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The role of eukaryotic initiation factor 5A in the control of cell proliferation and apoptosis

Review Article

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Summary. In the past years, the attention of scientists has mainly focused on the study of the genetic information and alterations that regulate eukaryotic cell proliferation and that lead to neoplastic transformation. An increasing series of data are emerging about the involvement of the initiation phase of translational processes in the control of cell proliferation. In this paper we review the novel insights on the biochemical and molecular events leading to the initiation and its involvement in cell proliferation and tumourigenesis. We describe the structure, regulation and proposed functions of the eukaryotic initiation factor 5A (eIF-5A) focusing the attention on its involvement in the regulation of apoptosis and cell proliferation. Moreover, we describe the modulation of its activity (through the reduction of hypusine synthesis) in apoptosis induced either by tissue transglutaminase or interferon α. Finally, we propose eIF-5A as an additional target of anti-cancer strategies.

Keywords: Amino acids – eIF-5A – Hypusine – tTGase – Apoptosis – IFNα – Tumour cells

Abbreviations: IF, initiation factor; eIF, eukaryotic IF; PHAS, phosphorylated heat and acid stable protein; 5′ UTR, 5′ untranslated region; EGF, epidermal growth factor; S6K, ribosomal S6 kinase; ERK, extracellular signal regulated kinase; PE, pseudomonas exotoxin A, TAA, tumour associated antigen; MAb, monoclonal antibody; JNK-1, NH2 terminal Jun Kinase-1

Introduction

In the past years, protein synthesis has not been considered as fundamental in the control of cell proliferation. However, data are emerging on the involve-

ment of this process in cell growth and tumourigenesis. Protein biosynthesis is a central process in all living cells. It is one of the last steps in the transmission of genetic information stored in DNA on the basis of which proteins are produced to maintain the specific biological function of a given cell. Protein synthesis takes place on ribosomal particles where the genetic information transcribed into mRNA is translated into protein. The process of protein synthesis on the ribosome consists of three phases: initiation, elongation and termination. The initiation phase appears to be the main target of regulatory messages within the cell and therefore is critical for the modulation of the species of mRNA transcripts (see also below). Here we briefly describe the molecular mechanisms involved in the initiation phase of protein synthesis.

Biochemical bases of the initiation phase

In prokaryotic protein biosynthesis the initiation phase is controlled by a small number of initiation factors (IFs), IF-1, IF-2 and IF-3. Among these, IF-2 is the most likely to form a ternary complex with GTP and initiator $tRNA^{Met}_{f}$. All three factors are involved in assembling the initiation complex of initiation factors, initiator $tRNA^{Met}_{f}$, the ribosomal subunits and mRNA. Eukaryotic initiation is much more complex and involves a large number of eukaryotic initiation factors (Hershey, 1991; Merrick, 1992; Pain, 1996). The 80S ribosomes dissociate, and 40S subunits are captured for initiation by binding eIF1A and eIF3; the size of the latter causes the particle to sediment at 43S (Gaspar et al., 1994; Hannig et al., 1993). Initiator tRNA binds, in the form of a ternary complex with eIF2 and GTP, to produce the 43S preinitiation complex (Gaspar et al., 1994; Hannig et al., 1993). The 43S preinitiation complex binds to mRNA at the 5′ terminal mGTP cap structure, and then migrates along the mRNA towards the AUG initiation codon (Gaspar et al., 1994; Hannig et al., 1993). The initial binding involves the factors eIF4E, eIF4G and eIF4A, which assemble at the 5′-end of mRNA, thus creating the conditions that allow the melting of intramolecular secondary structures within the mRNA that would otherwise prevent the binding of the 43S preinitiation complex (Hershey, 1991; Pain, 1996; Rhoads, 1993; Merrick, 1994; Rhoads et al., 1994). The term 48S preinitiation complex is frequently used, and refers to the 43S – globin ~ mRNA complex formed in the reticulocyte lysate (Bommer et al., 1991). When the 43S preinitiation complex stops at the initiation codon, the GTP molecule introduced as part of the eIF2 complex is hydrolysed to GDP, and this gives energy for the ejection of the initiation factors bound to the 40S ribosomal subunit (Price et al., 1992). The initiation factor eIF5 is involved in this process which is likely to accelerate the hydrolysis of GTP (Price et al., 1992). The release of these factors allows the association of a native 60S ribosomal subunit, to reconstitute a 80S ribosome at the initiation codon positioned to commence the elongation stage of translation. The continuity of initiation events requires the recycling of initiation factor molecules. eIF2 is released as a binary complex with GDP and requires a guanine nucleotide exchange factor, eIF2B, to

catalyse the regeneration of the eIF2*GTP complex (Bommer et al., 1991; Price et al., 1992).

