Expression of fragments of translation initiation factor eIF4GI reveals a nuclear localisation signal within the N-terminal apoptotic cleavage fragment N-FAG

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Accepted 16 January 2004
Journal of Cell Science 117, 2545-2555 Published by The Company of Biologists 2004
doi:10.1242/jcs.01106

Summary

The eukaryotic initiation factor eIF4GI plays a central role in the assembly of a competent initiation complex at the 5′ end of an mRNA. Five isoforms of eIF4G exist in cells, arising from alternative translation initiation. During picornaviral infection or apoptosis, eIF4GI is cleaved proteolytically to yield distinct fragments. Using HeLa cells, we have examined the fate of these proteins in the cell. We have found that while endogenous eIF4GI is predominantly cytoplasmic, a population can also be visualised in the nucleus. Furthermore, eIF4GI is localised primarily at the nuclear periphery in the vicinity of eIF4E and PABP1. Transient transfection of HeLa cells with different myc-tagged isoforms of eIF4GI did not result in any obvious differences in their localisation. However, expression of discrete fragments of eIF4GI corresponding to those generated after apoptosis or picornaviral infection generated a distinctive, but intricate localisation pattern. Our work shows that the N-terminal apoptotic cleavage fragment N-FAG contains a sequence of basic amino acids that can act as a nuclear localisation signal. In addition, the presence or absence of the sequence flanking and including the eIF4E binding site (residues 533-682) confers a distinct cellular distribution pattern for the central domain of eIF4GI.

Supplemental data available online

Key words: Apoptosis, Caspase, eIF4G, Localisation, Translation initiation

Introduction

During the initiation phase of eukaryotic protein synthesis, the ribosome is recruited to the 5′ untranslated region (5′ UTR) of the mRNA. Several eukaryotic initiation factors (eIFs) are required for this process. The activities of many of the eIFs can be up- or down-regulated, either by phosphorylation in response to numerous intracellular signalling pathways or by modification by specific cleavage events (Morley, 2001). These modifications can affect the decoding of all mRNAs (global translational control) or the translation of particular subsets of mRNAs (selective translational control).

mRNA recruitment to the ribosome is brought about by a group of initiation factors that interact to form the eIF4F complex (Hershey and Merrick, 2000), comprising the mRNA cap-binding protein (eIF4E), an ATP-dependent RNA helicase activity (eIF4A, in concert with eIF4B) and eIF4G, which is the central scaffold to which these and other factors bind (Gingras et al., 1999; Morley, 2001; Morley et al., 1997). The last protein exists as two homologues in humans (eIF4GI and eIF4GII) sharing 46% identity at the amino acid level. The original cDNA clone of eIF4GI obtained from a human brain cDNA library (Yan et al., 1992) was extended in a later study (Imataka et al., 1998) to identify an N-terminal poly(A) binding protein (PABP1) binding site. More recently, two research groups independently discovered a further N-terminal extension of 40 amino acids, finally identifying the longest possible open reading frame (ORF) of eIF4GI (Bradley et al., 2002; Byrd et al., 2002). These studies suggest that five isoforms of eIF4GI exist in cells, generated by alternative translation initiation. However, little is known about the function of these different isoforms in the cell. Interaction of the cytoplasmic PABP1 with the N terminus of eIF4G promotes interactions between the 5′ and 3′ ends of the mRNA (Imataka et al., 1998; Wells et al., 1998), whereas the kinase that phosphorylates eIF4E at Ser209 to modify its cap-binding properties (Mnk1) binds to the C terminus (Pyronnet et al., 1999). The formation of the 48S preinitiation complex on the mRNA is completed by binding of the central portion of eIF4G to the multisubunit eIF3, which is part of the 43S preinitiation complex together with a 40S ribosomal subunit and eIF2·GTP·initiator methionyl tRNA.

As eIF4G plays such a central role in the assembly of the preinitiation complex, it is perhaps unsurprising that modifications of eIF4GI and eIF4GII by physiological cleavage events can have drastic consequences on translation initiation. Cleavage of eIF4GI and eIF4GII by picornaviral proteases, such as the Leader (L) protease expressed by the aphthoviruses or the 2A protease from entero- and rhinoviruses, is used to hijack the translation machinery during viral infection (Belsham and Jackson, 2000). Such cleavage of eIF4GI leads to the separation of the region involved in mRNA cap-binding (designated Nt or CpN) (Lamphear et al., 1995; Ohlmann et al., 1996) from the ribosome binding portion (Ct or CpC) of the molecule (see

Fig. 2A). This results in a severe, selective inhibition of host protein synthesis while allowing translation of uncapped viral RNA by internal ribosome entry.

Further studies have demonstrated that both eIF4GI and eIF4GII are targets for caspase-3-mediated degradation during apoptosis (Clemens et al., 2000; Morley, 2001). In several cell types, activation of either the receptor-mediated or the mitochondrially mediated pathway of apoptosis leads to the inhibition of cap-dependent translation and the progressive degradation of eIF4GI and eIF4GII. However, the translation of some cellular mRNAs possessing an internal ribosome entry segment (IRES) is maintained (Henis-Korenblit et al., 2002; Stoneley et al., 2000). In contrast to the cleavage of eIF4G during picornavirus infection (Gradi et al., 1998), the loss of eIF4GI and eIF4GII during apoptosis occurs with similar kinetics (Bushell et al., 2000b; Clemens et al., 1998; Marissen et al., 2000; Morley et al., 2000). Caspase-3 cleaves eIF4GI at two sites, between amino acids 492 and 493 and between amino acids 1136 and 1137, with initial cleavage events at either of these two sites generating intermediate fragments of 120 and 150 kDa, respectively. The three distinct breakdown products of eIF4GI resulting from this proteolysis were termed Fragments of Apoptotic cleavage of eIF4G (FAGs) and designated N-FAG, M-FAG and C-FAG (Bushell et al., 2000a; Clemens et al., 1998).

It is becoming increasingly evident that components of the translation machinery show complex localisation patterns within cells. Biochemical fractionation experiments with a number of cell types have indicated the association of ribosomes and translation factors, as well as specific mRNAs, with the cytoskeleton (Bassell et al., 1999; Hesketh, 1994; Hesketh, 1996; Jansen, 1999; Lenk et al., 1977). Surprisingly, several initiation factors have also been detected in the nucleus. eIF4E has been reported to co-localise with splicing factors in speckles (Dostie et al., 2000b), interact with the RING domain of the promyelocytic leukaemia protein (PML) (Strudwick and Borden, 2002), and use a specific transporter protein (4E-T) to shuttle between the nucleus and the cytoplasm (Dostie et al., 2000a). Previous work in our laboratory and others has shown that a proportion of cellular eIF4GI is also nuclear (Kedersha et al., 2002; Kimball et al., 2003) and may play a role in mRNA processing and/or the 'pioneer' round of translation of newly exported mRNA (McKendrick et al., 2001).

Previously, our laboratory has shown that the Nt fragment of human eIF4GI is localised to the nucleus when expressed in the fission yeast, *Schizosaccharomyces pombe* (Hashemzadeh-Bonehi et al., 2003). However, a sequence slightly longer than the human Ct fragment (designated p100) remains cytoplasmic. This raises the question of whether the physiological cleavage of eIF4GI during apoptosis or picornavirus infection is followed by re-localisation of any of the resulting fragments. As part of an investigation to address this question we have examined the localisation of endogenous eIF4GI in comparison to that of each of the natural variants of the full-length protein following transfection into mammalian cells. These data indicate a distinctive but complex pattern of localisation of a number of fragments that result from specific proteolytic cleavages of this factor during viral infection or apoptosis and identifies a sequence within the eIF4GI sequence that can act as a nuclear localisation signal.

Materials and Methods

Expression of eIF4GI sequences

The oligonucleotides mycF and mycR (see supplemental data, Fig. S1. A, http://jcs.biologists.org/supplemental/) were annealed and digested with the restriction endonucleases *Nhe*I and *Kpn*I. This cDNA was ligated into pcDNA3.1(+) (Invitrogen) cut with the same enzymes, creating the vector pcDNAmyc. The *Nco*I site in the introduced sequence was used to create an N-terminal myc epitope tag (EQKLISEEDL) that was expressed from an initiation codon in the optimal context. All eIF4GI sequences were subcloned from the vector pSPORT4GI (a kind gift from Richard E. Lloyd, Baylor College of Medicine, Houston, TX, USA), which contained the entire eIF4GI cDNA (GenBank accession no. AY082886) using the high fidelity DNA polymerase *Pfu* Turbo (Stratagene Europe, Amsterdam, Netherlands). Oligonucleotide primers corresponding to the desired region of amplification (see supplemental data, Fig. S1. B and C) were designed so that 5′ primers contained a *Hin*dIII restriction site while 3′ primers had a stop codon followed by an *Xho*I site. These ensured that the cDNA fragment was ligated in the correct frame with the N-terminal myc epitope tag and that translational termination occurred after the appropriate residue. Each sequence was amplified and inserted into pcDNAmyc. The presence of the correct sequence was verified by restriction digest and automated sequencing. The KRRRK amino acid sequence was mutated to five alanines using the oligonucleotide primers 4G KRRRK 5′ and 4G KRRRK 3′ (see supplemental data, Fig. S1. D) and the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol.

