IRES-Mediated Protein Translation Overcomes Suppression by the p14ARF Tumor Suppressor Protein

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Abstract

Internal ribosome entry sites (IRES elements) have attracted interest in cancer gene therapy because they can be used in the design of gene transfer vectors that provide bicistronic co-expression of two transgene products under the control of a single promoter. Unlike cellular translation of most mRNAs, a process that requires a post-translational 5’ modification of the mRNA known as the cap structure, IRES-mediated translation is independent of the cap structure. The cellular conditions that may intervene to modulate IRES-mediated, cap-independent versus cap-dependent translation, however, remain poorly understood, although they could be critical to the choice of gene transfer vectors. Here we have compared the effects of the p14ARF (Alternate Reading Frame) tumor suppressor, a translational suppressor frequently overexpressed in cancer, on cap-dependent translation versus cap-independent translation from the EMCV viral IRES often used in bicistronic gene transfer vectors. We find that ectopic overexpression of p14ARF suppresses endogenous and ectopic cap-dependent protein translation, consistent with other studies. However, p14ARF has little or no effect on transgene translation initiated within an IRES element. This suggests that transgenes placed downstream of an IRES element will retain efficient translation of their gene products in the presence of high levels of ectopic or endogenous p14ARF, a finding that could be particularly relevant to therapeutic gene therapy strategies for cancer.

Key words: p14ARF, Internal ribosome entry site (IRES), cap-dependent, protein translation.

Introduction

Initiation of protein translation from an internal ribosome entry site (IRES) is an alternative type of protein translation in which ribosomes are recruited directly to an mRNA by virtue of its secondary structure, independently of the 5’ modification of the mRNA known as the cap structure, a modification required for the recruitment of the translation initiation factor protein (eIFs) to most cellular mRNAs (see review [1-3]). IRES often eliminates the involvement of eIFs, activated by binding to specific sets of proteins, referred to as IRES trans-acting factors (ITAFs). IRES-mediated translation was first described in poliovirus RNA and encephalomyocarditis virus (EMCV) RNA [4, 5], and is now thought to contribute to the translation of certain cellular RNAs as well, particularly under abnormal cellular states triggered by hypoxia or cellular stress [6]. So far, multiple IRES were identified with different mechanisms in translational regulation. They may or may not use eIFs and ITAFs to recruit the ribosome to the message.

IRES-mediated translation has become
increasingly attractive as a tool in biotechnology because it provides a means to achieve bicistronic co-expression of transduced genes under the control of a single transcriptional promoter. When an IRES segment is located between two genes in a eukaryotic mRNA molecule, it can drive translation of the downstream protein coding region independently of the 5’-cap structure bound to the message. Nevertheless, the regulation of IRES-mediated translation has not been fully explored and remains poorly understood.

In this study we asked how IRES-mediated translation is affected by the 14ARF (alternate reading frame) tumor suppressor, a translational repressor [7] induced in cells with activated oncogenes [8, 9]. p14ARF could inhibit the polysome formation and protein synthesis by interacting with the nucleolar 60 S preribosomal particle [6]. p14ARF expression is undetectable in most normal cells, but evidence suggests that its levels increase at an early stage of tumorigenesis [10] and elevated p14ARF levels have been observed in certain cancers [11-13]. This increased expression could therefore affect the outcome of some therapeutic gene transfer strategies.

Because of the differences in the mechanisms by which CAP-dependent and IRES-mediated, CAP-independent translation are initiated, it seemed possible that p14ARF could have differential effects on these two modes of translation that could be relevant to use of IRES-containing gene transfer vectors. We report here our finding that messages translated from IRES elements display little or no suppression by p14ARF, suggesting that IRES-mediated translation may be particularly useful for gene therapy applications in certain cancers where p14ARF expression is high.

