Distinct Regulation of Internal Ribosome Entry Site-mediated Translation following Cellular Stress Is Mediated by Apoptotic Fragments of eIF4G Translation Initiation Factor Family Members eIF4GI and p97/DAP5/NAT1*

Tara A. Nevins, Zdena M. Harder, Robert G. Korneluk‡, and Martin Holčík§

From the Solange Gauthier Karsh Molecular Genetics Laboratory, Children’s Hospital of Eastern Ontario, and Department of Pediatrics, University of Ottawa, Ottawa, Ontario K1H 8L1, Canada

Many cellular stresses lead to the inhibition of protein synthesis. Despite this, some cellular mRNAs are selectively translated under these conditions. It was suggested that the presence of internal ribosome entry site (IRES) sequences in the 5'-untranslated regions allow these mRNAs to be actively translated despite the overall cessation of protein synthesis. Here we tested the hypothesis that the IRES elements of genes that are involved in the control of cell survival are distinctly regulated by cellular stresses. We show that the transient conditions of cellular stress favor the translation of pro-survival IRES, while the severe apoptotic conditions support translation of pro-death IRES elements. Furthermore, activation of pro-death IRES during the etoposide-induced apoptosis is caspase-dependent and can be regulated by the expression of apoptotic fragments of two members of the eIF4G translation initiation factor family, p97/DAP5/NAT1 and eIF4GI. Our results suggest that the regulation of IRES translation during stress contributes to the fine-tuning of cell fate.

Many chemotherapeutic agents and irradiation induce apoptosis in human cancers. Induction of apoptosis leads to the selective cleavage of translation initiation factors that results in an inhibition of cap-dependent protein synthesis (1). Despite this, resistant tumor cells arise that up-regulate proteins promoting their survival. Therefore, it is critical to identify the proteins and the mechanism promoting their preferential translation.

Translational control is a final regulatory step in gene expression. Cellular mRNAs are translated by the so-called ribosome scanning mechanism (2). This mechanism involves the specific recognition of the 5'-end m7G structure by the cap-binding protein eIF4E. The eIF4E is a part of the larger cap-binding protein complex eIF4F that consists of eIF4A, eIF4G, and eIF4E. The binding of eIF4F to mRNA further recruits other initiation factors as well as the 40 S ribosomal subunit. This complex is then thought to proceed in the 5’ direction until an AUG initiation codon in a favorable context is encountered, and protein synthesis is initiated.

A broad range of cellular stresses lead to the inhibition of translation. This is accomplished by (i) the phosphorylation of some initiation factors and/or their regulators (3) or (ii) by the proteolytic cleavage of several initiation factors (1). The rapid inhibition of protein synthesis is believed to function as a protective homeostatic mechanism. In this context, it is noteworthy that mRNAs encoding several oncoproteins, survival factors, and proteins critically involved in apoptosis are preferentially translated by a poorly understood cap-independent mechanism under conditions of compromised translation initiation (4). These mRNAs contain IRES (internal ribosome entry sequence) elements in their respective 5'-UTR and were shown to be translated during apoptosis (5, 6), cell cycle (7, 8), development (9), amino acid availability (10), and endoplasmic reticulum stress (11). Cellular IRES elements are found in a limited but growing number of mRNAs (12). Interestingly, they are found preferentially in the mRNAs of genes involved in the control of cellular proliferation, survival, and death (e.g. FGF2 (13), PDGF (14), VEGF (15), IGFII (16), c-Myc (17), c-Jun (18), PITSLRE (8) XIAP (5), DAP5 (6), Apaf-1 (19), and bag-1 (20). It was therefore suggested that IRES-mediated translation plays a critical role in the regulation of cell fate (4). It is not clear, however, how cellular IRES facilitate translation or how this translation mechanism is regulated.

