

Serum-stimulated, rapamycin-sensitive phosphorylation sites in the eukaryotic translation initiation factor 4GI

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The eukaryotic translation initiation factor 4G (eIF4G) proteins play a critical role in the recruitment of the translational machinery to mRNA. The eIF4Gs are phosphoproteins. However, the location of the phosphorylation sites, how phosphorylation of these proteins is modulated and the identity of the intracellular signaling pathways regulating eIF4G phosphorylation have not been established. In this report, two-dimensional phosphopeptide mapping demonstrates that the phosphorylation state of specific eIF4GI residues is altered by serum and mitogens. Phosphopeptides resolved by this method were mapped to the C-terminal one-third of the protein. Mass spectrometry and mutational analyses identified the serum-stimulated phosphorylation sites in this region as serines 1108, 1148 and 1192. Phosphoinositide-3-kinase (PI3K) inhibitors and rapamycin, an inhibitor of the kinase FRAP/mTOR (FKBP12–rapamycin-associated protein/mammalian target of rapamycin), prevent the serum-induced phosphorylation of these residues. Finally, the phosphorylation state of N-terminally truncated eIF4GI proteins acquires resistance to kinase inhibitor treatment. These data suggest that the kinases phosphorylating serines 1108, 1148 and 1192 are not directly downstream of PI3K and FRAP/mTOR, but that the accessibility of the C-terminus to kinases is modulated by this pathway(s).

Keywords: eukaryotic translation initiation factor 4G/ FKBP12–rapamycin-associated protein/mRNA translation/phosphoinositide-3-kinase/protein synthesis

Introduction

A key regulatory step in protein synthesis is the recruitment of the translational machinery to the 5' end of mRNA. This bridging process is accomplished via multiple protein–protein and protein–RNA interactions, coordinated primarily by two large, modular scaffolding factors: the eukaryotic translation initiation factor 4G (eIF4G) proteins (for reviews, see Hentze, 1997; Morley *et al.*, 1997;

Gingras *et al.*, 1999b). eIF4GI was originally identified as a constituent of eIF4F, a tripartite complex also containing an mRNA 5' cap binding protein, eIF4E, and an RNA helicase, eIF4A (reviewed in Gingras *et al.*, 1999b). Viral proteases cleave the mammalian eIF4GI protein into three fragments (N-terminal, middle and C-terminal; Lamphear *et al.*, 1995); biochemical and functional analyses of these fragments have clarified the role that each plays in the formation of a functional translation initiation complex. The N-terminal fragment interacts directly with eIF4E (Lamphear *et al.*, 1995; Mader *et al.*, 1995) and the poly(A) binding protein (PABP; Imataka *et al.*, 1998). The middle fragment of eIF4GI possesses binding sites for eIF3, a multisubunit factor associated with the 40S ribosomal subunit, and eIF4A (Lamphear *et al.*, 1995; Imataka and Sonenberg, 1997). The middle fragment also possesses RNA binding activity (Pestova *et al.*, 1996). The C-terminal fragment of eIF4GI contains a second, independent binding site for eIF4A (Lamphear *et al.*, 1995; Imataka and Sonenberg, 1997), and interacts with Mnk1 (MAP kinase signal-integrating kinase 1; Fukunaga and Hunter, 1997; or MAP kinase-interacting kinase 1; Waskiewicz *et al.*, 1997), a MAP kinase-activated serine/threonine kinase that phosphorylates eIF4E *in vitro* (Waskiewicz *et al.*, 1997) and *in vivo* (Pyronnet *et al.*, 1999; Waskiewicz *et al.*, 1999). A second eIF4G protein, eIF4GII, was recently characterized (Gradi *et al.*, 1998a), which shares 46% identity with eIF4GI at the amino acid level. eIF4GI and eIF4GII are functional homologs, in that all of the features described above for eIF4GI are conserved in eIF4GII (Gradi *et al.*, 1998a; Imataka *et al.*, 1998; Pyronnet *et al.*, 1999).

Through the aforementioned protein–protein and protein–RNA interactions, the eIF4G proteins perform several critical functions in translation initiation, including: (i) recruitment of the 40S ribosomal subunit to the 5' end of mRNA via interactions with eIF4E and eIF3; (ii) relief of inhibitory secondary structure in the mRNA 5' UTR, by delivering the eIF4A helicase to this region (Rozen *et al.*, 1990); (iii) circularization of mRNA through interactions with both eIF4E and PABP (Wells *et al.*, 1998); and (iv) delivery of the kinase Mnk1 to its substrate, eIF4E (Pyronnet *et al.*, 1999; Waskiewicz *et al.*, 1999). How all of these functions are coordinated and regulated by the eIF4G proteins is not understood.

The human eIF4G family also includes two other proteins: p97 and PAIP-1. p97 is homologous only to the C-terminal two-thirds of the eIF4Gs, and does not possess a region corresponding to the N-terminal one-third of the eIF4Gs. Like the eIF4Gs, it contains binding sites for eIF3, eIF4A and Mnk1. However, p97 does not interact with eIF4E or PABP (Imataka *et al.*, 1997; Levy-Strumpf *et al.*, 1997; Yamanaka *et al.*, 1997), and inhibits both cap-dependent and cap-independent translation *in vivo*,

presumably by forming non-functional initiation complexes (Imataka *et al.*, 1997). A more distant eIF4G homolog, PAIP-1, possesses binding sites for PABP and eIF4A, and appears to act as a translational enhancer *in vivo* (Craig *et al.*, 1998).

eIF4G homologs have been identified in many species (e.g. Browning *et al.*, 1987; Goyer *et al.*, 1993; Morley *et al.*, 1997; Hernandez *et al.*, 1998). While all of the eIF4G-like proteins identified so far possess a region homologous to the middle fragment of the mammalian eIF4G proteins, the N- and C-terminal regions of these proteins diverge significantly. For example, an extended eIF4G C-terminus is a feature present only in certain organisms: the mammalian (Yan *et al.*, 1992; Lamphear *et al.*, 1993), *Drosophila* (Hernandez *et al.*, 1998) and putative zebrafish eIF4G homologs possess an elongated C-terminal region. However, a wheat eIF4G homolog (Browning *et al.*, 1987) possesses a much smaller C-terminal region, and the *Saccharomyces cerevisiae* (Goyer *et al.*, 1993) and *Schizosaccharomyces pombe* (Morley *et al.*, 1997) eIF4G homologs do not possess such a region at all.