The wild-type and AAAAA mutant forms of N-FAGa were amplified from the corresponding pcDNAmyc vectors with the oligonucleotide primers NaGFPF and NaGFPR (supplemental data, Fig. S1. E), whereas the oligonucleotides 5KRRRK5F and 5KRRRK5R, and 5AAAAA5F and 5AAAAA5R (supplemental data, Fig. S1. F) were combined and annealed. All the resulting cDNAs were digested with the restriction endonucleases *Xho*I and *Hin*dIII and then ligated into pEGFP-N1 (Clontech) cut with the same enzymes, ensuring the cDNA would be expressed in frame with enhanced green fluorescent protein (eGFP).

Cell culture and transfections

HeLa cells were maintained in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO_2. Cells were seeded on glass coverslips in 6-well plates at a density of 12,500 cells per well. 24 hours later, 1 μg of DNA was transfected into each well using FuGene 6 (Roche Molecular Biochemicals, Sussex, UK) according to the manufacturer's protocols. The transfection mixture was removed 3 hours later and the cells were washed twice with PBS before being incubated for a further 16 hours in fresh medium.

Antibodies

To detect exogenously expressed myc-tagged proteins from total cell lysates and within cells, the 9E10 mouse monoclonal antibody raised against the myc epitope (Sigma-Aldrich Company Ltd, Dorset, UK) was used. Proteins fused to eGFP were detected in total cell lysates with a mouse monoclonal antibody to eGFP, which was a kind gift from Dr Alison Sinclair, University of Sussex. Polyclonal rabbit antibodies used included those raised against: a bacterially expressed 6xHis tagged protein corresponding to amino acids 1-533 of eIF4GI**;** a C-terminal peptide of eIF4GI, RTPATKRSFSKEVEERSR (amino acids 1179-1206) (Bushell et al., 2000a); eIF4E, TATKSGSTTKNRFVV (amino acids 203-217) (Bushell et al., 2000a), and PABP1, IPQTQNRAAYYPPSQIAQLRPS (amino acids 413-434). All rabbit antisera were isolated from crude serum by affinity chromatography with the corresponding peptide using the SulfoLink kit (Perbio Science UK Ltd, Cheshire, UK) according to the manufacturer's instructions. For co-localisation studies, mouse

monoclonal antibody raised against eIF4E was obtained from Transduction Laboratories (Beckton Dickinson UK Ltd, Oxford UK) and mouse monoclonal antibody to PABP1 (clone 6B12) was a kind gift from Matthias Görlach (Institut für Molekulare Biotechnologie, Jena, Germany). For immunofluorescence studies, primary antibodies were diluted as follows into PBS containing 3% BSA; 9E10, anti-N-terminal eIF4GI, anti-C-terminal eIF4GI, and anti-PABP1 at 1:300; 6B12 at 1:100; rabbit anti-eIF4E at 1:50 and mouse anti-eIF4E at 1:1000. Goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) (DakoCytomation Ltd, Cambs., UK) or goat anti-rabbit IgG conjugated to rhodamine (Jackson Immunochemicals, West Grove, PA, USA) were used as secondary antibodies. The actin cytoskeleton was visualised with a phalloidin-TRITC or phalloidin-FITC conjugate (DakoCytomation) at a concentration of 100 ng/ml.

Immunofluorescence microscopy

For indirect immunofluorescence experiments, HeLa cells were seeded on glass coverslips in 6-well plates and transfected as described above. Cells were fixed in 4% paraformaldehyde (w/v) in PBS, pH 7.4 for 15 minutes, rinsed with PBS three times and permeabilised in PBS containing 0.8% Triton-X-100 (v/v) for 8 minutes according to the method of with modifications Harlow and Lane (Harlow and Lane, 1999). Non-specific binding was blocked by adding 3% BSA in PBS for a minimum of 20 minutes. Cells were incubated in the primary antibody solution for 60 minutes, washed extensively and then incubated with the appropriate secondary antibody and phalloidin-FITC or -TRITC conjugate for 45 minutes. Following further extensive washing, nuclei were stained with 12.5 ng/ml 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) (Sigma) for 5 minutes. After a further two washes, coverslips were mounted on microscope slides with Mowiol mounting solution (0.2 M Tris pH 8.5, 33% (w/v) glycerol, 13% (w/v) mowiol, 2.5% (w/v) 1,4-diazobicyol [2,2,2]-octane (DABCO)) and sealed with clear nail polish.

Cells transfected with plasmids expressing eGFP were fixed in paraformaldehyde then incubated twice with PBS for 10 minutes before being mounted and sealed with dark red nail polish.

Cells were analysed using a Zeiss Axioscop 2 microscope equipped with a 63× oil immersion objective and fitted with the appropriate filter sets. Images were captured with a Photometrics 'Quantix' digital camera. Images were processed using Metamorph imaging software (Universal Imaging Corp.). Greyscale images were pseudo-coloured to correspond to the red (TRITC/rhodamine), green (FITC) or blue (DAPI) fluorescence and the digital images were merged. Adobe Photoshop v5.5 was used to prepare images for publication. All images are to the same scale.

Results

Localisation of endogenous initiation factors in NIH3T3 and HeLa cells

Previous work in our laboratory has investigated the localisation of initiation factors in the fission yeast, *S. pombe* (Hashemzadeh-Bonehi et al., 2003). In the present study, we have examined the localisation of endogenous components of the mammalian translation initiation pathway in HeLa and NIH3T3 cell lines. For this, cells were grown on coverslips and prepared for immunofluorescence studies by paraformaldehyde fixation and permeabilisation with Triton X-100 (see Materials and Methods). The actin cytoskeleton was defined by the F-actin binding molecule phalloidin, conjugated to an appropriate fluorophore while nuclei were stained with DAPI. Affinity purified anti-peptide polyclonal rabbit antibodies were used to visualise the localisation of eIF4GI, eIF4E or PABP1 together with the actin cytoskeleton. The localisation of eIF4GI was also visualised with a rabbit polyclonal antibody raised against a bacterially expressed protein corresponding to amino acids 1-533 of eIF4GI. In both HeLa (Fig. 1A, upper panels) and NIH3T3 (data not shown) cells, eIF4GI was predominantly localised to the cytoplasm but did not directly co-localise with actin. In agreement with published data (McKendrick et al., 2001), low levels of eIF4GI were visualised in the nucleus in both cell types. eIF4E showed a similar pattern of localisation to eIF4GI, did not directly colocalise with actin and often exhibited abundant staining in the perinuclear region (Fig. 1A, lower left panel). PABP1 was observed in the nucleus and the perinuclear region (Fig. 1A, lower right panel), reflecting the ability of this protein to actively shuttle between the nucleus and cytoplasm (Afonina et al., 1998). Combinations of these factors were also visualised in both cell lines using rabbit anti-eIF4GI antisera and monoclonal antibodies specific for eIF4E or PABP (Fig. 1B). These data indicate that eIF4GI, eIF4E and PABP are enriched in the perinuclear region. At the exposure settings used here, goat anti-mouse antibody conjugated to FITC did not detectably react with rabbit antibodies, and swine anti-rabbit antibody conjugated to TRITC gave no discernible signal with mouse antibodies (data not shown).

Expression of exogenous eIF4GI

Recent work has shown that five isoforms of eIF4GI are expressed in mammalian cells, arising from translation initiation at alternative AUG codons (Bradley et al., 2002; Byrd et al., 2002). Individual roles for these isoforms [designated eIF4Gf, eIF4Ge, eIF4Gdc, eIF4Gb, eIF4Ga (Bradley et al., 2002)] have not yet been identified, and we wished to determine whether the differing N-termini of these proteins could cause a change in their subcellular localisation. To achieve this, cDNAs corresponding to the different eIF4GI isoforms (Fig. 2A) were subcloned into a vector containing an N-terminal myc epitope tag (see Materials and Methods and supplemental data, Fig. S1.; http://jcs.biologists.org/supplemental/). The initiation codon of the epitope tag was designed in an optimal context such that reinitiation at downstream codons would be reduced and therefore only myc-tagged proteins should be expressed. Immunoblotting of total HeLa cell lysates with an anti-myc monoclonal antibody (9E10; Fig. 2B) shows that the isoforms are not expressed equally. Indeed, the longest isoform [eIF4GIf (Bradley et al., 2002)] is particularly poorly expressed, possibly reflecting the high (30%) proline content within the first 40 amino acids of this protein. To visualise the myc-tagged isoforms of eIF4GI in HeLa and NIH3T3 cells we used the 9E10 anti-myc antibody and an anti-mouse antibody conjugated to FITC (Fig. 2C and data not shown). Only the data for HeLa cells are shown in this and subsequent figures, but in all cases very similar results were obtained with NIH3T3 cells. As in Fig. 1, the actin cytoskeleton was defined with phalloidin, in this case conjugated to TRITC, while nuclei were stained with DAPI. Comparison of the FITC staining with these fluorescent signals indicates the presence of a number of non-transfected cells, which provides an indication of the specificity of the anti-myc antibody used. For both cell lines, all exogenous eIF4GI isoforms were found in a cytoplasmic location, with some being detectable in the nucleus. Thus the localisation of each of the isoforms resembled the pattern seen with endogenous eIF4GI in Fig. 1. At the exposure setting used for the photographs