The initiation phase of protein synthesis is involved in the regulation of cell proliferation

There is presently a growing body of evidence which suggests the involvement of the initiation phase of protein synthesis and its translational factors in the regulation of cell proliferation and transformation.

Initially, eIF4E culminated in the unexpected finding that a moderate overexpression of this factor results in dramatic phenotypic changes, including rapid proliferation and malignant transformation (De Benedetti et al., 1999; Shantz et al., 1994; Raught et al., 1996; Rosenwald, 1996; Anthony et al., 1996; West et al., 1995). Conversely, the tumorigenic properties of cancer cells can be strongly inhibited by antisense-RNA against IF, or overexpression of the inhibitory proteins, such as 4E-BPs also called phosphorylated heat and acid stable proteins I-III (PHAS I-III) (De Benedetti et al., 1999; Shantz et al., 1994; Raught et al., 1996; Rosenwald, 1996; Anthony et al., 1996; West et al., 1995; Shantz et al., 1996). When the eIF4E is overexpressed in mammalian cells *in vitro* oncogenic transformation occurs (De Benedetti et al., 1999; Shantz et al., 1994; Raught et al., 1996; Anthony et al., 1996; West et al., 1995). Furthermore, it has been reported that c-myc expression stimulates cell growth by increasing expression of eIF-4E (Rosenwald, 1996) and overexpression of eIF4G also caused malignant transformation in NIH3T3 cells (Fukuchi-Shimogori et al., 1997). The involvement of eIF4E in carcinogenesis has been demonstrated also *in vivo*. In fact, eIF4E is overexpressed not only in all head and neck squamous cell cancers but also in some dysplastic lesions (De Benedetti et al., 1999; Nathan et al., 1999; Sorrels et al., 1999), in the germinal centers of reactive follicles of non Hodgkin's lymphomas (Wang et al., 1999), in active growing lymphocytes (Wang et al., 1999) and in colon tumours (Rosenwald et al., 1999). Beside IF overexpression, another mechanism capable of inducing cell transformation is the disregulation of the IF activity. In fact, mitogenic stimulation of protein synthesis is accompanied by an increase in eIF-4E phosphorylation that enhances its activity (Kleijn et al., 1995). In the last years other IFs have been correlated with tumorigenesis, such as eIF2alpha and eIF3-p40 (Rosenwald, 1996; Wang et al., 1999; Eberle et al., 1997; Nupponen et al., 1999). Moreover, their involvement in tumour cell survival and protection from cytotoxic agents has been shown. In fact, eIF4G is specifically degraded by caspases during the occurrence of apoptosis in lymphoma cell lines, suggesting its involvement in the protection of tumour cells from programmed cell death (Clemens et al., 1998). Another initiation translational factor involved in the regulation of cell growth and apoptosis is the Eukaryotic Initiation Factor 5A (eIF5A). This factor is peculiar because its activity is modulated by a series of post-translational modifications that culminates in the formation of the unusual amino acid hypusine.

The eukaryotic initiation factor 5A: a factor regulated by the formation of the unusual amino acid hypusine