These observations suggest that the middle region of eIF4G is the 'core' unit required for translation. Consistent with this hypothesis, the middle fragment alone is sufficient for cap-independent translation from the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES; Pestova *et al.*, 1996). The middle one-third of the protein plus the eIF4E binding site is the minimal fragment required for cap-dependent translation of the β -globin mRNA (Morino *et al.*, 2000), and the middle region alone, fused to iron regulatory protein-1, can direct translation of an RNA containing an iron response element (DeGregorio *et al.*, 1999). Thus, the termini of the mammalian eIF4GI protein are largely dispensable for translation. However, recent evidence suggests that both the N- and C-termini play important roles in enhancing and/or modulating translation in mammalian cells. For example, the eIF4G-PABP interaction, mediated by the eIF4G N-terminus (Imataka *et al.*, 1998), is posited to inhibit the synthesis of truncated proteins from mRNAs that are not full-length (Sachs *et al.*, 1997). The interaction between eIF4A and the eIF4GI C-terminus also appears to be important, as mutation of the C-terminal eIF4A binding site decreases the activity of eIF4GI in a reticulocyte lysate by several-fold (Morino *et al.*, 2000). How the activity of the eIF4G termini may be regulated is not understood.

The eIF4Gs are phosphoproteins (Duncan *et al.*, 1987; Morley and Traugh, 1989, 1990; Donaldson *et al.*, 1991; this study). However, the intracellular signaling pathways mediating eIF4G phosphorylation, and the location of the phosphorylation sites within the proteins, have remained uncharacterized. In this report, we map a set of phosphorylated residues in eIF4GI to the C-terminal one-third of the protein. Using mass spectrometry and mutational analyses, the serum-stimulated phosphorylation sites within this region were identified. An intracellular signaling pathway (or pathways) that may be dedicated to translational control (Brown and Schreiber, 1996) consists of phosphoinositide-3-kinase (PI3K), Akt [or protein kinase B (PKB)], and the FKBP12-rapamycin-associated protein/mammalian target of rapamycin (FRAP/mTOR; also known as RAFT1 or RAPT1; reviewed in Lawrence and Abraham,

1997; Thomas and Hall, 1997; Abraham, 1998; Gingras *et al.*, 1999b; Raught and Gingras, 1999). This pathway(s) regulates the activity of several translation factors. We demonstrate here that PI3K and FRAP/mTOR signaling modulates the serum-stimulated phosphorylation of the C-terminal eIF4GI sites. Interestingly, the eIF4GI N-terminus appears to modulate phosphorylation of the C-terminus, because phosphorylation of truncation mutants lacking the N-terminal region are resistant to kinase inhibitor treatment. Unexpectedly, although the related eIF4GII and p97 are also phosphoproteins, the corresponding C-terminal fragments in these proteins are not phosphorylated, suggesting that this region has undergone divergent evolution.

Results

Phosphopeptide mapping of eIF4GI

To study the regulation of eIF4GI phosphorylation, two-dimensional phosphopeptide mapping of ^{32}P -labeled, immunoprecipitated eIF4GI was conducted, essentially as described (Gingras *et al.*, 1998). Briefly, human embryonic kidney 293 cells were metabolically labeled with [^{32}P]orthophosphate. A subset of these cells was then stimulated with serum. The cells were lysed, and eIF4GI was immunoprecipitated using a polyclonal antiserum directed against the N-terminus of the protein (Craig *et al.*, 1998). Immunoprecipitated material was subjected to SDS-8% PAGE, and electrotransferred to a nitrocellulose membrane. The membrane was autoradiographed, and the region harboring the protein of interest excised. Membrane pieces were subjected to tryptic digestion, and liberated phosphopeptides visualized by two-dimensional (thin-layer electrophoresis/thin-layer chromatography; TLE/TLC) mapping.

A typical autoradiogram of ^{32}P -labeled, immunoprecipitated, gel-purified eIF4GI is shown in Figure 1A. Cerenkov counting of excised membrane pieces harboring ^{32}P -labeled eIF4GI revealed no significant difference in the quantity of ^{32}P incorporated into eIF4GI isolated from serum-starved (-S) versus serum-stimulated (+S) 293 cells ($n = 10$). However, two-dimensional tryptic phosphopeptide mapping revealed that the relative intensity of several phosphorylated peptides was significantly altered upon serum stimulation, indicating a change in the phosphorylation status of one or more amino acid residues. A highly reproducible pattern of phosphorylated peptides was observed for eIF4GI isolated from serum-starved cells (Figure 1B). Treatment of starved cells with serum (Figure 1C), insulin (data not shown) or phorbol ester (data not shown) resulted in an increase in the phosphorylation state of several tryptic peptides (serum-stimulated phosphopeptides 1-4), along with a concomitant decrease in the phosphorylation status of several others (serum-repressed phosphopeptides 7-9). No significant change in the intensity of the remaining major phosphopeptides (Figure 1C, phosphopeptides 5 and 6) was observed. Phosphopeptide mapping of eIF4GI from logarithmically growing cells yielded the same phosphopeptide pattern observed for serum-stimulated cells (data not shown). Immunoprecipitation with antiserum directed against the C-terminus of human eIF4GI yielded identical maps,