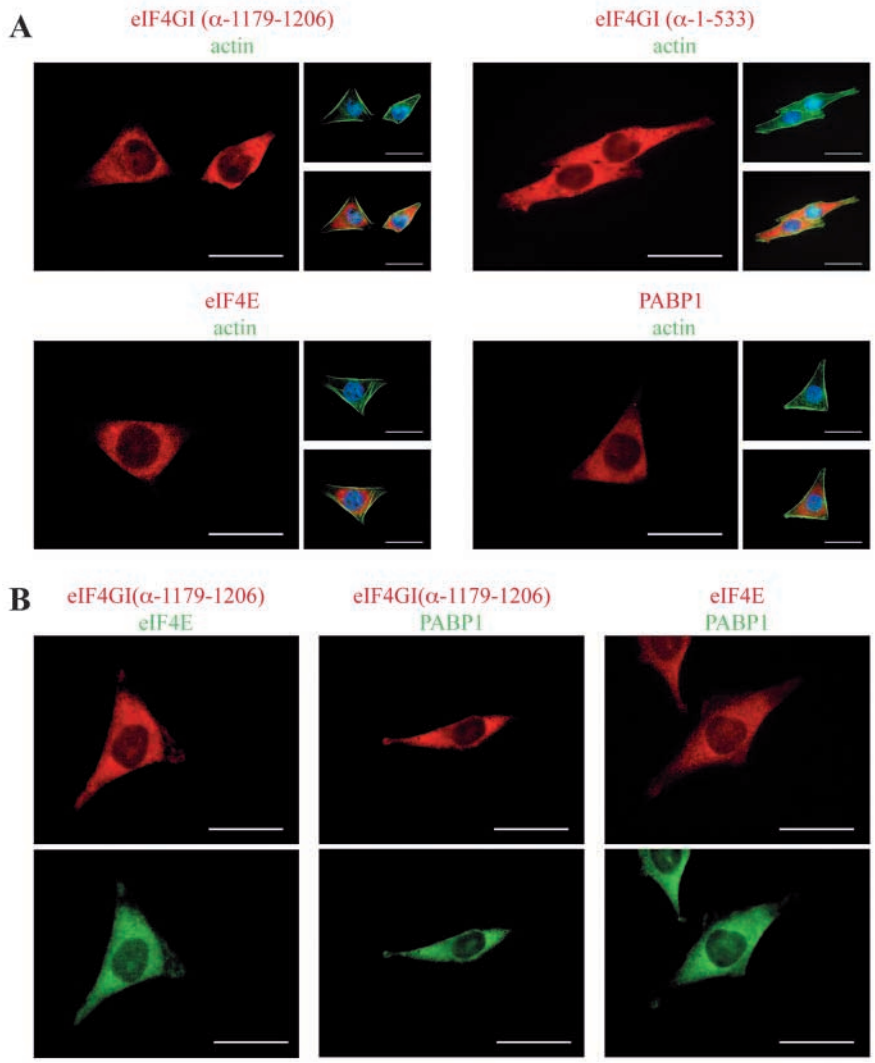

Fig. 1. eIF4GI, eIF4E and PABP are predominantly cytoplasmic and do not directly co-localise with the actin cytoskeleton. (A) HeLa cells seeded on glass coverslips were fixed in 4% paraformaldehyde for 15 min, rinsed with PBS three times and permeabilised in PBS containing 0.8% Triton-X-100 (v/v) for 8 minutes. Rabbit antisera recognising the C terminus of eIF4GI, the N terminus of eIF4GI, eIF4E and PABP1, followed by a secondary antibody of goat anti-rabbit IgG conjugated to rhodamine, were used to visualise the localisation of the endogenous proteins within these cells (pseudocoloured in red). Actin was visualised with phalloidin-FITC (pseudocoloured green) and nuclei with DAPI (pseudocoloured blue). Scale bars: 20 μm. (B) Cells were cultured and processed as in A. The localisation of the endogenous proteins within these cells was also visualised using combinations of rabbit and mouse antisera and secondary antibodies of goat anti-rabbit IgG conjugated to rhodamine (red) or goat anti-mouse IgG conjugated to FITC (green). Left panels: rabbit anti-eIF4GI and mouse anti-eIF4E; centre panels: rabbit anti-eIF4GI and mouse anti-PABP1; right panels: rabbit anti-eIF4E and mouse anti-PABP1. White bars represent 20 μm.

throughout this paper, the anti-myc antibody did not give a significant signal in the untransfected cells or those transfected with the parental pcDNAmyc vector, which expressed the myc tag alone (data not shown).

Expression of exogenous eIF4GI fragments in cells reveals a distinct nuclear localisation for N-FAG sequences

In an attempt to elucidate whether cellular stresses that lead to the cleavage of eIF4GI resulted in a re-localisation of the protein, we first used the cytotoxic ligand TRAIL to induce the receptor-mediated pathway of apoptosis. However, the cleavage of eIF4GI was incomplete, with a significant amount of the protein remaining intact, even after 8 hours of incubation with TRAIL when over 90% of cells had undergone apoptosis (Stoneley et al., 2000) (data not shown). This, coupled with the hallmark morphological changes of apoptosis, including breakdown of the nuclear membrane via cleavage of proteins such as the nuclear lamins (Oberhammer et al., 1994; Rao et al., 1996), makes it impossible to use immunofluorescence microscopy to draw any conclusions about the localisation of eIF4GI cleavage fragments generated in vivo during apoptosis.

To overcome this obstacle, we decided to express exogenous cDNA fragments encoding cleavage products of the eIF4GI molecule. To this end, vectors were constructed containing eIF4GI sequences corresponding to the fragments arising from cleavage by caspase-3 (N-, M- and C-FAG and NM and MC intermediates) or by picornaviral proteases (Nt and Ct, also designated CpN and CpC). We also constructed plasmids encoding proteins that have previously been used to map functional domains of eIF4GI (p100 and 4G-M) (Pestova et al., 1996). Each plasmid encoded a protein with an N-terminal myc tag.

We first examined the localisation of proteins corresponding to the N-terminal fragment generated during apoptosis (designated N-FAGf, N-FAGe, N-FAGdc, N-FAGb and N-FAGa), the two extremes of the N-terminal L protease cleavage fragment (Ntf and Nta) and the longest intermediate apoptotic cleavage fragment (NMf; Fig. 3A). These fragments were expressed in HeLa cells and total cell lysates resolved by SDS-PAGE. Immunoblotting analysis with the anti-myc antibody reveals once again that expression of the longest isoforms is relatively low and that all N-terminal sequences migrate more slowly than expected from their molecular mass (Fig. 3B).