eIF5A structure and function

Eukaryotic translation initiation factor 5A (eIF-5A), of m.w. 18-kD, is highly conserved from yeast to mammalian cells (Gordon et al., 1987; Park et al., 1984). The human cDNA that encodes the eIF-5A precursor has been cloned and sequenced (Smit-McBride et al., 1989). eIF-5A precursor [(ec-eIF-5A(lys)] is the only cellular protein known to contain a specific lysine residue which is transformed into the unique amino acid hypusine [N^{ε}-(4-amino-2-hydroxybutyl)-lysine]. This amino acid is formed by a series of post-translational reactions starting with the transfer of the butylamine moiety from spermidine to the ε-amino group of one of the lysine residues in the eIF-5A precursor protein, thus forming peptide-bound deoxyhypusine (Park et al., 1982). This intermediate is not accumulated in cells but it is immediately hydroxylated at C-2 of the incoming 4-aminobutyl moiety to form hypusine (Fig. 1) (Abbruzzese et al., 1985; Abbruzzese et al., 1986, 1988a,b). Hypusine plays a key role in the regulation of eIF-5A function because eIF-5A precursors, which do not contain hypusine, have little, if any, activity (Park et al., 1991). Moreover, the Lys50 → Arg variant is unable to stimulate methionyl-puromycin synthesis *in vitro* (Hershey et al., 1990; Beninati et al., 1995) and is inactive *in vivo* (Abbruzzese, 1988). These data suggest that hypusine synthesis is required for the biological activity of the protein and for interaction with the ribosome.

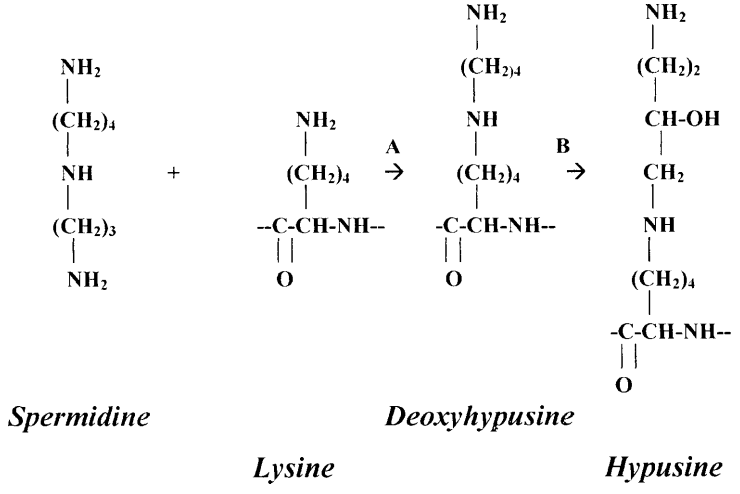

Fig. 1. The biosynthesis of the unusual amino acid hypusine. Hypusine [N^{ε}-(4-amino-2-hydroxybutyl)-lysine] is formed by a series of post-translatonal reactions starting with the transfer of the butylamine moiety from spermidine to the ε-amino group of one of the lysine residues in the eIF-5A precursor protein, thus forming peptide-bound deoxyhypusine. This reaction is catalyzed by deoxyhypusine synthase (**A**). This intermediate is not accumulated in cells but it is immediately hydroxylated by deoxyhypusine hydroxylase (**B**) at C-2 of the incoming 4-aminobutyl moiety to form hypusine

The polyamine-dependent post-translational modification of eIF-5A is involved in the control of cell growth and apoptosis

eIF-5A promotes the formation of the first peptide bond during the initial stage of protein synthesis (Hershey, 1991). The actual *in vivo* function of eIF-5A, however, is to date only partially known. A series of observations suggests that eIF-5A plays a role in cell growth and differentiation. In fact, the ec-eIF-5A(lys) modification is correlated with cell proliferation (Abbruzzese, 1988; Beninati et al., 1990; Caraglia et al., 1997) and is vital for *Saccharomyces cerevisiae* growth (Schnier et al., 1991). Moreover, agents that block the lys/hyp transformation (Park et al., 1984; Abbruzzese et al., 1991, 1989; Jakus et al., 1993) inhibit the growth of mammalian cells (Park et al., 1993) inducing reversible arrest at the G_1-S boundary of the cell cycle (Park, 1987; Lalande et al., 1990; Park et al., 1981). In fact, D,L-α-difluoromethylornithine depresses spermidine and, as a consequence, hypusine formation and produces a G_1-S block in 9L brain tumour cells (Park, 1987). In hydralazine-treated cloned mouse T cells, growth arrest in late G_1 and inhibition of deoxyhypusyl hydroxylation occur at the same time (Park, 1987). In Chinese hamster ovary cells, 2-(4-hydroxy-toluene-3-yl)-4,5dihydro-5-carboxythiazole causes both inhibition of hypusine biosynthesis and reversible cell cycle arrest in late G_1 and suppresses proliferation of human T lymphocytes *in vitro* at the G_1-S boundary (Lalande et al., 1990). Hypusine synthesis increases after mitogen treatment of human peripheral blood lymphocytes (Park et al., 1981). Moreover, both polyamine (Beninati et al., 1993) and hypusine levels (together with the enzymes that regulate their metabolism) (Abbruzzese et al., 1986, 1988a,b; Beninati et al., 1988; Schnier et al., 1991; Beninati et al., 1993) are correlated to normal and malignant growth. More recently a correlation has been found between the polyamine-dependent modification of eIF-5A and the triggering of apoptosis in tumour cells (Tome et al., 1997a). In fact, excess putrescine accumulation in hepatoma tissue culture DH23A/b cells induces apoptosis and suppresses the formation of hypusine-containing eIF-5A (Tome et al., 1997a). Moreover, DH23A cells overexpressing ornithine decarboxylase (ODC) accumulate putrescine with a consequent increase in apoptosis (Tome et al., 1997b). The latter effect induces an inhibition of the post-translational modification of eIF-5A that is also blocked by diaminoheptane, another strong inducer of apoptosis in DH23A cells (Tome et al., 1997b).