When multiple tumor suppressor genes are used in cancer gene therapy simultaneously, they sometimes interfere with each other. Our study proposed a model that when two tumor suppressor genes are constructed in one expression cassette containing IRES element, the IRES could protect the downstream gene from the inhibition induced by upstream gene, and this will make a common sense for other genes. Therefore, if we need to employ multiple tumor suppressor genes in gene therapy and to avoid the mutual interference, it is better to put genes in one cassette and introduce IRES between the genes, instead of applying two genes separately.

**Materials and methods**

**Cell culture**

All cultures were maintained at 37°C in 10% CO₂ in Dulbecco’s Modified Eagles Medium supplemented with nonessential amino acids, pyruvate, L-glutamine, gentamicin, and 10% FBS. DLD-1 human colon cancer cells were obtained from the American Type Culture Collection. OE33 human esophageal cancer cells originated from the European Collections of Cell cultures and were provided to us by Dr. Rebecca Fitzgerald, Cambridge University, UK. To obtain OE33-GFP and OE33-IRES-GFP, we stably modified OE33 cells with plasmid constructs pLXSN-GFP (for OE33-GFP) and pLXSN-IRES-GFP (for OE33-IRES-GFP), in which GFP message is translated in a cap-dependent or cap-independent manner, respectively. The GFP and IRES (ECMV) sequences were derived from pIRES-GFP (Clontech, Mountainview, CA). pLXSN was obtained from Clontech, Mountainview, CA. GFP-expressing clones were selected in G418 followed by cell sorting. Mouse Embryo Fibroblasts, nullizygous for p53 and mdm2, were generously provided by Dr. Guillermina Lozano, Department of Molecular Genetics, the University of Texas M.D. Anderson Cancer Center, Houston, TX.

**Vectors and treatment conditions**

The replication-defective adenoviral vectors, AdLuc, encoding firefly luciferase, and Adp53, encoding the human p53 tumor suppressor gene, were purchased from Vector Biolabs, Philadelphia, PA. The replication-defective adenoviral vectors, Adp14, encoding the human p14ARF (Alternate Reading Frame) tumor suppressor gene, and Adp14/p53, encoding both human p14ARF and p53 in a bicistronic configuration, have been described [14]. Cells were treated with adenoviral vectors for 4 hours with 50-100 plaque-forming units (pfu) per cell in culture medium containing 2% serum, as previously described [14]. Human mdm2 siRNA was purchased from Dharmacon (Chicago, Il). For siRNA treatments, 4 x 10⁶ adenoviral vector-treated cells were subjected to electroporation (200V) in the presence of 150 pmol siRNA.

**Western analysis**

Western analyses were carried out using 40 µg cell lysates as previously described [14, 15] using mouse monoclonal anti-human mdm2 SMP14 (Santa Cruz Biotechnology, Santa Cruz, CA).

**Metabolic labeling and fluorography**

GFP-expressing OE33-GFP and OE33-IRES-GFP cells were metabolically labeled for 1 hour with [³⁵S]-L-methionine (1175 Ci/mmol, MP Biomedicals, Solon, OH), followed by immunoprecipitation of GFP protein, SDS-PAGE and fluorography as previously described [16, 17]. Mouse monoclonal anti-GFP antibody was obtained from Santa Cruz...
Biopolis Biotechnology, Santa Cruz, CA).

**Polysome fractionation and analysis**

Post-mitochondrial cytoplasmic fractions were sedimented on 17 ml 10-50% sucrose gradients following published procedures [18]. 1.5 ml fractions were collected and RNA was extracted and analyzed by semi-quantitative RT-PCR as previously described [14, 15]. p53 primers have been previously described [19]. The RT-PCR products were resolved on a 1.0% agarose gel stained with ethidium bromide, and band intensities were quantitated digitally and represented as a percent of total intensity. Results of each polysome analysis were confirmed by at least one independent repeat experiment.