Surprisingly, when either HeLa or NIH3T3 cells were

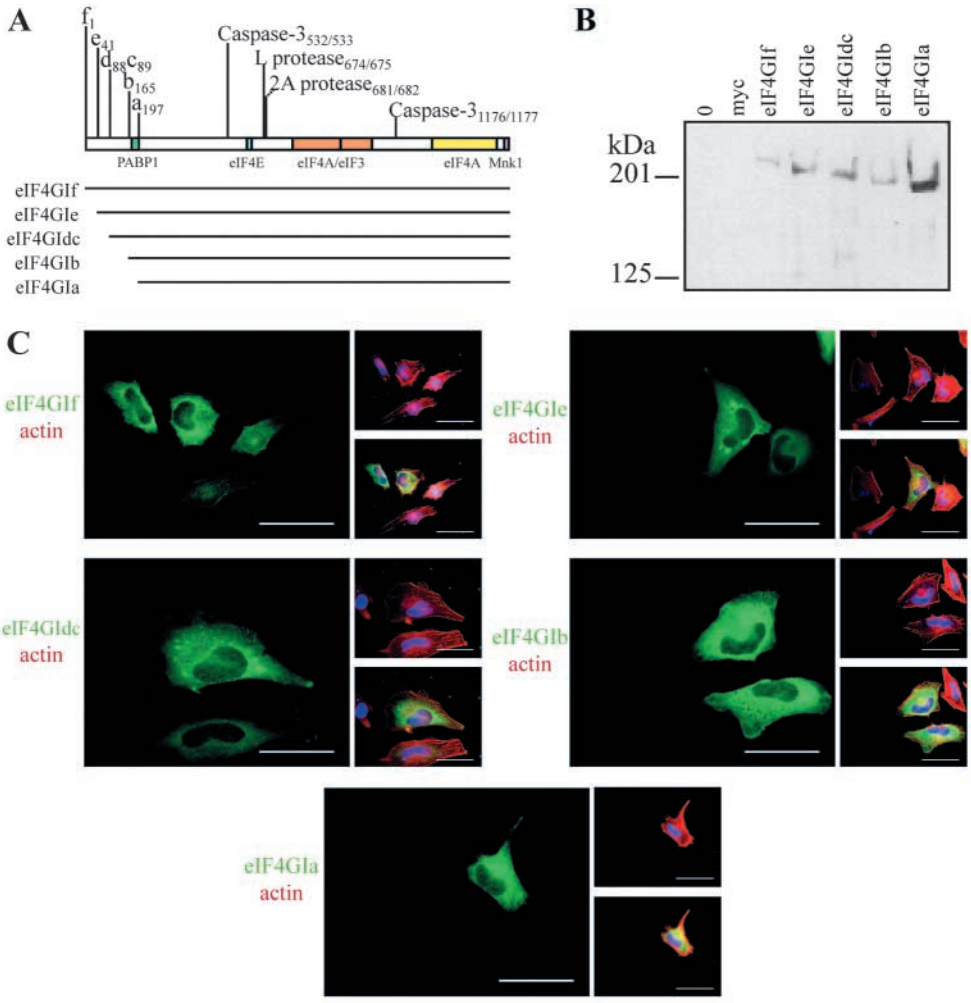

Fig. 2. Exogenously expressed myc-tagged isoforms of eIF4GI are localised to the cytoplasm. (A) Schematic representation of eIF4GI showing sites of alternative translation initiation and binding sites of other components of the translation initiation machinery (Bradley et al., 2002; Byrd et al., 2002; Morley, 2001). Sites of cleavage by caspase-3 and the picornaviral L and 2A proteases are indicated. Black lines indicate individual sequences used for expression in this figure. (B) Total cell lysates were prepared from HeLa cells transfected with plasmids containing eIF4GI cDNAs and resolved by SDS-PAGE. Expressed proteins were identified by immunoblotting using the 9E10 monoclonal antibody to the c-myc epitope; molecular mass markers are shown on the left. (C) Anti-myc monoclonal antibody (9E10) followed by goat anti-mouse IgG conjugated to FITC (green) was used to visualise the localisation of the tagged isoforms of eIF4G expressed in HeLa cells, as indicated. Actin was visualised with phalloidin-TRITC (red) and nuclei with DAPI (blue). Scale bars: 20 μm.

transiently transfected with expression vectors containing these myc-tagged N-terminal sequences, at least 90% of cells expressing the N-FAG variants showed a predominantly nuclear localisation (Fig. 3C and data not shown). In contrast, cells expressing the intermediate apoptotic cleavage fragment of eIF4GI, NMf, showed a predominantly cytoplasmic localisation whereas the N-terminal L-protease cleavage fragment, Nt, was observed in both the nuclei and the cytoplasm.

Since the longer N-FAG variants all contain the PABP1 binding site of eIF4GI, we investigated whether their expression may affect the localisation of endogenous PABP1. However, in spite of a dramatic localisation of N-FAG to the nucleus, this did not lead to a concomitant relocalisation of PABP1 (data not shown). While all N-FAG variants were nuclear, the two Nt variants showed nuclear, as well as cytoplasmic, localisation (Ntf and Nta; Fig. 3C).

Middle and C-terminal fragments of eIF4GI localise to the cytoplasm

Exogenous fragments corresponding to the middle and C terminus of eIF4GI (Fig. 4A) were also expressed in these cells and fragments were visualised with the anti-myc antibody (Fig. 4B). Lysates from cells transfected with eIF4GIa and N-FAGa were resolved concurrently to show the relative expression levels of all the eIF4GI sequences used in this study (compare Fig. 2B and Fig. 3B).

In general, the localisation of the expressed C-terminal fragments was predominantly cytoplasmic, although there were some individual variations, suggesting a complex pattern of regulation (Fig. 4C). The apoptotic cleavage fragment M-FAG, which contains both the eIF4E binding site and the central domain of eIF4G, showed a predominantly cytoplasmic localisation and did not co-localise with the actin cytoskeleton. The localisation pattern of MC resembled that of M-FAG, while C-FAG and 4G-M were localised to both the nucleus and the cytoplasm. Thus the comparison between the fate of Ct/p100, 4GM, M-FAG and MC indicates that the presence or absence of the sequence flanking, and including, the eIF4E binding site (residues 533-682) confers a distinct cellular distribution pattern for the central domain of eIF4GI.

In the course of this work we also found that expression of either the natural C-terminal cleavage product of picornaviral proteases (Ct), or the slightly longer cloned fragment frequently used as a model for this product (p100; see Fig. 4A), resulted in severe alterations in cell morphology with associated disruption of the actin cytoskeleton (Fig. 4C). This was seen in both HeLa and NIH3T3 cells, and resembles effects seen when either human p100 or its *S. pombe* counterpart were expressed in fission yeast (Hashemzadeh-Bonehi et al., 2003). However,

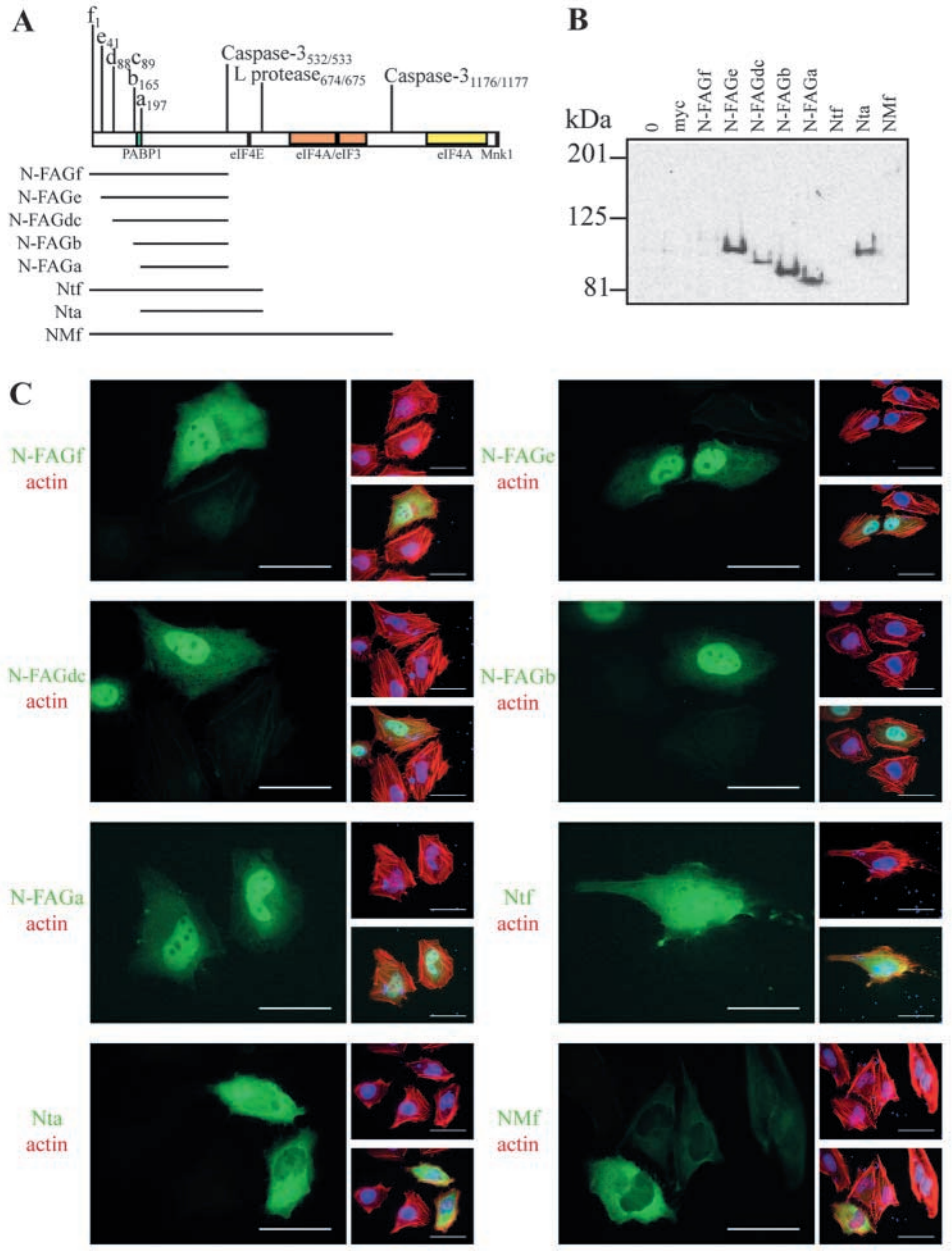

Fig. 3. All isoforms of exogenous myc-tagged N-FAG are localised to the nucleus. (A) Schematic representation of eIF4GI outlining the individual N-terminal sequences used for expression in this study. (B) HeLa cells were transfected with plasmids encoding myc-tagged isoforms of the N-terminal domains of eIF4GI. Sixteen hours after transfection, total cell lysates were prepared and proteins resolved by SDS-PAGE. eIF4GI and its N-terminal cleavage fragments were identified by immunoblotting using anti-myc antiserum; molecular mass markers are shown on the left. Owing to differences in expression levels and to aid visualisation, extracts from cells transfected with N-FAGdc, N-FAGb, N-FAGa and Nta were all diluted 1:2 with sample buffer; despite this, expression of Ntf is only detectable at extremely long exposures (data not shown). (C) Anti-myc antibody followed by goat anti-mouse IgG conjugated to FITC (green) was used to visualise the localisation of the tagged isoforms of the N-terminal domain of eIF4GI expressed in HeLa cells, as indicated. Actin was visualised with phalloidin-TRITC (red) and nuclei with DAPI (blue). Scale bars: 20 μm.

it is difficult to identify the parts of the molecule responsible for these responses, since both cell lines were able to tolerate expression of each of the individual domains (4G-M and C-FAG) that constitute the Ct/p100 fragments (Fig. 4A,C). In addition, cells tolerated the expression of the larger molecule MC, which corresponds to p100 with an additional N-terminal extension that includes the eIF4E binding site (Fig. 4C).