We have described previously that the IRES element of a key intrinsic inhibitor of apoptosis, XIAP, is actively translated during serum starvation and low dose γ-irradiation (5, 21). Importantly, the IRES-mediated translation of XIAP is critical for enhanced survival of cells under acute, but transient conditions of cellular stress, thus supporting the notion that IRES-mediated translation regulates cell fate (5). In this study we wished to investigate the XIAP IRES translation under different cellular stresses. Furthermore, we wished to test the hypothesis that the IRES elements of other cellular mRNAs that are involved in the cell survival are regulated by physiological stress. We demonstrate that XIAP IRES is active in the condi-
tions of transient stress. In addition, we find that while transient cellular stress favors the translation of pro-survival IRES, severe apoptotic conditions support translation of pro-death IRES. Importantly, the activation of Apaf-1 and DAP5 IRES elements during etoposide-induced apoptosis is caspase-dependent and correlates with the expression of apoptotic fragments of eIF4G initiation factor family members eIF4G1 and p97/DAP5/NAT1.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Human embryonic kidney (293T), human cervical carcinoma (HeLa), human bladder carcinoma (T24), human glialoblastoma (SF539), Chinese hamster ovary (CHO), and mouse fibroblast (NIH3T3) cell lines were cultured in standard conditions in Dulbecco’s modified Eagle’s medium (293T, HeLa, NIH3T3, SF539), F-12 (CHO), or McCoy’s 5A (T24) medium supplemented with 10% fetal calf serum, glutamate, and antibiotics. Transient DNA transfections were done using LipofectAMINE Plus (NIH3T3 and T24 cells) or LipofectAMINE 2000 (293T, HeLa, CHO, and SF539 cells) and the protocol provided by the manufacturer (Invitrogen). Briefly, cells were seeded at a density of 3 × 105 cells/ml in six-well plates and were transfected 24 h later in serum-free Opti-MEM medium (Invitrogen) with 2 µg of DNA. The protocol procedure was replaced 24 h later with fresh medium supplemented with 10% fetal calf serum (LipofectAMINE Plus) or left on the cells (LipofectAMINE 2000). Cells were collected for analysis 24 h post-transfection. For the cellular stress experiments 293T cells were seeded at a density of 3 × 105 cells/ml in six-well plates coated with poly-n-lysine (5 µg/ml) along with transfection mixture (12 µl of LipofectAMINE 2000 + 2 µg DNA in 500 µl of Opti-MEM per well). 24 h after seeding and transfection the cells were exposed to either anoxia (90% N2, 5% H2, 5% CO2 in a MACS-VA500 microaerophilic system), heat stress (42°C), or etoposide (26), and the CAT levels were determined using the CAT analysis 24 h post-transfection. For the cellular stress experiments the transfection mixture was replaced 3 h later with fresh DMEM/H11032.

Poly(A)angles were constructed from bicistronic construct by removing the IRES, severe apoptotic conditions support translation of pro-survival IRES. It is noteworthy that although the cellular IRES elements were not as dramatic as in this cell line than in any other cell line tested. These results suggest that the activity of cellular IRES elements vary among various cell lines and are not present or are present at low concentration in 293T cells. We therefore transfected the IRES-containing plasmids into five different cell lines (Fig. 2). In all cell lines tested the translation directed by IRES of DAP5, Apaf-1, c-Myc, BiP, and EMCV were PCR-amplified and inserted into this construct (Fig. 1). Bicistronic plasmids were transfected into 293T cells, and the activity of IRES-directed translation was determined. Surprisingly, the activity of Apaf-1, DAP5, BiP, and c-Myc IRES elements was only 3–4-fold above that of the control plasmid (each IRES element in the antisen orientation) In contrast, the IRES elements of XIAP and EMCV directed translation at −40- and 80-fold, respectively, above background (Fig. 2). The activity of all IRES elements in the antisen orientation was indistinguishable and was the same as that of the empty bicistronic vector (data not shown). It has been suggested that cellular IRES elements may require specific protein factors for their function (27). One reason why we observed very low levels of activity for Apaf-1, DAP5, BiP, and c-Myc IRES could have been that these auxiliary factors vary among various cell lines and are not present or are present at low concentration in 293T cells. We therefore transfected the IRES-containing plasmids into five different cell lines (Fig. 2). In all cell lines tested the translation directed by IRES of DAP5, Apaf-1, BiP, and c-Myc was very low when compared with EMCV and XIAP IRES elements. The exception was the T24 bladder carcinoma cell line where the translation of XIAP IRES was very weak. In contrast, translation of EMCV IRES was stronger in this cell line than in any other cell line tested. These results suggest that the activity of cellular IRES elements vary dramatically. In all cases the cellular IRES elements were not as efficient in directing the translation of the downstream cistron as the viral (EMCV) IRES. IRES-directed translation is cell line-dependent, which likely reflects the availability or status of transacting protein factors that are involved in the facilitation of IRES translation. It is noteworthy that although the cellular IRES elements used in this study originate from human mRNAs they were able to direct translation in human as well as rodent cell lines (CHO, NIH3T3), confirming that the mechanism of IRES-dependent translation is evolutionarily conserved.