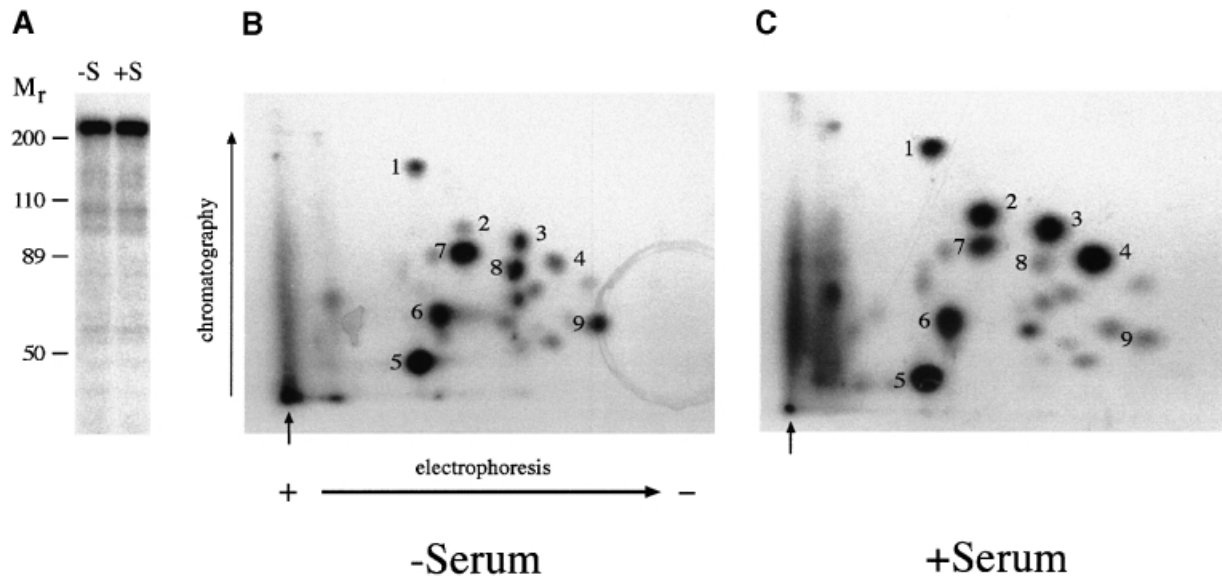


Fig. 1. Phosphorylation of specific sites in eIF4GI is modulated by serum. ^{32}P -labeled eIF4GI immunoprecipitated from 293 cells starved of serum for 36 h (-Serum or -S), or starved of serum for 36 h then stimulated with serum for 30 min (+Serum or +S), was subjected to (A) SDS-8% PAGE, then (B and C) to two-dimensional tryptic peptide mapping. The directions of chromatography (vertical) and electrophoresis (horizontal) as well as the loading origin (arrow) are indicated. Major phosphopeptides are numbered.

confirming that all of the observed phosphopeptides are derived from eIF4GI.

While the response of the phosphopeptides to serum stimulation was very consistent (i.e. peptides 1–4 increasing and peptides 7–9 decreasing in intensity), the degree of stimulation or repression varied from experiment to experiment. This variability appears to be due to a number of different reasons, including the confluence level and passage number of the cells, as well as the length of the starvation and stimulation periods. In general (as assessed by phosphoimaging and densitometry measurements), phosphopeptides 2 and 4 displayed the greatest sensitivity to serum stimulation (increasing in intensity ~3- to 10-fold), while peptides 3 (increasing ~2- to 5-fold) and 1 (increasing 1- to 3-fold; in some experiments no change was observed for peptide 1) were less responsive (data not shown). The intensity of peptide 7, in the serum-repressed group, also displayed acute sensitivity (decreasing by ~2- to 10-fold) to the presence of serum, whereas peptides 8 and 9 (both decreasing by ~2- to 5-fold) displayed a lower level of sensitivity. It is also noteworthy that while the eIF4GI phosphopeptide mapping pattern is quite reproducible, some variability is observed in the minor, unnumbered peptides as well as in peptides 4 and 6. This is most likely to be due to variations in the efficiency of tryptic digestion. Also, a streaked signal is observed directly above the loading origin in maps of endogenous eIF4GI (Figure 1B and C), suggesting that other peptides that are not clearly resolved by this method may be present. Nevertheless, the streaked signal and unnumbered phosphopeptides account for <10% of the total signal, as determined by phosphoimaging (data not shown).

Mapping of eIF4GI phosphorylation sites

To delineate the location of the eIF4GI phosphorylation sites, various influenza hemagglutinin (HA)-tagged eIF4GI protein fragments were expressed in 293T cells. (Phospho-

peptide maps of eIF4GI from 293T cells are identical to those from serum-stimulated 293 cells. However, since the transfection efficiency achieved in 293T cells is much greater than that for 293 cells, they were used in all transfection protocols.) Logarithmically growing 293T cells were transiently transfected (in the presence of 10% serum), and labeled with [^{32}P]orthophosphate. HA-tagged proteins were then immunoprecipitated with an anti-HA antibody, gel-purified and mapped. The constructs utilized in this study are shown in Figure 2F. HA-tagged eIF4GI protein fragments encompassing amino acids (aa) 614–1560 (Figure 2B) and 1045–1560 (Figure 2C) yielded phosphopeptide maps that were almost identical to the endogenous protein (Figure 2A). However, further N-terminal truncation to aa 1205 resulted in the loss of all phosphopeptides, except peptide 5 (Figure 2D). Mapping of a protein encompassing aa 1045–1372 yielded all phosphopeptides, except for peptides 5 and 6 (Figure 2E), and mapping of a fusion protein encompassing aa 1372–1560 yielded only peptide 5 (data not shown). Thus, all of the phosphopeptides mapped by this procedure were localized to the C-terminal one-third of the protein. The inability to localize peptide 6 is likely to result from differences in tryptic digestion efficiency between the larger and smaller eIF4GI fusion proteins. Interestingly, truncation of N-terminal sequences also resulted in a significant increase in the relative intensity of the serum-stimulated phosphopeptides (1–4) compared with the serum-repressed peptides (7–9; compare Figure 2A with E).