Identification of a monopartite basic nuclear localisation signal in N-FAG

The data in Fig. 4, together with those presented in Fig. 3 comparing the localisation patterns of N-FAG and Nt, suggest a complex interplay between sequences that confer either a nuclear or cytoplasmic localisation on eIF4GI. The only sequence in eIF4GI that resembles a nuclear localisation signal (NLS) is found near the C-terminal end of all possible N-FAG molecules between amino acids 513 and 525 (KRRRKIKELNKKE; Fig. 5A). Although this sequence is not conserved in eIF4GII, it is conserved in the rabbit and mouse eIF4GI sequences and in those in the database for rat and cat. This sequence does not exactly resemble the classic bipartite nuclear localisation signal exemplified in nucleoplasmin ($[KR]_2X_{10\text{-}12}[KR]_3$), although the first five lysine and arginine residues resemble those found in several NLSs such as that of the SV40 large T antigen (Kalderon et al., 1984) and the eIF4E kinase Mnk1 (Parra-Palau et al., 2003). We thought it possible that the first half of this sequence may be sufficient to confer nuclear localisation, as the sequence KRRR was able to maintain nuclear localisation of the EB2 mRNA export factor when the basic amino acids in the second half of a similar NLS within this protein were mutated to alanine residues (Hiriart et al., 2003). To determine the role of this putative NLS in the localisation of eIF4GI, the KRRRK sequence in plasmids

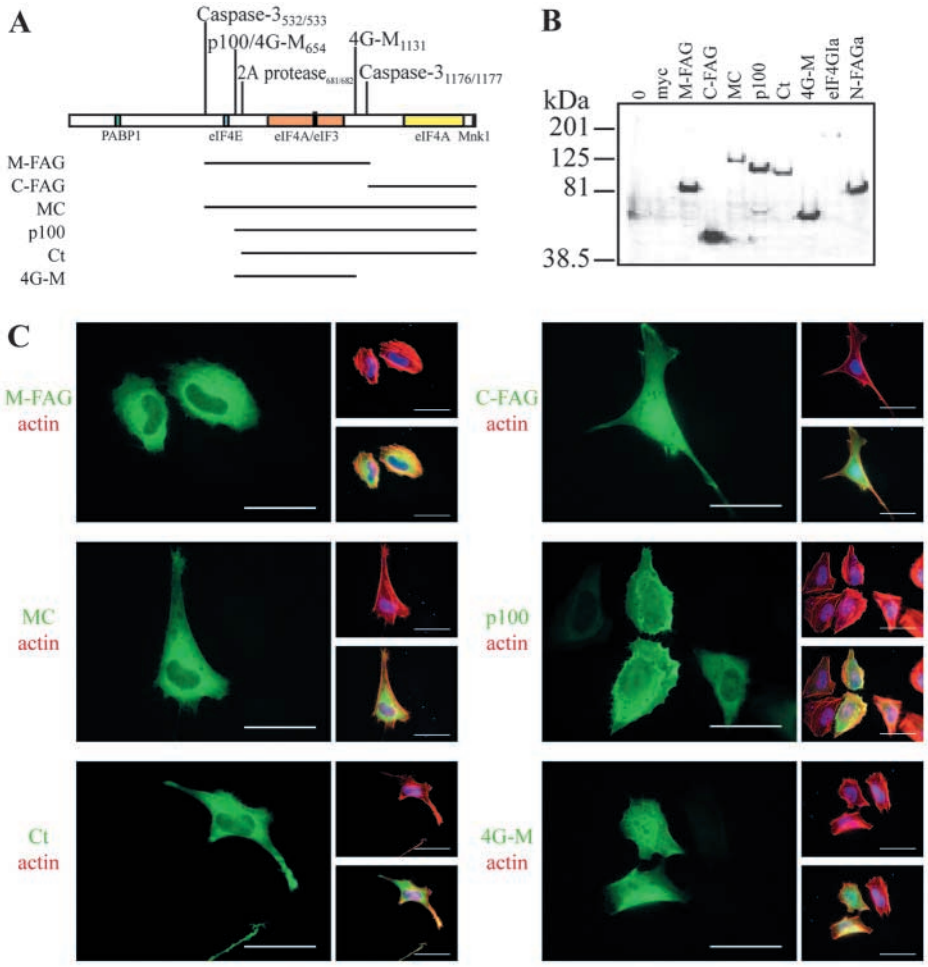

Fig. 4. Expressed M-FAG remains cytoplasmic while expressed Ct/p100 causes morphological defects and induces apoptosis. (A) Schematic representation of eIF4GI outlining the individual sequences used for expression in this study. **(**B**)** HeLa cells were transfected with plasmids encoding myc-tagged isoforms of the C-terminal domains of eIF4GI. Sixteen hours after transfection, total cell lysates were prepared and proteins resolved by SDS-PAGE. The fragments were identified by immunoblotting using anti-myc antiserum; molecular mass markers are shown on the left. Owing to differences in expression levels and to aid visualisation, extracts from cells expressing C-FAG were diluted 1:10 prior to analysis. (C) Anti-myc antibody followed by goat anti-mouse IgG conjugated to FITC (green) was used to visualise the localisation of the tagged isoforms of the C-terminal domains of eIF4GI expressed in HeLa cells, as indicated. Actin was visualised with phalloidin-TRITC (red) and nuclei with DAPI (blue). Scale bars: 20 μm.

expressing myc-tagged eIF4GIf, eIF4GIa, N-FAGf and N-FAGa was mutated to five alanines. Expression levels of the wild-type and mutant sequences were similar in HeLa cells (Fig. 5B), although the expression of the longest isoform is again much lower than that of the shortest (compare Fig. 2B and Fig. 3B). However, the change in amino acid composition does alter the migration of the proteins, an effect more easily visualised for the N-FAG forms than the slowly moving full-length eIF4G. The localisation of these fragments within HeLa cells was examined with the myc antibody (Fig. 5C).

Cells expressing wild-type and alanine mutant forms of eIF4GIf and eIF4GIa remain predominantly localised to the cytoplasm. However, mutation of the KRRRK sequence clearly alters the localisation of the myc-tagged N-FAG proteins from the nucleus to the cytoplasm. This result demonstrates that this basic sequence can indeed act as a nuclear localisation signal in the absence of other sequences in the middle and C terminus of eIF4GI. However, our results taken together indicate that the latter sequences may play a dominant role in defining the localisation of the intact eIF4G molecule.

The nuclear localisation signal in eIF4GI is necessary and sufficient to determine the subcellular localisation of a heterologous reporter gene

To ascertain whether the sequence KRRRK present in the N terminus of eIF4GI is sufficient to direct nuclear localisation, a short sequence containing the NLS plus the five amino acids that precede and succeed it (5KRRRK5) or the alanine mutant (5AAAAA5) were subcloned upstream of the N terminus of eGFP. The wild-type and mutant forms of N-FAGa were also fused in frame with eGFP (Fig. 6A).

Plasmids encoding these eGFP fusion proteins were transfected into HeLa cells, and 16 hours later, total cell lysates were probed with a monoclonal antibody to eGFP (Fig. 6B). Direct fluorescence of the eGFP moiety enabled the localisation of these chimeric proteins to be observed (Fig. 6C). When eGFP alone is expressed in HeLa cells, the protein is distributed throughout the nucleus and cytoplasm as expected from many studies (e.g. Ogawa et al., 1995; Rizzuto et al., 1995). In contrast, when expressed as a fusion with either N-FAGa or the sequence surrounding the NLS (5KRRRK5), eGFP becomes localised to the nucleus. The fusions containing the mutant NLS sequence localise to either the cytoplasm (N-FAGa$_{AAAAA}$) or have a diffuse distribution similar to that of eGFP alone (5AAAAA5).

Discussion

The work presented here has begun to uncover the complex nature of the control of the localisation of eIF4GI in mammalian cells. Fragments consisting of the N-terminal

