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Specific Isoforms of Translation Initiation Factor 4GI Show Differences in Translational Activity

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The eukaryotic initiation factor (eIF) 4GI gene locus (eIF4GI) contains three identified promoters, generating alternately spliced mRNAs, yielding a total of five eIF4GI protein isoforms. Although eIF4GI plays a critical role in mRNA recruitment to the ribosomes, little is known about the functions of the different isoforms, their partner binding capacities, or the role of the homolog, eIF4GII, in translation initiation. To directly address this, we have used short interfering RNAs (siRNAs) expressed from DNA vectors to silence the expression of eIF4GI in HeLa cells. Here we show that reduced levels of specific mRNA and eIF4GI isoforms in HeLa cells promoted aberrant morphology and a partial inhibition of translation. The latter reflected dephosphorylation of 4E-BP1 and decreased eIF4F complex levels, with no change in eIF2α phosphorylation. Expression of siRNA-resistant Myc-tagged eIF4GI isoforms has allowed us to show that the different isoforms exhibit significant differences in their ability to restore translation rates. Here we quantify the efficiency of eIF4GI promoter usage in mammalian cells and demonstrate that even though the longest isoform of eIF4GI (eIF4GII) was relatively poorly expressed when reintroduced, it was more efficient at promoting the translation of cellular mRNAs than the more highly expressed shorter isoforms used in previous functional studies.

Translational control plays a critical role in overall gene expression, allowing the rapid and reversible stimulation of protein synthesis from preexisting mRNAs, the fine-tuning of protein expression levels, and in some cases the production of proteins at specific sites in the cell (reviewed in reference 37). Eukaryotic protein synthesis comprises three stages: initiation, elongation, and termination. The initiation phase refers to the binding of the ribosomal subunits to the mRNA and positioning of the subunits at the first codon of the mRNA open reading frame. Several eukaryotic initiation factors (eIFs) are required for this process, and in most cases, initiation determines both the rate of translation of individual mRNAs and the overall rate of protein synthesis (17, 19, 32, 36). The inappropriate expression of several initiation factors has been noted for a number of diseases and cancers (1, 34), and the improper recruitment of mRNAs to the ribosome may also play a role in the deregulation of gene expression.

The multidomain factor eIF4G, expressed as two isoforms in mammalian cells sharing 46% identity at the amino acid level (eIF4GI/II), plays an essential role in mRNA recruitment by acting as a molecular focal point upon which the translation initiation complex is assembled to bring together the mRNA and the ribosome. eIF4G is part of the eIF4F complex (17, 36), which also comprises the mRNA cap-binding protein (eIF4E) and an ATP-dependent RNA helicase activity (eIF4A, in concert with eIF4B). The yeast, wheat, and mammalian homologs bind to RNA in a sequence-independent manner (6, 28, 30), although mammalian eIF4G also binds specifically to the 5′ untranscribed region of the eencephalomyocarditis virus RNA genome, which contains an internal ribosome entry segment (IRES) (30, 41). Interaction of the poly(A) binding protein (PABP) with the N terminus of eIF4F (23), the kinase that phosphorylates eIF4E (Mnk1) with the C terminus of eIF4F (45) and the multisubunit eIF3 (and therefore the 40S ribosomal subunit and eIF2-GTP-initiator methionyl tRNA complex) with the central portion of eIF4G, completes the formation of the 48S preinitiation complex (17, 37).

Consistent with an important regulatory role, the expression level of eIF4G is low in both mammalian and yeast cells, and its down-regulation by proteolysis is an early response to the induction of apoptosis or infection by some viruses (reviewed in reference 37). The importance of regulating the level of expression of eIF4G is demonstrated by the finding that amplification of overall eIF4G protein levels occurs in squamous cell lung carcinoma (3) and breast carcinoma (2), while overexpression of cDNA encoding the shortest isoform of eIF4G causes the malignant transformation of mammalian cell lines (14, 22). The original cDNA clone of eIF4G (52) was extended in a later study (23) to identify the PABP binding site. The independent discovery of a further N-terminal extension of 40 amino acids finally identified the longest possible open reading frame of eIF4G (7, 9), with these studies suggesting alternative translation initiation can generate five isoforms of eIF4G. The identification of three promoters within the eIF4G gene locus and the finding that mRNAs from one of these promoters are also alternately spliced (10) indicate that there are multiple levels at which the expression of eIF4G can be regulated. Until recently, no evidence had been found for differential activities between eIF4GI and eIF4GII, since the two proteins could functionally complement each other in various translation assays. However, an important difference has recently been revealed with the discovery that during megakaryocytic differentiation, eIF4GII is selectively recruited into eIF4F
complexes (11). In addition, eIF4G homologues differ in their phosphorylation status, with serum-stimulated sites of phosphorylation identified in the C terminus of eIF4GII but not in eIF4GI (44, 46), suggesting a possible isoform-specific role for this modification. In contrast, eIF4GII phosphorylation is increased primarily at the G2/M phase of the cell cycle (44, 46), when there is a transient decrease in overall translation rates, although it remains unclear how these individual phosphorylation events modulate translation.