The regions of eIF4GII and p97 corresponding to the eIF4GI phospho-region are not phosphorylated

Alignment of the region of eIF4GI containing most of the phosphorylation sites (aa 1045–1205; hereafter referred to as the phospho-region) with the corresponding segments of two other members of the human eIF4G family, eIF4GII and p97, revealed relatively poor conservation (Figure 3A),

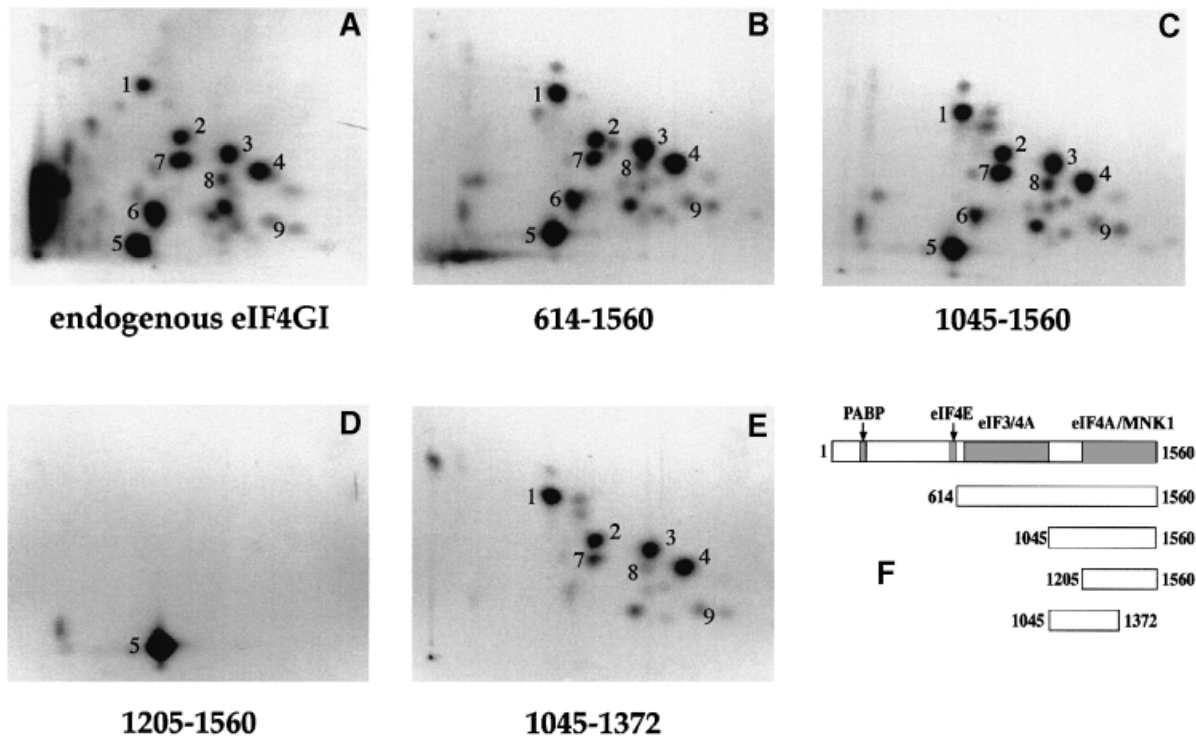


Fig. 2. Localization of eIF4GI phosphorylation sites. (A) Tryptic map of endogenous eIF4GI. (B–E) HA-tagged eIF4GI fusion protein fragments were expressed in 293T cells. Cells were metabolically labeled with ^{32}P , and HA-tagged proteins immunoprecipitated, gel purified, and subjected to two-dimensional tryptic mapping. (F) Protein fragments tested in this study. The various protein binding regions of eIF4GI are indicated.

especially compared with the surrounding regions. In fact, this region of human eIF4GI is much more closely related to the corresponding region of eIF4G proteins in other species than to the human eIF4GII and p97 proteins, sharing 93% identity with the rabbit eIF4GI protein (DDBJ/EMBL/GenBank accession No. L22090) and 47% identity with a putative zebrafish eIF4GI protein (DDBJ/EMBL/GenBank accession No. AI629389; data not shown). Thus, the newly defined phospho-region appears to have undergone divergent evolution within the human eIF4G family members.

Immunoprecipitation of eIF4GII and p97 from metabolically labeled 293 cells revealed that they are both phosphoproteins (data not shown). To determine whether eIF4GII and p97 are phosphorylated in the same region as eIF4GI, glutathione *S*-transferase (GST) fusion proteins encompassing fragments corresponding to the eIF4GI phospho-region (aa 1035–1206 for eIF4GI, aa 1057–1225 for eIF4GII, aa 395–547 for p97) were expressed in 293T cells, which were then metabolically labeled with [^{32}P]orthophosphate. The transfected 293T cells were lysed, and GST fusion proteins isolated via incubation with glutathione-coupled Sepharose beads. Western blotting, using anti-GST antiserum (Morino *et al.*, 2000), confirmed that all three GST fusion proteins were expressed (Figure 3B, upper panel), albeit at different levels. The same blot was then autoradiographed directly (Figure 3B, lower panel). Interestingly, only the GST–eIF4GI fusion protein incorporated significant amounts of ^{32}P . (A weak background signal at approximately the same location as the GST–eIF4GI and GST–eIF4GII proteins is detected in all lanes; however, the intensity of this signal did not

correlate with the amount of GST fusion protein expressed, and the signal did not co-migrate with GST–p97.) To ensure that all of the cells were efficiently metabolically labeled, endogenous eIF4GI was immunoprecipitated from the same lysates and found to incorporate similar amounts of ^{32}P in all samples (data not shown). Thus, while both eIF4GII and p97 are phosphoproteins, the regions corresponding to the eIF4GI C-terminal phospho-region do not appear to be phosphorylated significantly in these members of the eIF4G family.