**Results and Discussion**

The total level of a given protein subjects to two events, synthesis and degradation. It is known that p14ARF has a specific effect on p53 protein stability by binding with mdm2, the major inhibitor of p53 [20], therefore, our study focused on detection of the rates of p53 synthesis rather than total accumulation. Rates of protein are related to the number of ribosomes per message, with rapidly synthesized proteins being derived from messages that are heavily occupied with ribosomes and that sediment more rapidly. Alternatively, rates of protein synthesis rates are related to the incorporation of [35S]-methionine during a pulse.

We first investigated how overexpressed ectopic p14ARF affects the translation rate of an endogenous gene by examining the distribution of endogenous p53 message in polysomes from p14ARF-transduced DLD-1 human colon cancer cells, following sedimentation of post-mitochondrial cytoplasmic fractions on sucrose density gradients. A typical sedimentation profile of polysomes from DLD-1 cells, monitored by absorbance at 260 nm, is shown in Figure 1a, where those with heavier ribosome occupation appear in the rapidly sedimenting polysome region towards the right of the profile, and 60S ribosomal subunit of 80S initiation complex located in the left. The staining profile of ribosomal RNA is shown underneath it. Figure 1b shows the distribution of endogenous p53 message in polysomes of DLD-1 cells 24 hours post-treatment with 50 plaque-forming units (pfu) per cell of either a control replication-defective adenoviral vector encoding firefly luciferase (AdLuc) or a replication-defective adenoviral vector encoding p14ARF (Adp14). As we have previously reported, expression of p14ARF protein in DLD-1 cells is low to undetectable [14]. Treatment with Adp14 leads to about a 20-fold increase in p14ARF message levels by 24 hours post-treatment, and results in readily detectable levels of p14ARF protein on Western blots (data not shown). Suppression of cell viability by Adp14 treatment only becomes evident after 72 hours [14]. We found that in cells treated with AdLuc, endogenous p53 message was broadly distributed across the polysome profile, peaking in fraction 5, indicating efficient recruitment of message to the heavier polysome region and efficient translation. In contrast, in cells treated with Adp14, endogenous p53 message shifted to the left of the profile, peaking in fraction2, a region occupied by monosomes and small polysomes associated with slowly translated messages (Figure 1b). Figure 1c shows the correspondant polysome profiles from the cells treated with AdLuc and Adp14. Thus, endogenous p53 message is less efficiently translated in cells overexpressing ectopic p14ARF, consistent with previous reports that p14ARF can inhibit translation initiation [7].

However, a different result was observed when we examined ectopic expression of p53 following treatment of DLD-1 cells with a bicistronic adenoviral vector encoding both p14ARF and p53 in a single transcript (Adp14/p53), where the p53 open reading frame is situated downstream of an encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES). We found that in DLD-1 cells treated either with Adp14/p53 or with the Adp53 single gene vector (Figure 2a), the ectopic p53 message was broadly distributed across the polysome profile, both peaking in fraction 5. Relative polysome profiles for the vector treatment were shown in Figure 2b. Treatment conditions, as previously shown, were such that p53 message levels increased by some 20-fold by 24 hours post-treatment of DLD-1 cells with either Adp53 or Adp14/p53 [14]. Message levels of p14ARF also increased by some 20-fold in Adp14/p53-treated cells [14].