Since eIF4G plays such a central role in the assembly of the preinitiation complex, it is not surprising that modifications of eIF4GI and eIF4GII by physiological cleavage events have drastic consequences for translation initiation (reviewed in references 37 and 43). Cleavage of eIF4GI and eIF4GII by picornavirus proteases (leader or 2A protease) leads to the separation of the region involved in mRNA cap binding from the ribosome binding portion of the molecule (reviewed in reference 38). This results in a severe, selective inhibition of host protein synthesis, with the C-terminal fragment of eIF4G functioning in a modified preinitiation complex to allow translation of uncapped viral RNA and certain cellular IRES-containing mRNAs (5, 25). Human immunodeficiency virus type 1 protease-mediated cleavage of eIF4GI but not eIF4GII has also been observed (40, 51), with both eIF4GI and eIF4GII being targets for caspase-mediated degradation during apoptosis (37).

However, in spite of this knowledge, little is currently known about the partner binding capacities or function of the different isoforms and variants of eIF4G in translation initiation. To directly address the role for the different full-length isoforms of eIF4GI in vivo, we have used short interfering RNAs (siRNAs) expressed from DNA vectors (49) to reduce the expression of eIF4GI in HeLa cells (21), causing morphological changes and a partial inhibition of translation rates. The latter could be ascribed to a general dephosphorylation of 4E-BP1 and decreased eIF4F complex levels, with no change in eIF2a phosphorylation. Upon expression, the siRNA-resistant Myc-tagged eIF4GII isoforms were able to form eIF4F complexes in line with the binding sites present in the molecules. However, even though the eIF4GI isoform was relatively poorly expressed, it was significantly more efficient at promoting translation than the highly expressed, shortest isoform of eIF4GII used in previous studies.

MATERIALS AND METHODS

Plasmids expressing siRNA hairpins and eIF4G sequences. Details of oligonucleotide primers used are provided in the supplemental material. In all cases, the presence of the correct siRNA or eIF4G sequence was verified by restriction digestion and automated sequencing. A reference plasmid for the quantitative mRNA or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, using the QuantiTect RT-PCR assay was prepared from total RNA with the Improm II reverse transcription system (Promega) using random primers. The QuantiTect SYBR Green PCR kit (QIAGEN) was used to detect different species of eIF4GI mRNA or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, using the primers described in the supplemental material, which were designed using Primer Express Program v2.0 (Applied Biosystems). Amplification reactions were carried out and analyzed using an Applied Biosystems 7500 real-time PCR system.

Preparation of cell lysates and measurement of protein synthesis rates. Cells were harvested by scraping into ice-cold PBS containing 40 mM β-glycerophosphate and 2 mM benzamidine. Following centrifugation for 1 min at 10,000 × g, cell pellets were resuspended in buffer A (20 mM morpholinepropanesulfonic acid–KOH, pH 7.4; 25 mM KCl; 2 mM MgCl2; 2 mM benzamidine; 2 mM Na3VO4; 1 μM microcystin; 2 mM EGTA; 20 mM NaF; 0.1 mM GTP, 10% [vol/vol] glycerol, 1% Complete EDTA-free protease inhibitor cocktail [Roche] and lysed by the addition of 0.5% [vol/vol] Igepal and 0.5% [vol/vol] deoxycholate). Nuclear and insoluble protein were removed by centrifugation for 5 min at 10,000 × g and 4°C, and the resulting supernatant was snap frozen in liquid N2. To determine protein synthesis rates after 96 h of transfection, the conditioned medium was supplemented with 33 μCi/ml of [35S]methionine (ICN Biomedicals) for 60 min prior to harvesting cells as described above. Incorporation of [35S]methionine into protein was determined by precipitation with trichloroacetic acid and scintillation counting.

mG5-SEPHAROSE affinity isolation of eIF4E and associated factors. For the isolation of eIF4E and associated proteins, cell extracts of equal protein concentrations were subjected to mG5-SEPHAROSE chromatography (Amersham...
Biosciences, United Kingdom) and the resin washed twice with buffer B (20 mM morpholinepropanesulfonic acid–KOH, pH 7.4, 25 mM KCl, 2 mM CH₃H₂MgO₂, 2 mM benzamidine, 2 mM 2-mercaptoethanol, 0.1 mM GTP, and 0.25% [vol/vol] Igepal). Recovered protein was eluted directly into sample buffer.