RESULTS AND DISCUSSION

Cellular IRES Elements Exhibit Variable Strength and Cell Line Specificity—To allow direct comparison of translation mediated by various IRES elements we used the previously described bicistronic plasmid pgplα/CAT that uses β-galactosidase and chloramphenicol acetyltransferase as first and second cistrons, respectively (5). The IRES elements of XIAP, DAP5, Apaf-1, c-Myc, BiP, and EMCV were PCR-amplified and inserted into this construct (Fig. 1). Bicistronic plasmids were transfected into 293T cells, and the activity of IRES-directed translation was determined. Surprisingly, the activity of Apaf-1, DAP5, BiP, and c-Myc IRES elements was only 3–4-fold above that of the control plasmid (each IRES element in the antisen orientation). In contrast, the IRES elements of XIAP and EMCV directed translation at −40- and 80-fold, respectively, above background (Fig. 2). The activity of all IRES elements in the antisen orientation was indistinguishable and was the same as that of the empty bicistronic vector (data not shown). It has been suggested that cellular IRES elements may require specific protein factors for their function (27). One reason why we observed very low levels of activity for Apaf-1, DAP5, BiP, and c-Myc IRES could have been that these auxiliary factors vary among various cell lines and are not present or are present at low concentration in 293T cells. We therefore transfected the IRES-containing plasmids into five different cell lines (Fig. 2). In all cell lines tested the translation directed by IRES of DAP5, Apaf-1, BiP, and c-Myc was very low when compared with EMCV and XIAP IRES elements. The exception was the T24 bladder carcinoma cell line where the translation of XIAP IRES was very weak. In contrast, translation of EMCV IRES was stronger in this cell line than in any other cell line tested. These results suggest that the activity of cellular IRES elements vary dramatically. In all cases the cellular IRES elements were not as efficient in directing the translation of the downstream cistron as the viral (EMCV) IRES. IRES-directed translation is cell line-dependent, which likely reflects the availability or status of transacting protein factors that are involved in the facilitation of IRES translation. It is noteworthy that although the cellular IRES elements used in this study originate from human mRNAs they were able to direct translation in human as well as rodent cell lines (CHO, NIH3T3), confirming that the mechanism of IRES-dependent translation is evolutionarily conserved.
Distinct Regulation of IRES Translation during Cellular Stress—It has been proposed that IRES-mediated translation is preferentially utilized during cellular stress (4), because it employs different regulatory mechanisms than cap-dependent translation. In addition, IRES-mediated translation may enable selective regulation of IRES-driven genes in response to divergent cellular stress. Indeed, we have shown that translation of XIAP IRES is enhanced during serum starvation or low dose γ-irradiation (5, 21). Similarly, the IRES of c-Myc was shown to be maintained following genotoxic stress despite overall reduction of protein synthesis (28). To further test this hypothesis, transiently transfected 293T cell were subjected to three different types of cellular stress (heat stress, anoxia, and etoposide), and the efficiency of cap-dependent versus IRES-dependent translation was determined for each IRES element. While both heat stress and anoxia could be considered as transient conditions, the treatment of cells with etoposide will induce apoptosis and represents severe conditions of cellular stress. As summarized in Fig. 3, diverse treatments evoked distinct changes in the translation mediated by different IRES elements. Heat stress resulted in significant reduction of IRES translation. In particular, the translation mediated by the IRES of Apaf-1 and DAP5 was completely abolished. The anoxic conditions resulted in an initial reduction of IRES translation that was followed by partial (DAP5) or almost full (BiP, c-Myc) restoration of IRES activity. Significantly, translation mediated by XIAP and EMCV elements was enhanced following prolonged anoxic treatment. In contrast, translation mediated by the IRES element of Apaf-1 was completely repressed. The treatment of cells with etoposide resulted in the induction of cell death with only 15–20% of surviving cells at 48 h (data not shown). More importantly it also prevented the induction of translation of both Apaf-1 and DAP5 IRES elements, suggesting that the activation of caspases is required for the stimulation of IRES activity (Fig. 4A). Since the naturally occurring cellular mRNAs exist as monocistronic mRNAs, we next wished to test whether the enhanced translation of Apaf-1 and DAP5 IRES is also true in the monocistronic context. Treatment of transiently transfected cells with etoposide and/or caspase inhibitor zVAD resulted in the enhancement of Apaf-1 and DAP5 IRES-containing monocistronic reporter constructs to levels similar to those observed with the bicistronic constructs (Fig. 4A).