An additional function for eIF-5A: mRNA nuclear export

eIF-5A is found in the cytoplasm in two pools: one free and another bound to endoplasmic reticulum (probably its proper site of action) (Shi et al., 1996). However, it is also been recently reported that eIF-5A can accumulate at nuclear pore-associated intranuclear filaments in mammalian cells (Rosorius et al., 1999). Moreover, the factor interacts with the general nuclear export receptor CRM1 and is transported from the nucleus to the cytoplasm (Rosorius et al., 1999). More recently it has been identified a new exportin

(the exportin-4 belonging to the shuttle family called exportins) as the specific transporter of eIF-5A. Lipowsky et al. demonstrate that CRM-1 binds eIF-5A at least 1,000 times more weakly than exportin-4 (Lipowsky et al., 2000). The hypusine modification is apparently part of the signal that allows eIF-5A to access the exportin-4 pathway. In fact, recombinant eIF-5A that lacks the modification binds to exportin-4 35-fold more weakly than the fully modified protein. Deoxyhypusine can again partially, but not fully, substitute for hypusine. Exportin-4 requires a large part of eIF-5A molecule, however, for a stable binding. These findings open a new scenario in which eIF-5A may also function as nucleocytoplasmic shuttle protein of mRNAs eventually correlated with cell proliferation even if we do not yet know which RNAs eIF-5A normally interacts with (Lipowsky et al., 2000). eIF-5A could therefore function as an export adapter for these RNAs since it appears to be an RNA-binding protein with probably the hypusine modification and the C-terminal domain contributing to the interaction. However, a number of complications need to be considered. In fact, eIF-5A is present also in bacteria that not have a nucleus. Moreover, it is accumulated in the nucleus by passive diffusion. Alternatively, exportin-4 might mask the hypusine in order to prevent an interaction of eIF-5A with potential target in the nucleus. In fact, eIF-5A accumulates in the absence of exportin-4 in the nucleoli and such ultimely binding of eIF-5A to preribosomal particles might interfere with ribosomal biogenesis (Lipowsky et al., 2000). The involvement of eIF-5A in the transport of mRNAs from the nucleus to the cytoplasm is however an attractive hypothesis also in order to explain its role in the regulation of cell proliferation. Elevated protein synthesis may mark a critical transition in cancer progression since establishing a greater protein synthesis output may be a necessary step for cancer cells in order to support their rapid proliferation (De Benedetti et al., 1999; Rhoads et al., 1999). However, analysis of cells transformed by several components of the translational process revealed that the synthesis of only a few proteins was greatly enhanced, while synthesis of the most part of them was slightly increased. One possible explanation for this is the following: translational factors cause these effects by increasing the translational efficiency of several specific oncogene transcripts, leading to the overexpression of their products. The feasibility of this hypothesis was experimentally confirmed by identifying the interaction between signalling and translational components and by finding several growth regulating proteins, such as growth factors and proto-oncogenes, that are specifically upregulated (Rhoads et al., 1999). In fact, signalling pathways do not stimulate translation of all mRNAs equally and messenger RNAs differ widely in translational efficiency. Factors contributing to low efficiency of translation include a highly structured 5′ UTR, the presence of upstream AUGs, and poor sequence context for the initiating AUG (De Benedetti et al., 1999). Many proteins involved in cell growth and cell cycle progression are translated by m-RNAs with low efficiency of translation (De Benedetti et al., 1999). These mRNAs are poorly translated in quiescent cells but preferably recruited to ribosomes after a mitogenic signal (Darveau et al., 1985; Manzella et al., 1991; Nielsen et

al., 1995). The specificity of translated mRNAs involved in cell growth and survival could be an additional function for eIF-5A that can be explained on the basis of its properties of shuttle protein.