Immunoblotting and antibodies. Cell lysates adjusted to contain equal amounts of protein or obtained from m¹GTP-Sepharose affinity isolation were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, United Kingdom), and proteins were visualized with the antibodies described below. Appropriate secondary antibodies conjugated to horseradish peroxidase were used to visualize antibody complexes with enhanced chemiluminescence reagents (Perbio, United Kingdom). Polyclonal rabbit antibodies against epitopes in the C terminus of eIF4GI (residues 1179 to 1206), the N terminus of eIF4GII (residues 43 to 62), eIF4E (residues 203 to 217), and PABPI (residues 413 to 434) have been previously described (8, 12). Rabbits were immunized with the following peptides or bacterially expressed proteins to raise polyclonal antibodies against the N terminus of eIF4GI (residues 175 to 200; KREKRTRIRDNPGQGGKVIDEEI), against eIF4GII (residues 183 to 419; QLP.TPP), and against eIF4A (residues 348 to 369; DLPSRNY1 HRTGGRGQRGRK). All peptide antigens were isolated from crude serum by affinity chromatography with the corresponding peptide using the SulfoLink kit (Perbio, United Kingdom) according to the manufacturer’s instructions. The anti-Myc 4A6 monoclonal antibody was purchased from Upstate, and the anti-β-actin polyclonal antibody was from Sigma (United Kingdom). Polyclonal antibodies to 4E-BP1, phospho-4E-BP1, total eIF2α, phospho-eIF2α, and phospho-eIF4GI were from Cell Signaling Technology (United Kingdom). In all cases, care was taken to ensure that detection was within the linear response of the individual antisera to the protein.

Immunofluorescence microscopy. HeLa cells, seeded on glass coverslips as described above, were prepared for immunofluorescence microscopy by fixation in 4% (wt/vol) paraformaldehyde in PBS, pH 7.4, for 15 min and permeabilized in PBS containing 0.1% (vol/vol) Triton X-100 for 8 min. Following blocking, eIF4GI was detected with the C-terminus-specific antibody, followed by swine antirabbit immunoglobulin G conjugated to tetramethyl rhodamine isocyanate (Dako, United Kingdom), as previously described (12). Actin was detected by phalloidin conjugated to fluorescein isothiocyanate (Sigma, United Kingdom), while nuclei were stained with 4’6-diamidino-2-phenylindole hydrochloride (Sigma, United Kingdom). Cells were analyzed using a Zeiss Axioskop II microscope, as previously described (12).

Statistical analysis of data. For dual-luciferase and quantitative PCR experiments, data were collected from at least three independent experiments with assays performed in duplicate. For [35S]methionine incorporation into protein, data were collected from two independent experiments, with each assay performed in triplicate. Each chart shows the mean of these values, with error bars representing the standard error of the mean. Unpaired two-tailed t tests were performed using GraphPad Prism version 4.01 for Windows (www.graphpad.com).

RESULTS

The eIF4GI promoters are used with different efficiencies in human cell lines. Recent work has identified three promoters (α, β, and γ) (Fig. 1A) in the eIF4GI gene locus, with alternative splicing of the first eight exons giving rise to at least seven different mRNA species (Fig. 1B) (10). The mRNAs transcribed from the α and β promoters encode all possible eIF4GI proteins, whereas the single mRNA arising from the γ promoter encodes the isoform lacking the PABP-binding site (eIF4GIa). To measure the usage of the different eIF4GI promoters in different human cell lines, we have developed a quantitative RT-PCR assay. Total mRNA was reverse transcribed from three different cell lines (cervical carcinoma [HeLa], embryonic kidney cells [HEK293], and mammary carcinoma [MCF7]) (Fig. 1C), and the resulting cDNAs were amplified in the presence of the double-stranded DNA binding dye, SYBR Green. Using a reference plasmid containing a single copy of each ampiclon in the same assay enabled the relative abundances of the ampiclons in cellular mRNA to be determined compared to the abundance of total eIF4GI mRNA. In all cases, controls were carried out to ensure only spliced mRNA was being amplified and not unspliced RNA or genomic DNA. It must be noted that in all these assays, the total amount of mRNA quantified from the individual promoters always exceeded that of the “total” eIF4GI amplicon; this is most likely due to the additional transcripts detected not being full-length mRNAs. Figure 1C shows that the α and β promoters are used to transcribe around 50 to 60% of eIF4GI mRNA, while the γ promoter contributes only about 5 to 10% in HeLa and HEK293 cells. In MCF7 cells, the β promoter appeared to be used preferentially; however, this value always exceeded that obtained for total eIF4GI mRNA (Fig. 1C), possibly reflecting the presence of truncated transcripts in this cell line.