Apaf-1 and DAP5 IRES Are Activated by Caspase-mediated Fragments of the eIF4G Family of Translation Initiation Factors—Since induction of apoptosis leads to many intracellular changes, including activation of nucleases, we wished to determine whether the changes in Apaf-1 and DAP5 IRES activity could be due to activation of nucleases with subsequent production of functionally monocistronic reporter mRNAs. However, no differences in the integrity or the amount of the bicis-
Electronic RNA were observed between etoposide-treated and control cells excluding this possibility (Fig. 4B). Induction of apoptosis leads to the caspase-mediated cleavage eIF4G family members (eIF4GI, eIF4GII, and p97/DAP5/NAT1), which in turn inhibits translation (6, 25). It was suggested that the apoptotic fragments of the eIF4G proteins would allow preferential translation of IRES-containing mRNAs (1, 4, 29). Indeed, the initiation factor p97/DAP5/NAT1 is processed by caspases to produce a truncated form DAP5/p86, which can stimulate the translation of its own IRES thus establishing a positive feedback loop (6). Similarly, both eIF4GI and eIF4GII are cleaved by caspases to produce distinct fragments, although

Fig. 2. The relative translational efficiency of distinct IRES elements is cell line-specific. Human embryonic kidney (293T), human cervical carcinoma (HeLa), human bladder carcinoma (T24), human glioblastoma (SF539), CHO, and mouse fibroblast (NIH3T3) cell lines were transfected with indicated bicistronic plasmids as described under "Experimental Procedures," and the cells were collected for analysis 24 h post-transfection by washing in cold phosphate-buffered saline and lysing in the CAT ELISA kit lysis buffer (Roche Molecular Biochemicals), and cell extracts were prepared using the protocol provided by the manufacturer. β-Galactosidase enzymatic activity in cell extracts was determined by the spectrophotometric assay using o-nitrophenyl-β-D-galactopyranoside (26), and the CAT levels were determined using the CAT ELISA kit (Roche Molecular Biochemicals) and the protocol provided by the manufacturer. The relative IRES activity was determined as a ratio of CAT/β-gal. The activity of each IRES construct in an antisense orientation was set as 1 in each experiment. The bars represent the average ± S.D. of three independent experiments performed in triplicates.
their ability to support IRES translation has not been determined (25, 30, 31). While the caspase cleavage of eIF4GII produces several fragments that do not persist in the apoptotic cell (32), the middle part of the caspase-cleaved eIF4GI, termed M-FAG/p76, retains the core region capable of binding eIF3 and eIF4A that supports translation initiation (33) and is associated with ribosomes in apoptotic cells (25). Therefore, we wished to test whether apoptotic fragments of the eIF4G family could be responsible for the induction of cellular IRES elements, particularly Apaf-1 and DAP5, in our experimental system. 293T cells were co-transfected with IRES bicistronic plasmids and either GFP (pGFP), p97/DAP5/NAT1 (p97), DAP5/p86 (p86), N-FAG/p76, or M-FAG expression plasmids, and the relative activity of IRES translation was determined.

**Table I**

| Construct          | Relative IRES activity |
|--------------------|------------------------|
| Apaf-1 (control)   | 0.205 ± 0.029          |
| Apaf-1 (etoposide) | 0.129 ± 0.016          |
| DAP5 (control)     | 0.198 ± 0.019          |
| DAP5 (etoposide)   | 0.114 ± 0.012          |

**Fig. 3. IRES-mediated translation following cellular stress.** Transiently transfected 293T cells were exposed to either heat stress (A) (42 °C), anoxia (B), or etoposide (C) (200 μM) for 24 h (gray bars) or 48 h (white bars) and then collected for analysis as described under “Experimental Procedures.” The relative IRES activity in the untreated cells was set as 100 for each IRES (black bars). The bars represent the average ± S.D. of three independent experiments performed in triplicates (*, p < 0.05, one-way ANOVA). D, the stress conditions were verified by Western blot analysis using antibodies against marker proteins Hsp70, HIF-1α, and cleaved caspase-3.
We observed that the overexpression of p86/DAP5 resulted in a significant increase in the activity of Apaf-1 and DAP5, but not the other IRES elements. The overexpression of p97/DAP5/NAT1 had no effect on the translation of either IRES element tested. Similarly, the expression of M-FAG/p76 stimulated translation of Apaf-1 and DAP5 IRES elements, while the expression of the N-terminal fragment of eIF4GI, N-FAG, had no effect on IRES translation. It should be noted, however, that the p86/DAP5 fragment had a stronger effect on the IRES translation than M-FAG/p76.