Identification of the serum-inducible phosphorylation sites in eIF4GI

To identify the C-terminal eIF4GI residues phosphorylated in response to serum treatment, mass spectrometric and mutational analyses were performed. Briefly, phosphopeptide mapping was conducted as above, except that eIF4GI immunoprecipitated from 2×10^8 serum-stimulated 293 cells (1/20 of which were metabolically labeled with [^{32}P]orthophosphate) was loaded onto two TLC plates. The cellulose harboring peptides 1–4 was scraped from the plastic backing of the plates, and the phosphopeptides eluted and identified by capillary liquid chromatography–electrospray ionization tandem mass spectrometry (LC–MS/MS). The tandem mass spectrum for phosphopeptide 1 corresponds to EAALPPVSPLK, with the phosphorylation site being Ser1192 (Figure 4A). The tandem mass spectrum for phosphopeptide 3 corresponds to SFSSKEVEER, with the phosphorylation site being Ser1148 (Figure 4B). The tandem mass spectrum for phosphopeptide 4 corresponds to SSLSRRER, with the phosphorylation site being Ser1108 (data not shown).

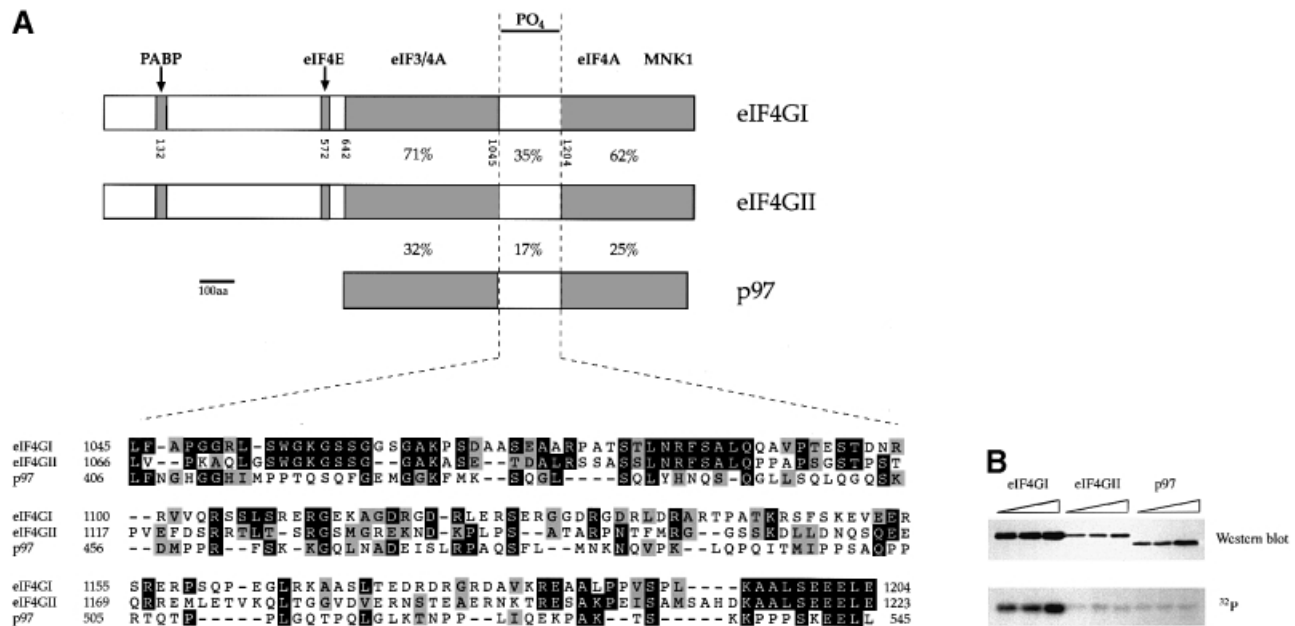


Fig. 3. The phospho-region of eIF4GI is poorly conserved within the human eIF4G family. (A) An alignment through the newly defined eIF4GI phospho-region of the members of the human eIF4G family. Locations of defined protein binding sites in eIF4G proteins are indicated, as well as percentage identity to the corresponding region in eIF4GI. Below the diagram is an alignment of the amino acid sequences of eIF4GI, eIF4GII and p97 in this region. Identical residues are boxed in black, similar residues are boxed in gray. (B) Fragments encompassing the phosphorylated region of eIF4GI (aa 1035–1206) and the corresponding regions of eIF4GII (aa 1057–1225) and p97 (aa 395–547) were expressed in 293T cells (5, 10 and 20 μ g of DNA transfected) as GST fusion proteins. Cells were metabolically labeled with ³²P. Proteins isolated with glutathione-coupled Sepharose beads were washed, gel-purified, and transferred to nitrocellulose. Upper panel, Western blot using anti-GST antiserum. Lower panel, direct autoradiogram of the same nitrocellulose membrane.

Mass measurements for phosphopeptide 2 predicted the peptide SSLSR (data not shown), indicating that peptide 2 is an alternative cleavage product of peptide 4. The identity of the phosphorylated residue could not be determined for this peptide.

To confirm the mass spectrometric results, site-directed mutagenesis of serines 1108, 1148 and 1192, along with several other phosphorylation site candidates, was performed. To simplify the cloning and mapping procedures these mutants were constructed in the background of the GST fusion protein containing only the eIF4GI phospho-region (aa 1035–1206). A typical two-dimensional phosphopeptide map of the GST–eIF4GI phospho-region protein is shown in Figure 5A. As observed for the HA-1045–1372 fragment (Figure 2E), phosphorylation of the GST fusion protein is almost exclusively limited to peptides 1–4, while serum-repressed peptides 7 and 8 are almost undetectable. A phosphopeptide that migrates in the same region as peptide 9 remains visible on most maps. A novel phosphopeptide (denoted by an asterisk, Figure 5A–D) that does not co-migrate with any endogenous eIF4GI peptides is observed in maps of these proteins, and is most likely to be derived from the GST moiety.