The five isoforms of the eIF4GI protein arising through alternative translation initiation can be detected without in vitro proteolytic cleavage. With the discovery of the longest possible open reading frame of eIF4GI mRNA (7, 9), five protein variants of eIF4GI were found to exist in cells, generated by alternative translation initiation at various AUG codons (Fig. 1D). It has been reported that IRES sequences are present in the different eIF4GI mRNA species (9, 10, 15, 16), and it is possible that these are also being utilized in these cell lines. Previous studies to resolve the different variants of eIF4GI and eIF4GI/ from cell extracts relied on proteases to bifurcate eIF4GI (7, 9). However, we have been able to use precast 4% Novex Tris-glycine gels (Invitrogen) and immunoblotting with specific antisera to resolve the eIF4GI isoforms directly from cell extracts (Fig. 1E). Using the scheme designated in reference 7, the two longest isoforms (eIF4GIb and eIF4GIe) were the most abundant in the three human cell lines examined, with the shortest isoform being less plentiful. This correlates with the greater amounts of transcripts from the α and β promoters (Fig. 1B and C). Immunoblotting for eIF4GI phosphorylated at Ser1148 (48) indicated that all the isoforms of eIF4GI were phosphorylated to a similar extent during logarithmic growth in the different cell types studied.

Plasmid-derived siRNA can be used to knock down eIF4GI mRNA and protein expression in HeLa cells. To investigate whether the different eIF4GI isoforms have different translational activities and binding partners, we used three siRNAs to reduce the expression of endogenous eIF4GI in HeLa cells (Fig. 2A). To first enable the fidelity of silencing to be measured, HeLa cells were transfected with vectors expressing the three different siRNA hairpins alongside a reporter construct (psiCHECK-2; Promega) expressing Renilla luciferase mRNA fused to the eIF4GI N-terminal RNA sequence. At 48 h after transfection, a dual-luciferase assay was used to determine the amount of Renilla luciferase in the cells relative to expression of control firefly luciferase transcribed from the same plasmid (Fig. 2B). These data show that transfecting HeLa cells with vectors expressing three different siRNA hairpins that target the 5’ end of the eIF4GI mRNA caused a similar significant (P < 0.001) reduction in the levels of Renilla luciferase (Fig. 2B). This down-regulation of Renilla luciferase expression was not observed with the vector backbone alone (empty), a vector expressing a random siRNA hairpin (siNEG), or vectors expressing siRNA hairpins containing mismatches to the eIF4GI sequence (Fig. 2A). This eliminates the possibility of nonspecific micro-RNA effects being responsible for changes
FIG. 1. The three eIF4GI promoters are used with different efficiencies in HeLa cells. A. Diagrammatic representation of the eIF4GI gene locus, showing the locations of the three promoters, designated α, β, and γ. Coding exons are shown in gray, and noncoding exons are shown in black. B. Diagrammatic representation of the eIF4GI mRNAs showing alternative splice variants generated from the different promoters (adapted from reference 10). C. Total RNA from the cell types shown was reverse transcribed and then amplified with primers specific to eIF4GI mRNAs. To quantify mRNAs arising from the α promoter, a primer pair that amplified only sequences containing exon 1 was created. The amplicon to quantify the mRNAs arising from the β promoter utilized a forward primer with complementarity to the β exon and a reverse primer specific for exon 3, while the γ promoter activity was quantified with a primer pair that amplified mRNAs containing exons γ and 9. Finally, a forward primer specific for exon 28 and a reverse primer complementary to exon 29 were used to quantify the total amount of eIF4GI mRNA in cells. Values obtained by quantitative RT-PCR were normalized to those obtained from an amplicon which detected all eIF4GI mRNA. Data were collected from three independent experiments, with assays each performed in triplicate, and error bars represent the standard errors of the means. D. Schematic representation of the eIF4GI protein. This figure shows the sites of alternative translation initiation, binding sites of other components of the translation initiation machinery (7, 10, 36), and epitopes used to generate antisera used in these studies. Sites of cleavage by caspase-3 and the picornaviral (L/2A) proteases are indicated. E. Equal amounts of extract from the cell types indicated were subjected to SDS-PAGE using 4% acrylamide precast gels and proteins transferred to PVDF. The membrane was then probed with the antibodies shown to visualize the eIF4GI isoforms (indicated on the left and right of the figure).
in *Renilla* luciferase, and thus eIF4GI, levels (50). Having shown that this effect was specific for the eIF4GI sequence, we isolated total RNA from HeLa cells transfected for 48 h or 72 h with either control vectors or the siRNA-expressing plasmids. The siRNA-mediated knockdown of eIF4GI was measured by a quantitative RT-PCR assay using the amplicon to determine total eIF4GI mRNA levels relative to GAPDH mRNA. When expressed as a ratio, these data show that relative to results...
with the random siRNA hairpin (siNEG), eIF4GI mRNA was efficiently targeted in this system (Fig. 2C). This result was further confirmed by Northern blotting using a radiolabeled cDNA probe to the 3' end of eIF4GI (Fig. 2D, lanes 4, 6, and 8). To allow the eIF4GI protein to turn over following the reduction in mRNA levels, cell extracts were made 72 h and 96 h after transfection. Immunoblotting using the antibody raised against a C-terminal peptide of eIF4GI demonstrated...
that siRNA31 was by far the most potent at reducing eIF4GI protein (Fig. 3A). Semiquantitative densitometrical analyses of eIF4GI expression (versus actin levels) showed that siRNA31 reduced levels of the eIF4GI protein to less than 10% ± 3% (n = 3) of that presented in the untransfected control at both times. The efficacy of siRNA31 probably reflects the fact that it targets all eIF4GI-encoding mRNA species (Fig. 2A).