eIF-5A is a substrate of tTGase, another protein involved in the apoptotic process

Several data support the hypothesis about the involvement of eIF-5A in the protection of eukaryotic cells from apoptosis. A biochemical link between eIF-5A and the onset of apoptotic process has been recently found by several authors. In fact, we have evidenced that eIF-5A can be *in vitro* modified through a reaction catalyzed by transglutaminases (E.C. 2.3.2.13, TGase) (Beninati et al., 1995). Moreover, Singh and coworkers have reported that the GDP-bound form of tissue TGase (tTGase) associates with eIF-5A in HeLa cells and that this interaction is promoted by Ca^{++}, Mg^{++} and retinoic acid (Singh et al., 1998). TGases are calcium-dependent enzymes that catalyze post-translational modifications of proteins by the formation of covalent crosslinks between γ-carboxamido groups of glutamine endoresidues and ε-amino groups of lysine endoresidues (Folk et al., 1977). Monoamines and polyamines can also act as amino group donors. tTGase or type II TGase is an ubiquitous member of the transglutaminase enzyme family. This protein, however, is expressed at very high levels in endothelial cells and condrocytes and is localized mainly at cytoplasmic level (Thomazy et al., 1989). Several studies show that the tTGase gene expression is regulated by retinoids and cAMP (Chiocca et al., 1988; Murthaugh et al., 1984; Zhang et al., 1995). Moreover, growth factors (i.e. EGF and TGFβ) (Katoh et al., 1996; George et al., 1990) and cytokines (i.e. IL-6) (Suto et al., 1993) can regulate tTGase gene expression in cancer cells. Previous data showed that tTGase activity is directly involved in some cellular activities, i.e. receptor mediated endocytosis (Davies et al., 1980), programmed cell death or apoptosis (Fesus et al., 1988) and tumor cell proliferation (Johnson et al., 1994; Nakaoka et al., 1994; Gentile et al., 1992). Since we have found that tTGase catalyzes the *in vitro* formation of crosslinks between dimethylcasein and eIF-5A (Beninati et al., 1995), we have investigated whether tTGase could regulate the *in vivo* activity of eIF-5A and, consequently, interfere with the biological behaviour of eukaryotic cells. In details, we studied the *in vivo* effect of tTGase expression and activity on hypusine synthesis, its metabolic precursors polyamines and γ-glutamyl polyamine and hypusine derivatives formation in a Balb-C 3T3 derived cell line in which we stably transfected the gene for human tTGase (Beninati et al., 1998). We have found that part of the intracellular polyamines of transfected cells was converted to a conjugated form and the free polyamine pool was drastically depleted, likely as a consequence of an enhanced excretion of the cations (Melvin et al., 1980). The mechanism by which tTGase could interfere with polyamine excretion is not clear. It could be hypothesized that the enzyme could affect the function of the channels that

regulate polyamine transport through the plasma membrane by the formation of cross-links with other cellular substrates. Moreover, we found a significant increase of the formation of γ-glutamyl polyamine conjugates in transfected cells (Beninati et al., 1998). The modification of intracellular polyamine levels was paralleled by an about 100-fold reduction of the hypusine levels in the transfected cells likely due both to the reduction of intracellular polyamine levels and to the formation of γ-glutamyl-derivatives. These biochemical intracellular changes induced by the tTGase transfection caused relevant biological effects. In fact, the biochemical modulation of the eIF-5A activity and of polyamine levels occurred together with significant change of Balb-C 3T3 cell proliferation. Balb C-3T3 cells, stably transfected with the gene for the human tTGase, showed an about 50% retardation of their proliferation and a 50% increase of apoptotic events (Beninati et al., 1998 and data not published). Therefore, tTGAse could induce cell death and apoptosis, at least in part, through the inactivation of eIF-5A reducing the levels of the hypusine-containing form. The latter effect could hamper the translation of proteins that are critical for the survival and proliferation of eukaryotic cells. Our mechanistical hypothesis is represented in a cartoon (Fig. 2).