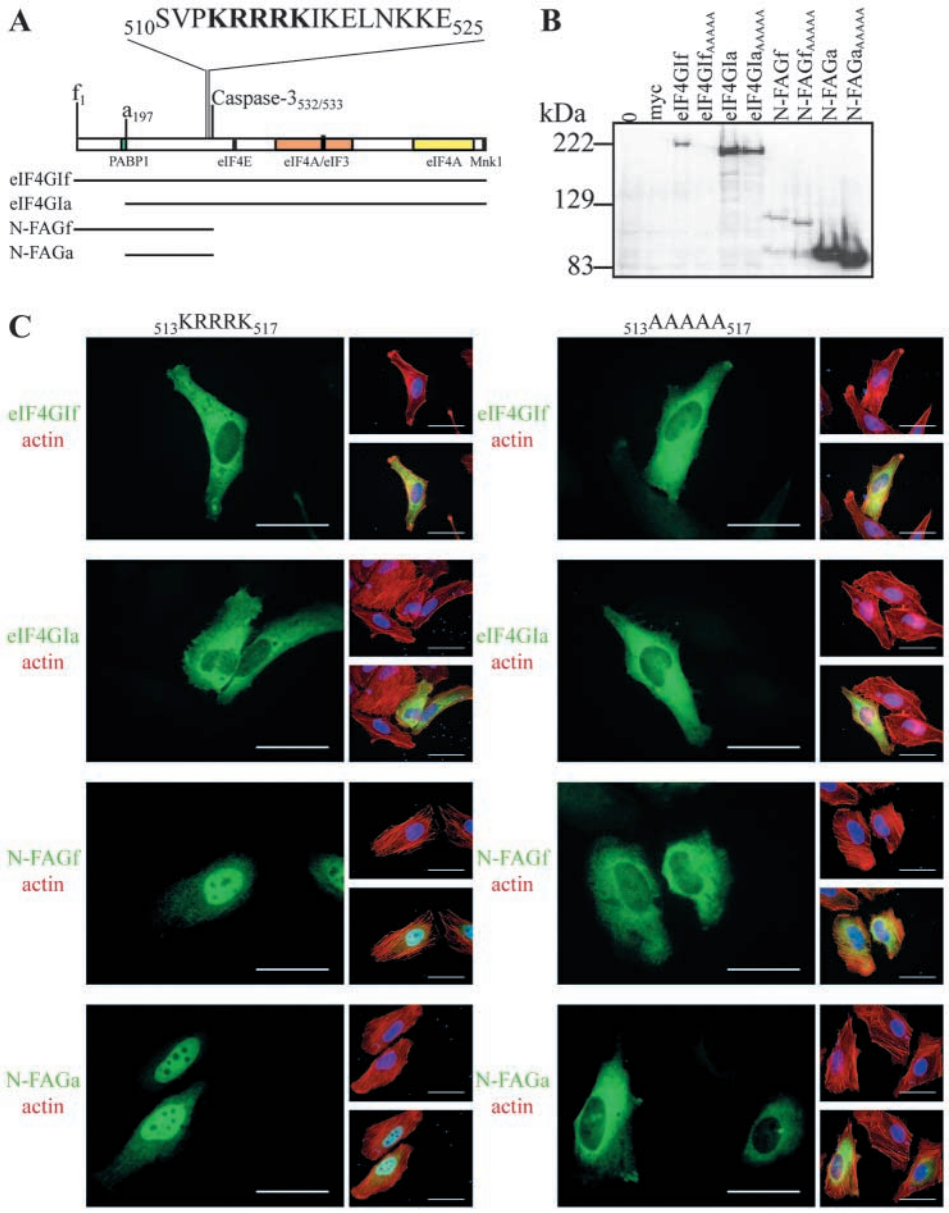

Fig. 5. Mutation of a putative NLS in N-FAG changes its localisation from nuclear to cytoplasmic. (A) Schematic representation of eIF4GI showing the basic region that may act as a nuclear localisation signal and the individual sequences used in this study. (B) HeLa cells were transfected with plasmids encoding the wild-type or $_{513}$KRRRK$_{517}$-$_{513}$AAAAA$_{517}$ mutated myc-tagged eIF4GI sequences indicated. 16 hours after transfection, total cell lysates were prepared and proteins resolved by SDS-PAGE. eIF4GI and its N-terminal cleavage fragments were visualised by immunoblotting using anti-myc antiserum; molecular mass markers are shown on the left. Expression of eIF4GIf$_{AAAAA}$ is only detectable at extremely long exposures (data not shown). (C) HeLa cells were probed with anti-myc antibody followed by goat anti-mouse IgG conjugated to FITC (green) to visualise the localisation of wild-type N-terminal apoptotic cleavage fragments of eIF4GI, or those in which a basic sequence was mutated to alanines, as indicated. Actin was visualised with phalloidin-TRITC (red) and nuclei with DAPI (blue). Scale bars: 20 μm.

region of eIF4GI are nuclear, and this behaviour is dependent on a basic region between residues 513 and 517. The movement of proteins between the nucleus and cytoplasm involves several families of shuttling proteins that bind to defined sequences in their substrates. Examples of nuclear localisation signals (NLS) are the monopartite sequence of basic residues [KR]$_{4-6}$ originally found in the SV40 large T antigen (Kalderon et al., 1984) or the bipartite basic sequence [KR]$_2$X$_{10-12}$[KR]$_3$ first identified in nucleoplasmin (Robbins et al., 1991). We show here that when this monopartite KRRRK sequence in N-FAG is mutated to alanines, the nuclear localisation of N-FAG sequences is abrogated (Fig. 5C). The heterodimeric importin proteins (reviewed by Jans et al., 2000; Kaffman and O'Shea, 1999; Komeili and O'Shea, 2001) responsible for transport of eIF4GI through the nuclear pore complex have yet to be identified.

The basic region of lysine and arginine residues is conserved in eIF4GI sequences from rabbit, mouse, rat and cat, but is not conserved (KTWKK) in human eIF4GII. One might therefore predict that eIF4GII would be exclusively cytoplasmic, but this is a question that cannot be answered at present, as the existing antibodies to eIF4GII are unsuitable for immunofluorescence studies.

These studies extend previous observations indicating the presence of a proportion of cellular eIF4GI in the nucleus (Kedersha et al., 2002; Kimball et al., 2003; McKendrick et al., 2001). In addition its binding partner, eIF4E, has also been shown to have a nuclear component (Dostie et al., 2000a; Dostie et al., 2000b; Strudwick and Borden, 2002). However, definite roles for these initiation factors in the nuclei of cells have still to be identified. It has been suggested that some translation may occur in the nucleus (Iborra et al., 2001), but there remains considerable controversy about the validity of these studies (Bohnsack et al., 2002; Dahlberg et al., 2003; Nathanson et al., 2003). Another attractive possibility is that the recruitment of eIF4G into a cap-binding complex in the

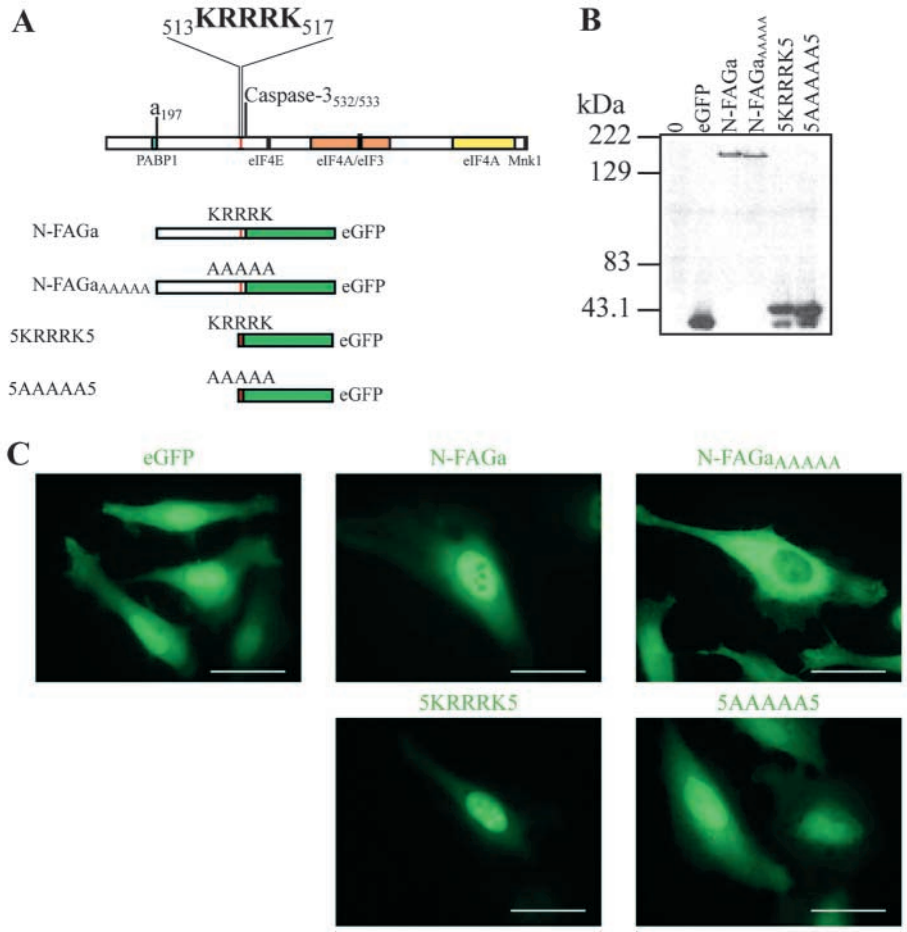

Fig. 6. The eIF4GI nuclear localisation signal is sufficient to direct a change in the subcellular localisation of eGFP. (A) Schematic representation of eIF4GI outlining the individual sequences used for expression in this figure. (B) Total cell extracts from HeLa cells expressing eGFP fusion proteins were resolved by SDS-PAGE. Expressed proteins were visualised by immunoblotting using a monoclonal antibody raised against eGFP; molecular mass markers are shown on the left. (C) HeLa cells expressing eGFP fusion proteins were grown on glass coverslips and fixed with paraformaldehyde. eGFP was visualised directly using the appropriate fluorescence filters. Scale bars: 20 μm.

nucleus may play an important part in the export of mRNAs, a process that may itself be closely associated with a 'pioneer' round of translation that monitors the integrity of newly synthesised mRNA (Ishigaki et al., 2001; McKendrick, 2003). However, further work is needed to establish whether eIF4GI is involved in nucleocytoplasmic shuttling of mRNA, or whether there are separate cytoplasmic and nuclear pools of this initiation factor with distinct functions.