Decreased levels of eIF4GI protein expression result in a partial reduction in translation rates. To determine the effect of reducing eIF4GI protein levels on translation rates, cells were pulse-labeled with [35S]methionine as described above. Figure 3B shows that when assayed after 96 h of transfection with the siRNA2 and -31-expressing plasmids, there was a significant, but incomplete, reduction in translation rates, reflecting a shift of ribosomes from polysomes to monosomes, indicative of a possible disruption of the initiation of translation (Fig. 3B). However, this may reflect that ongoing translation involving recycling of ribosomes on a single mRNA is less dependent upon eIF4G (27, 39) or that there is a certain critical threshold where the amount of eIF4GI is sufficient for translation initiation machinery, equal amounts of extracts prepared from cells transfected with siRNA2, siRNA5, or siRNA31 for 72 or 96 h were resolved by SDS-PAGE and proteins visualized by immunoblotting. A reciprocal effect on eIF4G expression has been reported when eIF4E levels were reduced in HeLa cells using antisense RNA (13), suggesting that there may be some sort of coregulation of these two initiation factors. As shown in Fig. 4A, depletion of eIF4GI with any of these siRNAs had little impact on the total levels of PABP or eIF4A. However, levels of eIF4GII and eIF4E were consistently reduced in cells transfected with siRNA31 (Fig. 4A, right panel). This was not observed with siRNA5 (Fig. 4A, middle panel), suggesting that at least part of the lack of effect on translation rates under these conditions may reflect a compensatory effect of eIF4GII or the maintained presence of eIF4Gdc and eIF4GIdc. While the phosphorylation of the α subunit of eIF2 at Ser51 was not reproducibly increased with any of the siRNAs, the phosphorylation status of the inhibitory eIF4E binding protein, 4E-BP1, showed differences in cells transfected with the different siRNAs. Relative to control siRNAs, with siRNA2 (left panel) and siRNA31 (right panel), immunoblotting with anti-eIF4E-BP1 antiserum suggested that the protein was markedly dephosphorylated at 96 h, going from the predominantly hypophosphorylated (γ) to the hypophosphorylated (α) form. These data were confirmed by using antiserum which recognizes 4E-BP1 phosphorylated on Thr70 (Fig. 4A) and Ser65 (data not shown), events associated with its release from eIF4E (17). In contrast, this was seen to a lesser extent with siRNA5 (middle panel); in conjunction with the maintained expression of eIF4GII and eIF4Gdc, this may also impact on translation rates in these cells (17, 36). As expected, isolation of eIF4E and associated proteins by m7GTP-Sepharose chromatography showed that the reduction of total eIF4GI levels also caused a decrease in eIF4G1, PABP, and eIF4A copurified as part of the eIF4F complex, with a concomitant increase in recovery of 4E-BP1 (Fig. 4B). This effect was observed with siRNA2 (right panel) but was most dramatic with siRNA31 (left panel), where levels of eIF4GI were reduced to a greater extent. These
findings of reduced levels but not complete absence of eIF4F correlate well with the reduced translation rates observed in cells transfected with siRNA2 and -31 (Fig. 2A). In contrast, with siRNA5, the association of eIF4G, PABP, and eIF4A with eIF4E was not decreased, suggesting that eIF4F levels were maintained. In addition, at 96 h, the binding of 4E-BP1 to eIF4E was not increased over that observed with the control siRNA (middle panel), consistent with a lack of effect of siRNA on 4E-BP1 phosphorylation (Fig. 4A) and with the lack of effects on translation rates in these cells (Fig. 3B).