Mutation of Ser1108 to alanine resulted in the loss of both peptides 2 and 4 (Figure 5B). Thus, tryptic cleavage after arginine 1109 appears to be relatively inefficient (most likely to be due to the presence of a phosphate group near the cleavage site), resulting in the formation of two tryptic peptides (SSLSR and SSLSRER) containing the same phosphoserine. Elimination of phosphopeptides 2 and 4 was specific for the Ser1108Ala mutant, as maps of Ser1105Ala and Ser1106Ala mutants (other serines

present in the same tryptic peptide) retained peptides 1–4 (data not shown). Mutation of Ser1148 to alanine specifically abolished phosphopeptide 3 (Figure 5C), and mutation of Ser1192 to alanine eliminated phosphopeptide 1 (Figure 5D). Mutation to alanine of serines 1085, 1095, 1125, 1155, 1160 and 1170, or of threonines 1093, 1096 and 1140 had no effect on the phosphopeptide pattern (see Figure 5E for a summary of the mutational analysis). Thus, using a combination of mass spectrometric and mutational analyses, the serum-stimulated phosphorylation sites were identified as Ser1108, Ser1148 and Ser1192.

PI3K and FRAP/mTOR signaling modulates eIF4GI phosphorylation

Several translation regulatory factors have been identified as targets of the PI3K–FRAP/mTOR signaling pathway(s) (Thomas and Hall, 1997; Abraham, 1998; Gingras *et al.*, 1999b). It was thus pertinent to test whether signaling to eIF4GI is also mediated by this pathway. Specific kinase inhibitors may be used to determine whether phosphorylation of a given protein is modulated by these kinases. Wortmannin inhibits PI3K by covalently binding to its catalytic subunit (reviewed in Ui *et al.*, 1995), and LY294002 is a structurally unrelated, reversible PI3K inhibitor (Vlahos *et al.*, 1994). Rapamycin is a fungal macrolide that, in a complex with the immunophilin FKBP12, binds to FRAP/mTOR to inhibit its kinase activity (Abraham, 1998).

Serum-starved 293 cells metabolically labeled with [³²P]orthophosphate (Figure 6A) were pre-treated (for 20 min) with kinase inhibitors, then serum was added to the culture media to a final concentration of 10%. After