We ruled out a possible role of the mdm2 protein in maintaining p53 translation rates in Adp14/p53-treated cells. The mdm2 gene is induced by p53 [21], and mdm2 protein can in turn stimulate p53 translation [22]. Induced mdm2 could therefore potentially offset the possible negative effects of over-expressed ectopic p14ARF. However, we found that siRNA treatment to downregulate mdm2 protein levels in Adp14/p53-treated cells by 48 hours post-treatment (Figure 2c, lane 4 versus lane 3) had no effect on the 48 hour polysome distribution of ectopic p53 (Figure 2a). This indicates that the translation of p53 from the bicistronic construct is intrinsically less susceptible p14ARF-mediated translational suppression than is translation of the endogenous p53 transcript, independently of mdm2.
Figure 1. Effect of p14ARF on endogenous p53 translation in DLD-1 human colon cancer cells. (a) Typical O.D.260 profile of fractionated polysomes and ribosomal RNA staining profile from 2 x 10^7 DLD-1 cells. Arrow indicates direction of sedimentation. (b) Distribution of endogenous p53 message in fractions of polysomes, prepared from 2 x 10^7 DLD-1 cells 24 hours post-treatment (50 pfu/per cell, 4 hours) with AdLuc control vector or Adp14. Fractions (1.5 ml) were collected from the top of the gradient, RNA was purified, and subjected to semi-quantitative RT–PCR analysis of p53. (c) Relative polysome profiles from the cells treated with AdLuc and Adp14.

Figure 2. Effect of p14ARF on ectopic p53 translation in DLD-1 human colon cancer cells. Distribution of ectopic p53 message in various polysome fractions from 4 x 10^6 DLD-1 cells, 48 hours post-treatment (50 pfu/cell, 4 hours) with Adp53, or Adp14/p53 bicistronic vector, or with Adp14/p53 bicistronic vector followed by electroporation with human mdm2 siRNA. (b) Relative polysome profiles for all the adenovirus treatment, separately. (c) Western analysis of human mdm2 protein expression in DLD-1 cells, 48 hours after treatment with the indicated vectors +/- mdm2 siRNA.

We therefore considered the possibility that the failure of p14ARF to suppress ectopic p53 translation in Adp14/p53-treated cells derived from the characteristics of the bicistronic vector itself, and in particular, the presence of the IRES in the bicistronic vector and the fact that translation from an IRES element was cap-independent, while translation of the endogenous message was cap-dependent. To test this, we used p14ARF and p53 single gene and bicistronic adenoviral vectors that would allow us to compare these two modes of translation for ectopic p53 under similar conditions (see diagram in Figure 3a). Translation of ectopic p53 message was monitored in p53/mdm2 doubly nullizygous mouse embryo fibroblasts (DN-MEFs), which lack endogenous p53 message. Polysome analyses were carried out 24 hours post-treatment with (a) Adp53 alone, where p53 translation is cap-dependent, or (b) with a combination of Adp53 plus Adp14, or (c) with the Adp14/p53 bicistronic vector, where p53 translation is cap-independent. Treatment conditions for Adp14 or Adp14/p53 were 100 pfu/cell and produced the
same level of p14ARF protein (Figure 3b). We found that ectopic p53 message in Adp53-treated DN-MEFs was efficiently translated, as indicated by the broad distribution of message across the polysome profile, peaking in fraction 5 (Figure 3c). The results are consistent with the results with Adp14-treated DLD-1 cells (Figure 2a). We found that ectopic p53 message was less efficiently translated in Adp53 + Adp14-treated DN-MEFs, as indicated by the shift in the polysome distribution of ectopic p53 message toward the left of the gradient, peaking in fraction 2 (Figure 3c). Thus, cap-dependent translation of ectopic p53 is less efficient in the presence of overexpressed ectopic p14ARF. In contrast, we found that ectopic p53 message in Adp14/p53-treated DN-MEFs, was efficiently translated, despite the overexpression of p14ARF, as indicated by the polysome profile peaking in fraction 5 (Figure 3c). The profile is similar to that observed with Adp53-treated DN-MEFs and Adp14/p53-treated DLD-1 cells. Thus, IRES-mediated, cap-independent translation of ectopic p53 is efficient, even in the presence of overexpressed ectopic p14ARF.