Cells depleted of eIF4GI exhibit a multinucleated morphology. To complement the biochemical analysis, we also wanted to determine the effects on morphology when cells were depleted of eIF4GI. Accordingly, HeLa cells were transfected with the plasmid encoding siRNA31 for 72 h or 96 h (only half the amount of siRNA plasmid was used here in order to be able to distinguish transfected from untransfected cells) and prepared for immunofluorescence microscopy as described.

eIF4GI was subsequently detected using a C-terminus-specific, affinity-purified antibody (12). As shown in Fig. 5A and in agreement with our previous studies (12, 33), while some eIF4GI resides in the nucleus, the staining pattern for eIF4GI in untransfected cells was predominantly cytoplasmic, with the majority of eIF4GI localized to the perinuclear region. However, in those cells which had reduced levels of eIF4GI, the staining pattern was more nuclear, possibly reflecting differential turnover rates of cytoplasmic and nuclear pools of eIF4GI. One of the most striking phenotypes observed in some cells depleted of eIF4GI, both with siRNA31 (Fig. 5A) and siRNA2 (data not shown), was that they were very large and often multinucleated, possibly reflecting an uncoupling of mitosis and cytokinesis as a consequence of reduced translation rates.

To further this work and to identify transfected cells more efficiently, we developed an alternative siRNA expression vector which also encoded enhanced green fluorescent protein (eGFP). The eGFP open reading frame, along with the cytomegalovirus promoter and simian virus 40 poly(A) signal from the vector peGFP-N1 (Clontech, United Kingdom), were inserted into the pGEM T-easy vector (Promega, United Kingdom). The sequence encoding the H1 promoter, siRNA hairpin, and termination signal from the various pSilencer plasmids was then subcloned into this vector. Cells were then transfected for 72 or 96 h with either of these plasmids (Fig. 5B, lanes 3, 4, 7, and 8) or the pSilencer plasmids (lanes 1, 2, 5, and 6), and the silencing of eIF4GI and expression of eGFP were monitored by immunoblotting. As shown in Fig. 5B, eGFP was efficiently expressed from these vectors without affecting the ability of the siRNA to reduce the level of eIF4GI. Therefore, asynchronous, mid-log-phase HeLa cells were transfected with these vectors and prepared for immunofluorescence microscopy (Fig. 5C). The difference in eIF4GI staining (red) is clearly evident between those cells which have been transfected with the plasmid (and therefore express eGFP [green]) and those which have not. Again, a number of cells depleted for eIF4GI and contributing to the inhibition of protein synthesis became enlarged and multinucleated. This vector was also utilized in live cell imaging, where the different events leading to multinucleation can be observed in more detail (see the supplemental material). These data show that some cells depleted for eIF4GI did not divide at all, possibly because they were unable to synthesize essential proteins required for cell cycle transition. In addition, other cells divided normally, with daughter cells then fusing before dividing again, leading to a multinucleated phenotype. In other examples, cells divided into very unequal daughter cells, the smaller of which eventually underwent apoptosis, while the larger cell survived to divide into three cells. These data confirm that perturbations in protein synthesis caused by depletion of eIF4G1 below a critical threshold, had severe effects on protein synthesis, the control of cell cycle progression, and cytokinesis.