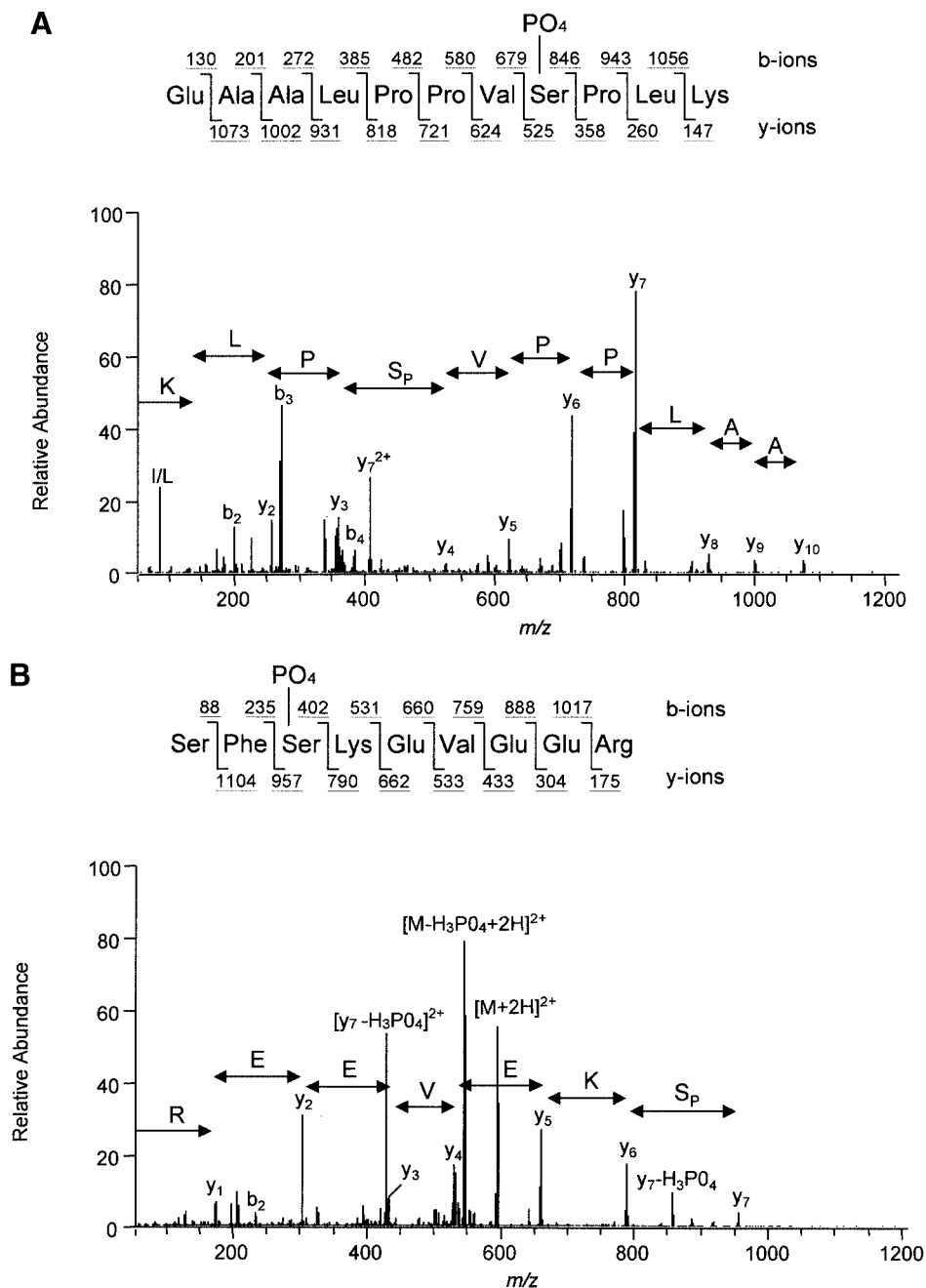


Fig. 4. Identification of serum-stimulated phosphorylation sites by mass spectrometry. (A) Tandem mass spectrum resulting from the analysis of excised phosphopeptide 1 from the map in Figure 1C. This spectrum contained sequence information for a single phosphopeptide. Two separate ion series were recorded simultaneously; b- and y-ion series represent sequencing inward from the N- and C-termini, respectively. The computer program Sequest (Eng *et al.*, 1994) was utilized to match this tandem mass spectrum to the sequence shown, with the serine residue being phosphorylated. (B) Tandem mass spectrum resulting from the analysis of excised phosphopeptide 3. The position of the phosphorylated serine residue is indicated.

30 min in the presence of serum (Figure 6B), or serum plus inhibitor (Figure 6C and D), cells were lysed and eIF4GI was subjected to phosphopeptide mapping. LY294002 (5 μ M; Figure 6C), wortmannin (100 nM; data not shown) and rapamycin (25 nM; Figure 6D) abrogated the serum-induced hyperphosphorylation of peptides 1–4, and prevented the hypophosphorylation of peptides 7–9. (In this experiment, peptide 3 responded only weakly to rapamycin addition.) Addition of solvent [dimethylsulfoxide (DMSO) for wortmannin and LY294002, ethanol for rapamycin] alone had no effect on the phosphopeptide pattern (data not shown). Thus, serum-induced modulation

of eIF4GI phosphorylation appears to be mediated by PI3K and FRAP/mTOR signaling.

The N-terminus confers kinase inhibitor sensitivity to the phospho-region

Two models may be proposed to explain the effects of kinase inhibitors on the phosphorylation state of eIF4GI: (i) the kinases phosphorylating this region are rapamycin- and LY294002-sensitive; or (ii) the kinases themselves are not sensitive to these inhibitors, but a change in the conformation of eIF4GI (due to alterations in intramolecular interactions, or in the interaction of eIF4GI with