New multimodal therapeutic strategies based on the inhibition of eIF-5A activity

Interferon-α, an anticancer agent, induces apoptosis and affects hypusine synthesis in tumour cells

The involvement of eIF-5A in the protection of tumour cells from apoptosis is demonstrated by additional data on the modulation of hypusine synthesis in cancer cells exposed to the proapoptotic anti-cancer agent interferon α (IFNα). In fact, we have demonstrated that IFNα induces apoptosis on KB cells and upregulates the receptor for epidermal growth factor (EGF-R) that appeared to mediate an anti-apoptotic signal (Budillon et al., 1991; Caraglia et al., 1995, 1999). In details, we have found that IFNα induces apoptosis through the activation of NH_2 terminal Jun Kinase-1 (JNK-1) that is counteracted by EGF when the growth factor antagonizes IFNα-induced apoptosis (Caraglia et al., 1999). These results suggest that the modulation of apoptosis by IFNα and EGF likely targets JNK-1 as a signalling molecule (Caraglia et al., 1999). The described effects are paralleled by modulation of hypusine synthesis. In fact, IFNα induces a strong reduction in hypusine synthesis that is restored when IFNα-treated KB cells are exposed to EGF for 12 h (Caraglia et al., 1997). Since post-translational modifications of eIF-5A are essential for its activity, by reducing hypusine synthesis, IFNα is likely to inhibit the function of the translational factor that is restored by EGF. Therefore, the occurrence of apoptosis in this experimental model correlates with the depression of eIF-5A activity. We can conclude that apoptosis either due to tTGase overexpression in fibroblast cells or induced by IFNα in epidermoid cancer cells correlates with reduced intracellular hypusine levels (Fig. 2).