siRNA-resistant eIF4G is incorporated into eIF4F and leads to partial restoration of translation rates. To facilitate the expression of eIF4G1 cDNAs in cells in which levels of endogenous eIF4G1 had been reduced, the three mismatches used to create the control siRNA (si31M) were introduced into Myc-tagged eIF4G1 cDNAs (12). This rendered them siRNA resistant without changing the amino acid sequences of the eIF4G1 isoforms. Other cDNAs encoding the middle caspase...
cleavage fragment of eIF4GI (M-FAG) and eIF4GIIdc cDNAs (18) were expressed without further manipulation. Plasmids encoding siRNA31 and the resistant Myc-tagged eIF4GI isoforms (Fig. 6A) or eIF4GII (Fig. 6B) were cotransfected into HeLa cells for 96 h and the levels of endogenous and exogenous eIF4G monitored by immunoblotting. Figures 6A and B show that endogenous eIF4GI was efficiently reduced by siRNA31 (lane 2 versus lane 1), with the different, resistant isoforms of eIF4GI expressed to different levels (Fig. 6A, lanes 3 to 7), without any effect on total PABP or eIF4A. In agreement with previous data (Fig. 4A), 4E-BP1 was dephosphorylated in response to siRNA31 (lane 2 versus lane 1). When monitored with anti-eIF4GI serum, we consistently found that eIF4GIf (lane 3), eIF4Gle (lane 4), and eIF4Gidc (lane 5) were expressed at levels similar to those of the endogenous protein, while eIF4Gleb (lane 6), eIF4Gla (lane 7), and MFAG (lane 8) were expressed at much higher levels, as observed previously (12). Although levels of eIF4E were decreased by siRNA31, expression of eIF4GI isoforms largely prevented this decrease (lanes 3 to 7 versus lane 2). In addition, as shown with both antisera to the total protein and that recognizing 4E-BP1 phosphorylated at Thr70, expression of all isoforms of eIF4GI (Fig. 6A, lanes 3 to 8) or eIF4GII (Fig. 6B, lane 4 versus lane 2) resulted in the phosphorylation of 4E-BP1, albeit to differing degrees. Myc-eIF4GII was consistently expressed at levels similar to that of Myc-eIF4GIf (Fig. 6B, lane 4 versus lane 3), representing expression to a much higher level than the endogenous protein (lane 3 versus lane 1). The incorporation of each of the expressed siRNA-resistant eIF4GI proteins or eIF4GII into eIF4F complexes was also examined by m7GTP-Sepharose chromatography (Fig. 6A and B, lower panels). As expected, siRNA31 reduced the recovery of eIF4GIf, eIF4A, and PABP in association with eIF4E (lanes 2 versus lanes 1). Coexpression of the Myc-eIF4GI proteins (Fig. 6A, lanes 3 to 8) or eIF4GII (Fig. 6B, lanes 3 and 4) resulted in the incorporation of eIF4GI into eIF4F complexes to a level reflecting their total level of expression (upper panel). The recruitment of PABP into eIF4F was partially restored in cells expressing eIF4GIIdc (Fig. 6B, lane 4) or eIF4GII with the ability to bind PABP (Fig. 6A, lanes 3 to 6). However, these data clearly show that the levels of PABP and eIF4A associated to eIF4E were not fully restored upon reintroduction of eIF4G isoforms but were increased over the levels seen when eIF4GI was depleted (Fig. 6A, lanes 3 to 8 versus lane 2, and Fig. 6B, lanes 3 and 4 versus lane 2). In contrast, those cells expressing eIF4GIa (lane 7) or M-FAG (lane 8) showed PABP levels similar to those observed in the siRNA31-transfected cells (lane 2). The recovery of eIF4A in the eIF4F complex was increased in all the cells expressing Myc-eIF4GII, although for eIF4GIf (lane 7) and M-FAG (lane 8) this was not as great as would be predicted from their expression levels.

Translation rates were assayed in parallel by measuring the incorporation of [35S]methionine into total protein and were expressed relative to the rate measured in untransfected cells (Fig. 6C). As before (Fig. 3B), siRNA31-mediated reduction of eIF4GI levels resulted in a partial inhibition of translation that was significantly prevented by coexpression of low levels of eIF4GIf (Fig. 6A, lane 3 versus lane 2; \( P < 0.001 \)) but not back to the level obtained with the siRNA31 control. eIF4GIfc (lane 4) and eIF4Glb (lane 6) were as effective as eIF4GIf (lane 3) in this response, but eIF4GIdc (lane 5) and eIF4Gla (lane 7) were significantly less able (\( P < 0.005 \)), in spite of the latter being expressed to relatively high levels. High levels of M-FAG (lane 8) and eIF4GII (lane 9) were able to induce a small increase in the rate of translation over that in the silenced cells, but not to the level observed with eIF4H (\( P < 0.05 \)). Although there were no obvious qualitative differences in translation assessed by SDS-PAGE, work is currently under way to establish just how the isoforms of eIF4GII and eIF4GIIdc differ in their ability to recruit different mRNAs and proteins to active translation complexes.

**DISCUSSION**

Mammalian eIF4GI is a family of modular proteins, consisting of three domains of approximately 500 amino acids each (as defined by proteolytic cleavage), separated by unstructured linker regions (37, 43). eIF4GI has an important role in translation initiation, allowing multiple initiation factors to assemble at the 5′ cap of an mRNA molecule, thereby promoting its recruitment by the small ribosomal subunit (reviewed in reference 37). Recent studies with Saccharomyces cerevisiae have also suggested that eIF4G may have an undetermined but rate-limiting function at a step downstream of 48S complex assembly in vivo (24). In spite of this knowledge, little is known about how the level of eIF4G is regulated at the transcriptional and translational levels, and IRES sequences present in the different eIF4G mRNA species may also play a role in expression under different growth conditions (9, 10, 15, 16, 47).