Attempts have been made by ourselves and others to elucidate the effects on translation of overexpressing the apoptotic cleavage fragments in cells that are still expressing endogenous, intact eIF4GI (our unpublished results) (Nevins et al., 2003). Overexpression of N-FAG in these cases had no detectable effect on protein synthesis rates or expression of reporter plasmids. This result was somewhat unexpected as one might predict that the presence of an N-terminal fragment of eIF4GI might affect translation by sequestering cytoplasmic PABP1, as suggested from in vitro studies (Imataka et al., 1998). Our demonstration that exogenously expressed N-FAG is sequestered in the nucleus may provide an explanation for the lack of effect of N-FAG on translation initiation in the cytoplasm. However, 293 cells stably over-expressing N-FAG containing the mutated monopartite NLS ($_{513}$KRRRK$_{517}$ to $_{513}$AAAAA$_{517}$) show no obvious changes in ongoing translation (S.J.M. and M.J.C., unpublished data). Further biochemical work is underway to determine the reasons behind this observation.

While the presence of this N-terminal motif is necessary and sufficient to exert a nuclear localisation on the apoptotic fragment N-FAG (or on a fused reporter protein), it is clear that intact eIF4G has a predominantly cytoplasmic localisation within the cell (Fig. 1 and Fig. 2C), which is not substantially altered by mutation of the putative NLS (Fig. 5C). Analysis of the rather complex pattern of distribution of the central and C-terminal fragments of eIF4GI (Fig. 4) indicates that the most completely cytoplasmic localisation is exhibited by those fragments that contain the eIF4E binding motif and the sequences surrounding it (amino acids 534-674). This region is relatively proline rich (13.5%) and includes the target sequences for cellular and viral proteases that attack protein in situ during apoptosis or picornavirus infection (Bushell et al., 2000a; Bushell et al., 2000b; Clemens et al., 1998; Lamphear et al., 1995; Marissen and Lloyd, 1998; Morley, 2001). It is therefore probable that an, as yet, undefined sequence within this region conferring cytoplasmic localisation or nuclear export would be exposed in the intact molecule.

In addition, the M-FAG and C-FAG sequences each contain motifs with a degree of homology to leucine-rich nuclear export signals (NES). Leucine-rich nuclear export sequences (NES) with the consensus sequence $LX_{2-3}[LIVFM]X_{2-3}LX[LI]$ (La Cour et al., 2003) were first found in the protein kinase A inhibitor (PKI) (Wen et al., 1995) and the Rev protein of HIV (Fischer et al., 1995) and are bound

to and shuttled by the Crm1/exportin1 protein (Gorlich and Mattaj, 1996; Ullman et al., 1997). A relative of the importin family of proteins, transportin, is involved in shuttling of proteins containing a third signalling sequence, the M9 nucleocytoplasmic shuttling sequence (NS) that can act as both an NLS and NES. This sequence was first found in hnRNP A1 and is also found in other proteins involved in mRNA transport (Michael, 2000). The LNRQLEKLL sequence in C-FAG (1444-1452) exhibits a much higher degree of homology to the consensus NES motif (37) than the LFRRVRSILNKL sequence in M-FAG (761-772) although only 36% of experimentally validated nuclear export signals conform to this consensus (La Cour et al., 2003). However, the cellular distribution of either endogenous or exogenous full-length eIF4GI (McKendrick et al., 2001) or of the exogenous fragments of eIF4GI used in this work (data not shown) is insensitive to leptomycin B (LMB) treatment, which inhibits the Crm1 export receptor (Kudo et al., 1998). As described previously (McKendrick et al., 2001; Parra-Palau et al., 2003), when the eIF4E kinase, Mnk1, was expressed from the pcDNAmyc vector, this cytoplasmic protein became sequestered in the nucleus in response to LMB, demonstrating that this compound was active in our cells. These data strongly support the findings that Crm1-dependent transport of eIF4GI is not important for its localisation. Further work is required to identify the nuclear export signals responsible for the predominantly cytoplasmic localisation of intact eIF4G and an LMB-independent pathway responsible for the shuttling of this protein between the nucleus and the cytoplasm.

We thank Richard E. Lloyd for the kind gift of the pSPORT4GI plasmid and Matthias Görlach for the monoclonal PABP1 (6B12) antibody. Research in our laboratory is funded by grants (40800, 56778, 58915, 57494) from The Wellcome Trust and S.J.M. is a Senior Research Fellow of The Wellcome Trust.

References

Afonina, E., Stauber, R. and Pavlakis, G. N. (1998). The human poly(A)-binding protein 1 shuttles between the nucleus and the cytoplasm. *J. Biol. Chem.* **273**, 13015-13021.

Bassell, G. J., Oleynikov, Y. and Singer, R. H. (1999). The travels of mRNAs through all cells large and small. *FASEB J.* **13**, 447-454.

Belsham, G. J. and Jackson, R. J. (2000). Translation initiation on Picornavirus RNA. In *Translational Control of Gene Expression* (ed. N. Sonenberg, J. W. B. Hershey and M. B. Mathews), pp. 869-900: Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Bohnsack, M. T., Regener, K., Schwappach, B., Saffrich, R., Paraskeva, E., Hartmann, E. and Gorlich, D. (2002). Exp5 exports eEF1A via tRNA from nuclei and synergizes with other transport pathways to confine translation to the cytoplasm. *EMBO J.* **21**, 6205-6215.

Bradley, C. A., Padovan, J. C., Thompson, T. C., Benoit, C. A., Chait, B. T. and Rhoads, R. E. (2002). Mass spectrometric analysis of the N-terminus of translation initiation factor eIF4G-1 reveals novel isoforms. *J. Biol. Chem.* **277**, 12559-12571.

Bushell, M., Poncet, D., Marissen, W. E., Flotow, H., Lloyd, R. E., Clemens, M. J. and Morley, S. J. (2000a). Cleavage of polypeptide chain initiation factor eIF4GI during apoptosis in lymphoma cells: characterisation of an internal fragment generated by caspase-3-mediated cleavage. *Cell Death Differ.* **7**, 628-636.

Bushell, M., Wood, W., Clemens, M. J. and Morley, S. J. (2000b). Changes in integrity and association of eukaryotic protein synthesis initiation factors during apoptosis. *Eur. J. Biochem.* **267**, 1083-1091.

Byrd, M. P., Zamora, M. and Lloyd, R. E. (2002). Generation of multiple isoforms of eukaryotic translation initiation factor 4GI by use of alternate translation initiation codons. *Mol. Cell Biol.* **22**, 4499-4511.

Clemens, M. J., Bushell, M., Jeffrey, I. W., Pain, V. M. and Morley, S. J. (2000). Translation initiation factor modifiations and the regulation of protein synthesis in apoptotic cells. *Cell Death Differ.* **7**, 603-615.

Clemens, M. J., Bushell, M. and Morley, S. J. (1998). Degradation of eukaryotic polypeptide chain initiation factor (eIF) 4G in response to induction of apoptosis in human lymphoma cell lines. *Oncogene* **17**, 2921-2931.

Dahlberg, J. E., Lund, E. and Goodwin, E. B. (2003). Nuclear translation: What is the evidence? *RNA* **9**, 1-8.

Dostie, J., Ferraiuolo, M., Pause, A., Adam, S. A. and Sonenberg, N. (2000a). A novel shuttling protein, 4E-T, mediates the nuclear import of the mRNA 5′ cap binding of the mRNA 5′ cap-binding protein, eIF4E. *EMBO J.* **19**, 3142-3156.

Dostie, J., Lejbkowicz, F. and Sonenberg, N. (2000b). Nuclear eukaryotic initiation factor 4E (eIF4E) colocalizes with splicing factors in speckles. *J. Cell Biol.* **148**, 239-247.

Fischer, U., Huber, J., Boelens, W. C., Mattaj, I. W. and Luhrmann, R. (1995). The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell* **82**, 475-483.

Gingras, A. C., Raught, B. and Sonenberg, N. (1999). eIF4 initiation factors: Effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu. Rev. Biochem.* **68**, 913-963.

Gorlich, D. and Mattaj, I. W. (1996). Nucleocytoplasmic transport. *Science* **271**, 1513-1518.

Gradi, A., Imataka, H., Svitkin, Y. V., Rom, E., Raught, B., Morino, S. and Sonenberg, N. (1998). A novel functional human eukaryotic translation initiation factor 4G. *Mol. Cell Biol.* **18**, 334-342.

Harlow, E. and Lane, D. (1999). *Using Antibodies: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Hashemzadeh-Bonehi, L., Curtis, P. S., Morley, S. J., Thorpe, J. R. and Pain, V. M. (2003). Overproduction of a conserved domain of fission yeast and mammalian translation initiation factor eIF4G causes abberrant cell morphology and results in disruption of the localization of F-actin and the organization of microtubules. *Genes Cells* **8**, 163-178.

Henis-Korenblit, S., Shani, G., Sines, T., Marash, L., Shohat, G. and Kimchi, A. (2002). The caspase-cleaved DAP5 protein supports internal ribosome entry site-mediated translation of death proteins. *Proc. Natl. Acad. Sci. USA* **99**, 5400-5405.