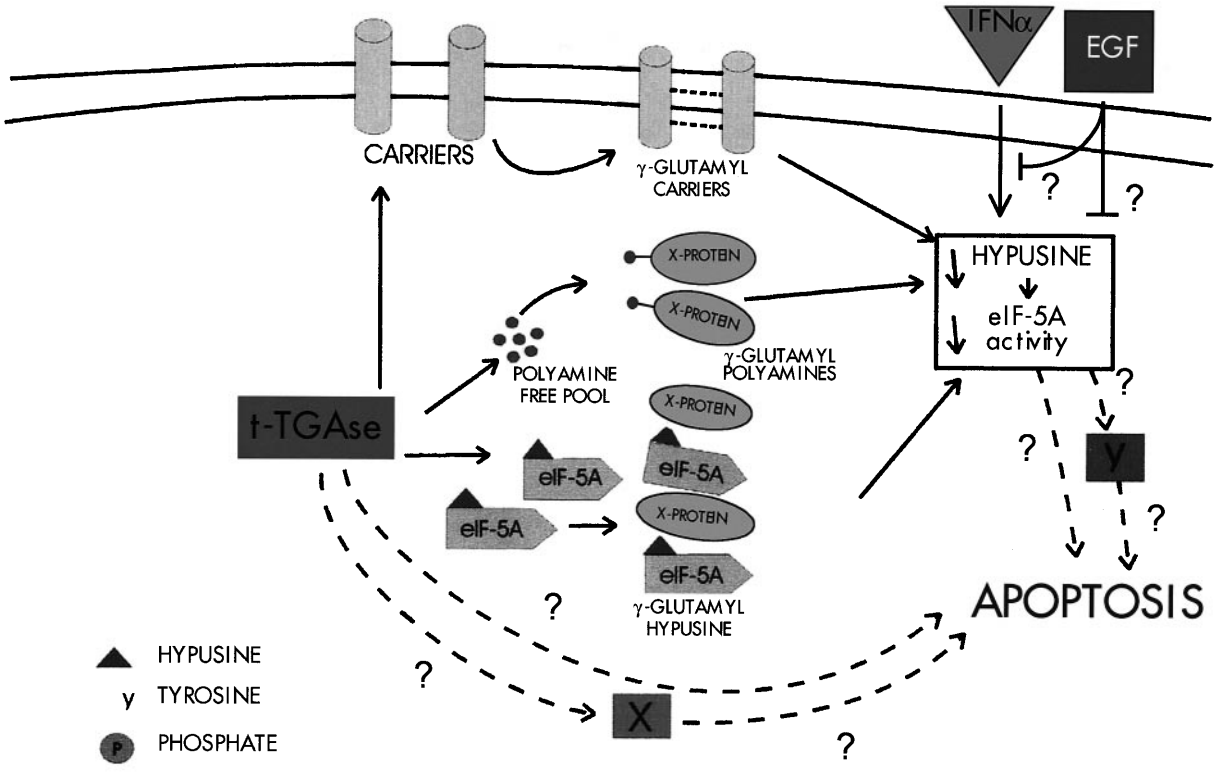

Fig. 2. Correlation between eIF-5A activity and apoptosis. tTGase is implicated in the induction of apoptosis in eukaryotic cells. We have found that the transfection of tTGase in murine fibroblasts BALB-C 3T3 causes apoptosis and decreases hypusine levels. The latter effect is likely due to the depletion of intracellular free polyamine pool that is in turn caused either by the γ-glutamyl-conjugate formation or by the inhibition of polyamine uptake (carrier cross-link formation?) mediated by tTGase. Therefore tTGase induces apoptosis, at leats in part, through the reduction of eIF-5A activity. Moreover, IFNα induces apoptosis and reduction of intracellular hypusine levels in human epidermoid cancer cells. Both effects are counteracted by EGF. Therefore, apoptosis onset requires reduced eIF-5A activity in cancer cells

eIF-5A as a target to potentiate apoptosis and cell death

On the basis of the described data we have hypothesized that eIF-5A could be a useful target in order to potentiate the efficacy of anticancer therapy. Therefore, we have studied if the use of a fusion protein that targets EGF-R (that is also upregulated by IFNα) and inhibits protein synthesis through a downstream step (EF2) could increase the antiproliferative activity of IFNα. We have indeed found that IFNα increases by about 27-fold the effect of TP40, formed by the fusion of transforming growth factor α (a ligand of EGF-R) and the modified toxin of *Pseudomonas Aeruginosa* (Caraglia et al., 1997). This effect could be attributed both to IFNα-induced EGF-R upregulation and to IFNα- and TP40-induced inhibition of multiple steps of protein synthesis. A possibility to enhance apoptosis in cancer cells, that should be investigated, is the concomitant increase of the activity of the stress dependent JNK path-

way evoked by IFNα and block of eIF5A induced by the cytokine itself or by the transfection of anti-sense oligoDNAs. Moreover, it could be suggested the selection of pharmacological inhibitor/s of eIF5A achieved through the analysis of the three dimensional structure of the hypusine containing site and successive computer-based screening of drugs predicted to bind the site. The use of plasmid/s encoding for dominant negatives of eIF5A in combination with IFNα or other proapoptotic agents could be an additional approach for the increase of the inhibition of cell proliferation. Therefore, eIF5A, on the basis of its intrinsic biochemical properties, could represent a useful target in combined approaches for the potentiation of apoptosis in cancer cells.

Conclusions

As our understanding of mechanisms of malignant transformation and tumor proliferation improves, new molecular targets are continuously revealed, thus providing support to the hope of developing more effective and selective anti-cancer therapies. On this topic the academic as well as biotechnology company research programs have already produced several clinical grade products, some of which on clinical studies. It is now reasonable to consider that eIF-5A could represent a still unexplored target of anti-cancer intervention. In fact, several strategies to modulate its activity can be designed since its function is specifically regulated by a post-translational modification of a single amino acid. eIF-5A expression or function could be specifically inhibited through the use of anti-sense oligo DNAs or pharmacological agents (such as IFNα or N(1)-guanyl-1, 7-diaminoheptane) that inhibit hypusine synthesis. Moreover, new drugs, that compete for the hypusine-containing site thus inhibiting eIF-5A activity, could be selected through computer-based screening. Finally, hypusine synthesis can be additionally regulated through the stimulation of tTGase activity (retinoic acid or strategies based on gene transfer).

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