To address this, we have quantified eIF4GI mRNA levels from a number of mammalian cell lines. Figure 1C shows that the α and β promoters are used to transcribe around 50 to 60% of eIF4GI mRNA, while the γ promoter contributes only about 5 to 10% in HeLa and HEK293 cells. We have also shown that it is possible to resolve the eIF4GI isoforms directly from cell extracts by using 4% Novex Tris-glycine gels and immunoblotting with specific antisera (Fig. 1E). Western blotting con-

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**FIG. 5. HeLa cells with reduced expression of eIF4GI often show a multinucleated morphology.** A. HeLa cells were grown on glass coverslips and after transfection for 72 h (top panels) or 96 h (bottom panels) with siRNA31 were processed for immunofluorescence microscopy, as described for panel A. These data are from a single experiment but are representative of those obtained in at least three separate experiments.

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**Table 1.** Western Blotting Analysis of eIF4GI isoforms in HeLa cells expressing Myc-eIF4GI. 

<table>
<thead>
<tr>
<th>Myc-eIF4GI Isoform</th>
<th>Western Blotting Protein</th>
<th>% of Total Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>eIF4GIf</td>
<td>4E-BP1</td>
<td>75%</td>
</tr>
<tr>
<td>eIF4Gle</td>
<td>4E-BP1</td>
<td>80%</td>
</tr>
<tr>
<td>eIF4GIdc</td>
<td>4E-BP1</td>
<td>90%</td>
</tr>
</tbody>
</table>

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Prior to the harvest after 96 h of transfection, cells were incubated with radioactive methionine for 1 h, extracts prepared, and the incorporation of

radioactive methionine into protein determined as described in the text (expressed as cpm/μg total protein). Unpaired two-way t tests were performed separately, comparing eIF4GI with si31 (***, P < 0.001; n = 3) and comparing eIF4GI with other isoforms of eIF4GI (*, P < 0.05; **, P < 0.01; ns, P > 0.05; n = 3), with error bars representing standard errors of the means.
of ribosomes on a single mRNA is less dependent upon intact eIF4G (27, 39). A recent study has shown that translation compartmentalization to the endoplasmic reticulum (ER) is resistant to cleavage of eIF4GI by Coxsackie B virus (29), raising the possibility that ongoing translation is maintained on the ER in siRNA31-treated cells, although staining of eIF4GI in the siRNA31-transfected cells does not reveal a pattern consistent with ER staining. Alternatively, it may be that there is a threshold where the amount of eIF4GII is sufficient for translation to be maintained or that the less-abundant isoform of eIF4GII, eIF4GIb, can compensate for eIF4GI under these conditions. While eIF4GIb levels were increased with siRNA2 treatment at 96 h (Fig. 3C), it is unlikely to be masking an effect on down-regulating translation, since a similar level of inhibition was observed with siRNA31, which had no effect on eIF4GIa levels (Fig. 3B).

In all cases, the failure to rescue translation to the control level may reflect that we are examining a mixed cell population. For instance, as shown in Fig. 5 (and reference (12), some cells express high levels of Myc-eIF4GI, others lower levels, and others none at all, but with analysis by SDS-PAGE, we observed a reasonable, overall expression. It is also possible that not all silenced cells were cotransfected with the siRNA-resistant eIF4G cDNAs. We are currently attempting to address this by studying the rescue of translation in eGFP-positive cells sorted by fluorescence-activated cell sorting. Due to the phenotype associated with reduced expression of eIF4GI (35) and which binds to eIF4E, eIF4A, and eIF3, was unable to rescue translation to the same extent as the N-terminal extension rendered eIF4GIe and eIF4GIb statistically more efficient at rescuing translation rates than eIF4GIa. Similarly, overexpression of M-FAG, the minimum fragment of eIF4GII able to participate in in vitro cap-dependent translation (35) and which binds to eIF4E, eIF4A, and eIF3, was unable to rescue translation to the same extent as the longest isoforms of eIF4GI, possibly reflecting the poorer incorporation of eIF4A into eIF4G complexes in these cells. The effects of reintroduction of eIF4GI cDNAs lacking partner binding sites on general protein synthesis have also been tested (Hinton et al., submitted). These data suggest that the PABP interaction site is not required for the rescue of translation, suggesting a novel function for the N-terminal extension of eIF4GI. Using the techniques developed in this study, we are currently in the process of characterizing proteins which interact with this sequence and attempting to further delineate eIF4G isoform-specific effects on cap-dependent and cap-in-

Differential activity of eIF4G isoforms

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