Hershey, J. W. B. and Merrick, W. C. (2000). Pathway and mechanism of initiation of protein synthesis. In *Translational Control of Gene Expression* (ed. N. Sonenberg, J. W. B. Hershey and M. B. Mathews), pp. 33-88. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Hesketh, J. (1994). Translation and the cytoskeleton: a mechanism for targeted protein synthesis. *Mol. Biol. Rep.* **19**, 233-243.

Hesketh, J. (1996). Sorting of messenger RNAs in the cytoplasm: mRNA localization and the cytoskeleton. *Exp. Cell Res.* **225**, 219-236.

Hiriart, E., Farjot, G., Gruffat, H., Nguyen, M. V., Sergeant, A. and Manet, E. (2003). A novel nuclear export signal and a REF interaction domain both promote mRNA export by the Epstein-Barr virus EB2 protein. *J. Biol. Chem.* **278**, 335-342.

Iborra, F. J., Jackson, D. A. and Cook, P. R. (2001). Coupled transcription and translation within nuclei of mammalian cells. *Science* **293**, 1139-1142.

Imataka, H., Gradi, A. and Sonenberg, N. (1998). A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation. *EMBO J.* **17**, 7480-7489.

Ishigaki, Y., Li, X., Serin, G. and Maquat, L. E. (2001). Evidence for a pioneer round of mRNA translation: mRNAs subject to nonsense-mediated decay in mammalian cells are bound by CBP80 and CBP20. *Cell* **106**, 607-617.

Jans, D. A., Xiao, C. Y. and Lam, M. H. (2000). Nuclear targeting signal recognition: a key control point in nuclear transport? *Bioessays* **22**, 532-544.

Jansen, R. P. (1999). RNA-cytoskeletal associations. *FASEB J.* **13**, 455-466.

Kaffman, A. and O'Shea, E. K. (1999). Regulation of nuclear localization: a key to a door. *Annu. Rev. Cell Dev. Biol.* **15**, 291-339.

Kalderon, D., Roberts, B. L., Richardson, W. D. and Smith, A. E. (1984). A short amino acid sequence able to specify nuclear location. *Cell* **39**, 499-509.

Kedersha, N., Chen, S., Gilks, N., Li, W., Miller, I. J., Stahl, J. and Anderson, P. (2002). Evidence that ternary complex (eIF2-GTP-tRNA(i)(Met))-deficient preinitiation complexes are core constituents of mammalian stress granules. *Mol. Cell Biol.* **13**, 195-210.

Kimball, S. R., Horetsky, R. L., Ron, D., Jefferson, L. S. and Harding, H. P. (2003). Mammalian stress granules represent sites of accumulation of

stalled translation initiation complexes. *Am. J. Physiol. Cell Physiol.* **284**, C273-C284.

Komeili, A. and O'Shea, E. K. (2001). New perspectives on nuclear transport. *Annu. Rev. Genet.* **35**, 341-364.

Kudo, N., Wolff, B., Sekimoto, T., Schreiner, E. P., Yoneda, Y., Yanagadi, M., Horinouchi, S. and Yoshida, M. (1998). Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1. *Exp. Cell Res.* **242**, 540-547.

La Cour, T., Gupta, R., Rapacki, K., Skriver, K., Poulsen, F. M. and Brunak, S. (2003). NESbase version 1.0: a database of nuclear export signals. *Nucleic Acids Res.* **31**, 393-396.

Lamphear, B. J., Kirchweger, R., Skern, T. and Rhoads, R. E. (1995). Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. *J. Biol. Chem.* **270**, 21975-21983.

Lenk, R., Ransom, L., Kaufmann, Y. and Penman, S. (1977). A cytoskeletal structure with associated polyribosomes obtained from HeLa cells. *Cell* **10**, 67-78.

Marissen, W. E., Gradi, A., Sonenberg, N. and Lloyd, R. E. (2000). Cleavage of eukaryotic translation initiation factor 4GII correlates with translation inhibition during apoptosis. *Cell Death Differ.* **7**, 1234-1243.

Marissen, W. E. and Lloyd, R. E. (1998). Eukaryotic translation initiation factor 4G is targeted for proteolytic cleavage by caspase 3 during inhibition of translation in apoptotic cells. *Mol. Cell. Biol.* **18**, 7565-7574.

McKendrick, L. (2003). What's new in translation initiation? The first translation determines the fate of mRNA. *Cell. Mol. Life Sci.* **60**, 1-9.

McKendrick, L., Thompson, E., Ferreira, J., Morley, S. J. and Lewis, J. D. (2001). Interaction of eukaryotic translation initiation factor 4G with the nuclear cap-binding complex provides a link between nuclear and cytoplasmic functions of the m(7) guanosine cap. *Mol. Cell Biol.* **21**, 3632-3641.

Michael, W. M. (2000). Nucleocytoplasmic shuttling signals: two for the price of one. *Trends Cell Biol.* **10**, 46-50.

Morley, S. J. (2001). The regulation of eIF4F during cell growth and cell death. *Prog. Mol. Subcell Biol.* **27**, 1-37.

Morley, S. J., Curtis, P. S. and Pain, V. M. (1997). eIF4G: Translation's mystery factor begins to yield its secrets. *RNA* **3**, 1085-1104.

Morley, S. J., Jeffrey, I., Bushell, M., Pain, V. M. and Clemens, M. J. (2000). Differential requirements for caspase-8 activity in the mechanism of phosphorylation of eIF2alpha, cleavage of eIF4GI and signaling events associated with the inhibition of protein synthesis in apoptotic Jurkat T cells. *FEBS Lett.* **477**, 229-236.

Nathanson, L., Xia, T. and Deutscher, M. P. (2003). Nuclear protein synthesis: A re-evaluation. *RNA* **9**, 9-13.

Nevins, T. A., Harder, Z. M., Korneluk, R. G. and Holcik, M. (2003). 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. *J. Biol. Chem.* **278**, 3572-3579.

Oberhammer, F. A., Hochegger, K., Froschl, G., Tiefenbacher, R. and Pavelka, M. (1994). Chromatin condensation during apoptosis is accompanied by degradation of lamin A+B, without enhanced activation of cdc2 kinase. *J. Cell Biol.* **126**, 827-837.

Ogawa, H., Inouye, S., Tsuji, F. I., Yasuda, K. and Umesono, K. (1995). Localization, trafficking, and temperature-dependence of the Aequorea green fluorescent protein in cultured vertebrate cells. *Proc. Natl. Acad. Sci. USA* **92**, 11899-11903.

Ohlmann, T., Rau, M., Pain, V. M. and Morley, S. J. (1996). The C-terminal domain of eukaryotic protein synthesis initiation factor (eIF) 4G is sufficient to support cap-independent translation in the absence of eIF4E. *EMBO J.* **15**, 1371-1382.

Parra-Palau, J. L., Scheper, G. C., Wilson, M. L. and Proud, C. G. (2003). Features in the N and C termini of the MAPK-interacting kinase Mnk1 mediate its nucleocytoplasmic shuttling. *J. Biol. Chem.* **278**, 44197-44204.

Pestova, T. V., Shatsky, I. N. and Hellen, C. U. T. (1996). Functional dissection of eukaryotic initiation factor 4F: the 4A subunit and the central domain of the 4G subunit are sufficient to mediate internal entry of 43S preinitiation complexes. *Mol. Cell. Biol.* **16**, 6870-6878.

Pyronnet, S., Imataka, H., Gingras, A. C., Fukunaga, R., Hunter, T. and Sonenberg, N. (1999). Human eukaryotic translation initiation factor 4G (eIF4G) recruits mnk1 to phosphorylate eIF4E. *EMBO J.* **18**, 270-279.

Rao, L., Perez, D. and White, E. (1996). Lamin proteolysis facilitates nuclear events during apoptosis. *J. Cell Biol.* **135**, 1441-1455.

Rizzuto, R., Brini, M., Pizzo, P., Murgia, M. and Pozzan, T. (1995). Chimeric green fluorescent protein as a tool for visualizing subcellular organelles in living cells. *Curr. Biol.* **5**, 635-642.

Robbins, J., Dilworth, S. M., Laskey, R. A. and Dingwall, C. (1991). Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell* **64**, 615-623.

Stoneley, M., Chappell, S. A., Jopling, C. L., Dickens, M., MacFarlane, M. and Willis, A. E. (2000). c-Myc protein synthesis is initiated from the internal ribosome entry segment during apoptosis. *Mol. Cell. Biol.* **20**, 1162-1169.

Strudwick, S. and Borden, K. L. (2002). The emerging roles of translation factor eIF4E in the nucleus. *Differentiation* **70**, 10-22.

Ullman, K. S., Powers, M. A. and Forbes, D. J. (1997). Nuclear export receptors: from importin to exportin. *Cell* **90**, 967-970.

Wells, S. E., Hillner, P. E., Vale, R. D. and Sachs, A. B. (1998). Circularisation of mRNA by eukaryotic translation initiation factors. *Mol. Cell* **2**, 135-140.

Wen, W., Meinkoth, J. L., Tsien, R. Y. and Taylor, S. S. (1995). Identification of a signal for rapid export of proteins from the nucleus. *Cell* **82**, 463-473.

Yan, R., Rychlik, W., Etchison, D. and Rhoads, R. E. (1992). Amino acid sequence of the human protein synthesis initiation factor eIF-4 gamma. *J. Biol. Chem.* **267**, 23226-23231.