In this study we have shown that distinct cellular IRES elements are regulated by different cellular mechanisms. We tested the hypothesis that IRES elements of cellular mRNAs involved in the control of cell survival are differentially regulated by physiological stress. It was anticipated that physiological conditions favoring translation of pro-death mRNAs such as DAP5, Apaf-1, or c-Myc would suppress translation of pro-survival molecules such as XIAP and vice versa. Indeed, we observed that different conditions favored distinct IRES elements. Although heat stress reduced activity of all IRES ele-

(Fig. 4, C and D, and Table II).
ments studied, XIAP, c-Myc, and BiP were affected to a lesser extent than Apaf-1 or DAP5. Conversely, following 48 h of anoxia the activity of XIAP IRES was enhanced, while the activity of Apaf-1 and DAP5 IRES were reduced. The most dramatic difference was seen following the treatment of cells with the apoptosis-inducing agent etoposide. This resulted in the significant increase in Apaf-1 and DAP5 IRES activity, while XIAP, BiP, and c-Myc IRES were repressed. Importantly, these results suggest that the cellular IRES elements may belong to separate and distinct classes that will likely share either common regulatory mechanisms or trans-acting factors. For example, both Apaf-1 and DAP5 IRES were severely inhibited by heat stress and induced by the etoposide treatment, while other IRES were not. Similarly, XIAP and EMCV elements were the only two IRES induced by anoxia. The identification of the shared elements among various IRES elements awaits further experiments.

To further understand the mechanisms of regulation of IRES translation during cellular stress, we investigated the etoposide-induced enhancement of IRES translation in more detail. We found that the activation of Apaf-1 and DAP5 IRES elements is caspase-dependent, since the pretreatment of cells with caspase inhibitor zVAD.fmk prevented IRES translation enhancement. Significantly, we found that this specific activation of Apaf-1 and DAP5 IRES elements correlated with the expression of apoptotic fragments of the eIF4G family of proteins (34). However, in contrast we did not observe any effect of overexpression of DAP5/p86 on the translation of XIAP and c-Myc IRES elements. Furthermore, in our experiments we observed 6-fold and 21-fold induction of Apaf-1 and DAP5 IRES, respectively, while Henis-Korenblit et al. (34) reported modest 2- and 3-fold increases in the activity of the same IRES elements. In addition, our results oppose the finding of Henis-Korenblit et al. (34) that the overexpression of M-FAG/p76 has no effect on IRES translation. While it is difficult to determine the reason for these discrepancies, it is possible that the use of different reporter systems and/or the levels of overexpressed proteins could contribute to these differences. However, in our study the in vivo caspase-dependent induction of Apaf-1 and DAP5 IRES elements by etoposide parallels the induction seen by overexpression of either DAP5/p86 or N-FAG/p76. This suggests that only these two IRES elements are physiologically inducible by the apoptotic fragments of eIF4G family of proteins. Furthermore, the p97/DAP5/NAT1 is a promoter of apoptosis (6), and therefore it would be expected to enhance translation of pro-death (Apaf-1 and DAP5) but not the pro-survival (XIAP) IRES elements.

Our data support the hypothesis that IRES-mediated translation escapes the control mechanisms that regulate cap-dependent translation during the conditions of cellular stress. In addition, conditions of transient cellular stress, such as anoxia, favor translation of pro-survival IRES, such as XIAP, while the severe apoptotic conditions result in the activation of pro-death IRES of Apaf-1 and DAP5. While the exact molecular pathways that regulate IRES translation in transient cellular stress need to be further investigated, our results indicate that the apoptotic fragments of eIF4G translation initiation factor family mediate up-regulation of pro-death IRES elements during apoptotic stress and may contribute to the acceleration of cell death.

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