To confirm and extend these observations to another cell line and to another gene, and to use a different method to evaluate protein synthesis rates, we prepared stably-modified variants of the OE33 human esophageal adenocarcinoma cell line, in which the Green Fluorescent Protein (GFP) gene was placed either downstream of a viral LTR promoter (OE33-GFP, providing cap-dependent translation of GFP) or an IRES (OE33-IRES-GFP, providing cap-independent translation of GFP). The two modified clones were treated with Adp14 (100 pfu/cell) and 2 days later the rate of GFP protein synthesis was monitored by pulsing cells with $[^{35}S]$:methionine, followed by SDS/PAGE and fluorography of immunoprecipitated GFP. A typical fluorograph of $[^{35}S]$:methionine incorporation into GFP is shown in Figure 4a, where each lane represents $10^5$ cell equivalents. A bar graph representing an average of two experiments is shown in Figure 4b. A Western blot confirming that p14ARF was induced to similar levels 24 hours post-infection of both cell lines is shown in Figure 4c. The results show that cap-dependent translation of GFP in OE33-GFP cells modified with an LTR-GFP construct is suppressed on the average by some 90% in Adp14 treated cells compared to AdLuc-treated cells (Figure 4b, black bar), while cap-independent translation of GFP in OE33-IRES-GFP cells modified with an IRES-GFP construct shows only weak to no suppression by Adp14 (Figure 4b, gray bar). The ability of IRES-mediated protein translation to escape suppression by p14ARF appears therefore to be a general phenomenon with potential relevance to any gene transfer protocol involving p14ARF.
Multiple tumor suppressor genes and molecular pathways have been disrupted in cancer. However, the current strategy of cancer therapy usually focuses on repairing a particular gene or pathway, leading to a limited therapeutic effect. p53 and p14ARF have been reported to be loss of function in about 50% and 40% of cancer patients separately. In our study, we choose p53 and p14ARF as our selected target, and our results suggest that for gene transfer applications where p14ARF is co-expressed with another transgene, it may be necessary to place the second transgene downstream of an IRES element to insure maximum expression of its gene product. Similarly, for cancer cells with a disabled apoptotic pathways, and characterized by high level expression of endogenous p14ARF due to chronic oncogene activation, it may be necessary to employ gene transfer vectors that incorporate IRES elements in order to ensure maximum transgene expression following gene transfer. However, we didn’t check other viral IRES types, whether our conclusion applied to EMCV IRES only, or other IRES elements, is still unknown.

Further studies will be needed to determine whether selective suppression of cap-dependent translation by p14ARF involves a specific interaction of p14ARF with a translational regulatory protein, and whether cellular IRES elements are also resistant to p14ARF-mediated translational suppression. In preliminary work, we have been unable to find evidence for the binding of p14ARF to the eIF4E binding protein, an upstream regulator of the eIF4E translation initiation factor, suggesting that p14ARF does not exert its differential effects via the eIF4E initiation complex (Huang Y., et al, unpublished observations).

**Conclusions**

From the conclusion of our study, we can use the Adp14/p53 in which two tumor suppressor genes were expressed in one cassette including IRES for colon cancer treatment, and achieve a significant effect of tumor suppression. Also, the ability of p14ARF to selectively suppress cap-dependent translation could have broader relevance to cellular regulation. CAP-independent translation from cellular IRES elements, though normally weak [23], is emerging as an important cellular mechanism for maintaining expression of critical gene products under conditions where cap-dependent translation is suppressed, for example during the cellular stress response [24]. Thus the induction and subcellular redistribution of p14ARF in response to certain types of DNA damage [17, 25] could have a dual consequences for the cellular stress response, the first involving its well-described role in stabilizing the p53 protein, and the second involving selective suppression of cap-dependent protein translation followed by a redirection of cellular translation.
towards IRES-mediated translation of stress response proteins.

**Abbreviations**

ARF, alternate reading frame; IRES, Internal ribosome entry site; EIF, eukaryotic translation initiation factor; ITAF, IRES trans-acting factor; EMCV, encephalomyocarditis virus; Luc, luciferase; DN, double null; MEF, mouse embryonic fibroblast

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**Competing Interests**

The authors have declared that no competing interest exists.

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