is heavily edited in the liver (29) and in a functional screening assay to identify modulators

of interferon γ-induced apoptosis (30). DAP5/p97 shares significant homology to eIF4G; however, this homology is restricted to the central and C-terminal portions of eIF4G (31). Like eIF4G, DAP5/p97 can interact with eIF3, eIF4A and Mnk-1 (29,32,33). Unlike eIF4G, however, DAP5/p97 lacks the amino-terminal portion that includes the eIF4E and PABP-binding domains. Therefore, it is believed that DAP5/p97 cannot support cap-dependent translation initiation. Consistent with this idea, overexpression of DAP5/p97 was found to repress both cap-dependent and EMCV IRES-dependent translation, presumably by sequestering eIF3 and eIF4A in inactive complexes (28,29). In contrast, however, DAP5/p97-null ES cells have normal levels of global protein synthesis and show no differences in the activities of several IRES (34). Furthermore, many studies have shown that the caspase-cleaved fragment of DAP5/p97 (termed DAP5/p86), but not the full-length DAP5/p97, is capable of specifically enhancing the translation mediated by the Apaf-1, c-myc, XIAP, and HIAP2 IRES elements (10–13). Thus a model has emerged in which DAP5/p97 is activated by caspase cleavage and the resulting DAP5/p86 fragment functions as a specific translation initiation factor for cellular IRES, in particular during conditions of pathophysiological stress (4,7,11,31).

However, several recent publications have challenged this model. It was shown that the addition of exogenous DAP5/p97 can stimulate the activity of the XIAP, c-myc, DAP5/p97 and HIAP2 IRES elements in a HeLa-based cell-free translation system depleted of eIF4G (14). More recently, it was shown that DAP5/p97 can function as an activator of translation *in vivo* and this function of DAP5/p97 does not necessarily requires proteolytic processing (27,35). In these experiments, DAP5/p97 was found to be associated with polysomes, overexpression of DAP5/p97 resulted in the activation of cap-dependent reporter mRNA translation and global protein synthesis and siRNA-mediated knockdown of DAP5/p97 led to a reduction of global protein synthesis. These observations are all consistent with the role of DAP5/p97 as an activator of translation. In addition, reduced DAP5/p97 levels result in a reduction in cell viability and embryonic lethality in both zebrafish and mouse (27,34,35). Our data confirm the observation that DAP5/p97 knockdown is not compatible with cell survival. However, in contrast to the data of Lee and McCormick (15), we find that a reduction in DAP5/p97 levels does not affect global protein synthesis (Figure 4A). The likely explanation for this discrepancy is that, while Lee and McCormick assessed protein synthesis 48 h post- DAP5/p97 knockdown, in our experiments polysome profiling was performed 24 h after siRNA treatment because we observed a significant loss of cell viability after this timepoint. It should also be noted that DAP5/p97-null ES cells do not display a defect in global protein synthesis (15,34) suggesting that the role of DAP5/97 in the regulation of global translation may be cell-type specific.

We and others have shown previously that overexpression of the p86 fragment of DAP5/p97 is sufficient to activate the DAP5/p97 and HIAP2 IRES elements (10–12). Here we have extended these observations by demonstrating that both DAP5/p97 and HIAP2 IRES-mediated translation are dependent on DAP5/p97, since the knockdown of p97 significantly reduces translation of DAP5/p97 itself and HIAP2 during ER stress by precluding activation of their respective IRES elements. However, in contrast to the requirement of caspase-mediated cleavage of DAP5/p97 to the p86 isoform for induction of HIAP2 IRES activity (11), we find that the translational induction of DAP5/p97 following ER stress does not require caspase activity. Since DAP5/p97 is absolutely required for the translational induction of DAP5/p97 during ER stress, we conclude that DAP5/p97 enhances the activity of its IRES in a caspase-independent manner. These data support the hypothesis that DAP5/p97 can function as a translational activator in the absence of proteolytic processing. It is possible that other post-translational modifications of DAP5/p97 during ER stress control its ability to modulate IRES activity. Indeed, phosphorylation of DAP5/p97 at threonine 508 was identified by a large-scale proteomics study (36) and it has been suggested that phosphorylation of DAP5/p97 controls its activity in response to growth factor signaling (27). Further investigations of the signaling pathways that modulate DAP5/p97 activity during cellular stress or following growth factor stimulation should prove to be enlightening.

We sought to mechanistically determine how full-length DAP5/p97 differentiates between distinct mRNA molecules such as DAP5/p97 and HIAP2. Our data indicate that both full-length DAP5/p97 and the truncated p86 fragment are capable of binding DAP5/p97 and HIAP2 endogenous mRNAs equally well. Thus, the simple model whereby DAP5/p97 associates with a particular pool of cellular mRNAs while its DAP5/p86 truncated isoform associates with a different cohort of mRNAs is unlikely. Our observations raise the possibility that full-length DAP5/p97 preassembles with several mRNAs, but is only able to enhance IRES-dependent translation following a particular post-translational modification, which may be different depending on the target mRNA molecule. For example, caspase cleavage of DAP5/p97 to generate the DAP5/p86 isoform enhances both DAP5/p97 and HIAP2 IRES activity, whereas other post-translational modifications (such as phosphorylation) may be sufficient to allow full-length DAP5/p97 to enhance the activity of its own IRES.

In summary, our data provide direct evidence that DAP5/p97 can function as a translational activator of at least two IRES-containing cellular mRNAs during conditions of pathophysiological stress. Together with previously published observations (35), these findings support the hypothesis that DAP5/p97 controls translation of select, rather than all, mRNAs. Importantly, the fact that the DAP5/p97 IRES itself is activated by DAP5/p97 during ER stress suggests the existence of a positive feedback loop that ensures elevated levels of DAP5/p97 to support IRES-dependent translation of select mRNAs during conditions of reduced global protein synthesis.

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