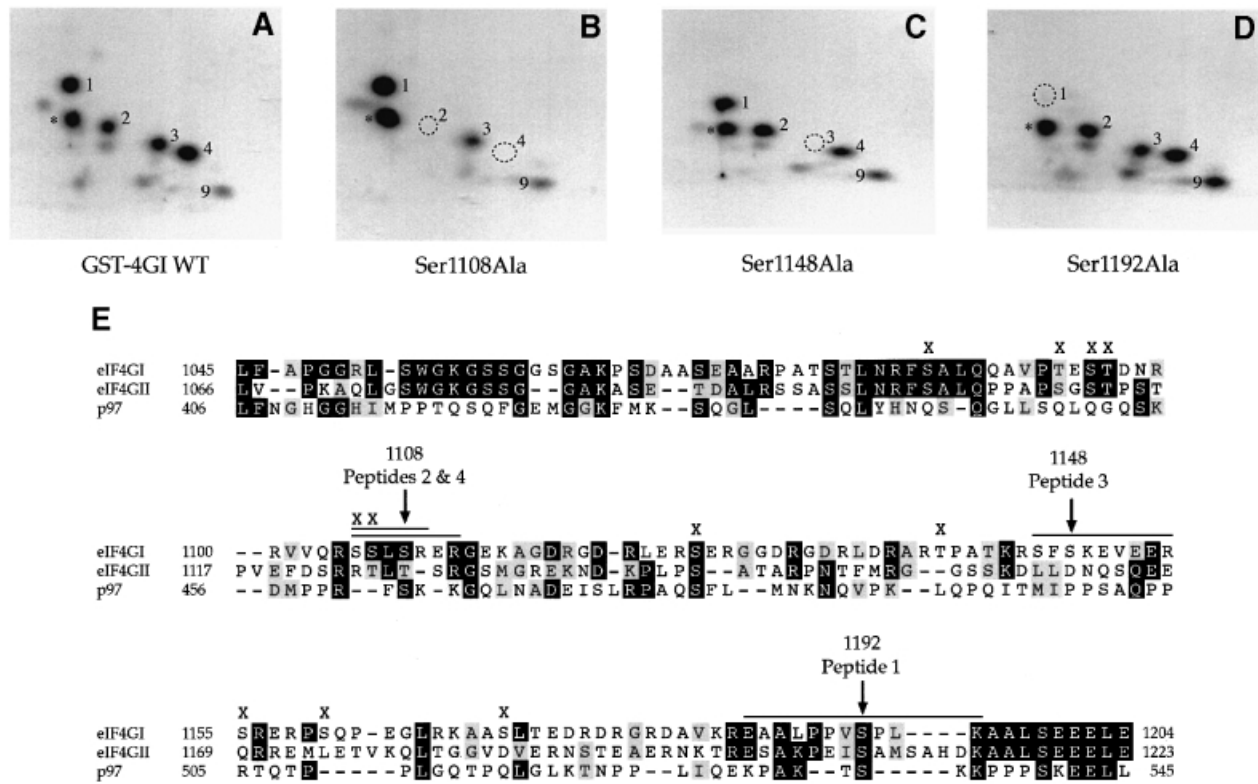


Fig. 5. Identification of serum-inducible phosphorylation sites by mutational analysis. Mutations of serine residues identified by mass spectrometry as phosphorylation sites were introduced into the eIF4GI phospho-region (aa 1035–1206)–GST fusion proteins. Proteins were expressed in 293T cells, ³²P-labeled, isolated and mapped. Tryptic maps of the GST–eIF4GI (aa 1035–1206) wild-type protein (**A**), and point mutants Ser1108Ala (**B**), Ser1148Ala (**C**) and Ser1192Ala (**D**). A novel phosphopeptide that does not co-migrate with any endogenous eIF4GI peptide is indicated with an asterisk. (**E**) Complete mutational analysis summary. An alignment of the eIF4GI phospho-region with the corresponding regions of eIF4GII and p97. Identical residues are boxed in black, similar residues are boxed in gray. eIF4GI residues mutated to alanine are indicated by an ‘x’ or arrow. Locations of the serum-stimulated, phosphorylated residues, as well as the phosphopeptides identified in this analysis, are also indicated.

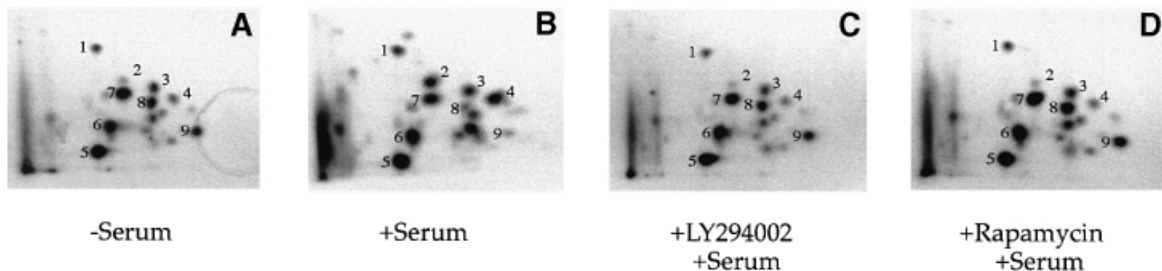


Fig. 6. Serum-induced phosphorylation of eIF4GI is sensitive to inhibitors of PI3K and FRAP/mTOR. Two-dimensional tryptic peptide mapping of endogenous eIF4GI isolated from 293 cells (**A**) starved of serum, (**B**) then treated with serum for 30 min, (**C**) pre-treated with LY294002 for 20 min, then treated with serum for 30 min, or (**D**) pre-treated with rapamycin for 20 min, then treated with serum for 30 min. The map in (**A**) is the same as that shown in Figure 1B.

other binding partners) modulates the accessibility of the C-terminal phosphorylation sites. In this regard, the N-terminal truncation mutants used in this study were no longer sensitive to kinase inhibitor treatment. Instead, phosphorylation of the truncation mutants was constitutively in the ‘serum-stimulated’ state (data not shown). These data argue for the second model, and suggest that the N-terminal region of eIF4GI modulates the phosphorylation state of the C-terminal phospho-region.

It was of particular interest to determine whether this N-terminal regulatory region could be the eIF4E binding site, since binding of eIF4E to eIF4G is also rapamycin- and LY294002-sensitive, and because eIF4E binding induces a conformational change in the eIF4GI protein

(Haghighat *et al.*, 1996; Ohlmann *et al.*, 1997; Hershey *et al.*, 1999) that could theoretically alter the accessibility of the C-terminus to kinases. To address this question, HA-tagged eIF4GI fragments aa 550–1560 (consisting of the eIF4E binding site + middle + C-terminus) and aa 614–1560 (consisting only of the middle + C-terminus) were expressed in 293T cells. Cells were metabolically labeled, treated with kinase inhibitors, and both the HA-tagged eIF4GI fragments and endogenous eIF4GI were immunoprecipitated from the cell lysates and mapped (Figure 7). Both of the HA-tagged fragments were insensitive to rapamycin- (compare Figure 7B and D with A and C) and LY294002-treatment (data not shown); serines 1108, 1148 and 1192 remained phosphorylated, while

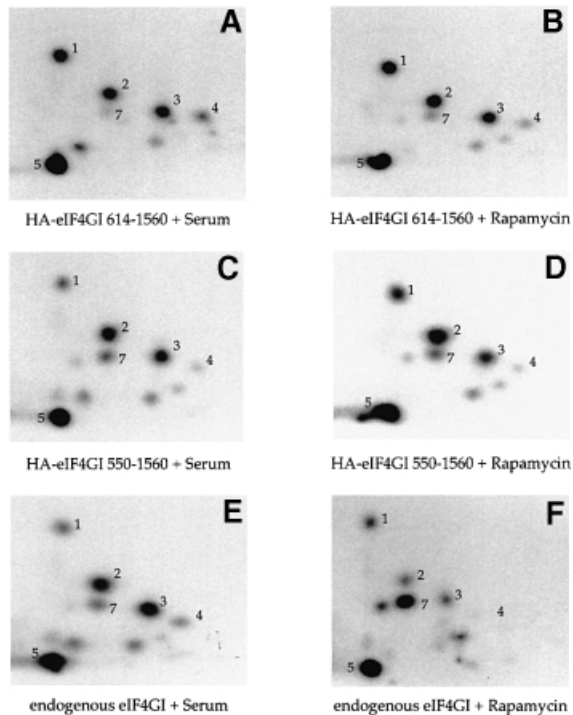


Fig. 7. N-terminal sequences confer kinase inhibitor sensitivity to the C-terminal phospho-region. HA-tagged eIF4GI fragments were expressed in 293T cells. Cells were metabolically labeled and incubated in 10% serum (A, C and E) or 10% serum + 25 ng/ml rapamycin (B, D and F) for 1 h. Following lysis, immunoprecipitation was conducted sequentially with anti-HA and anti-eIF4GI antisera. Isolated proteins were gel-purified and mapped. The HA-eIF4GI 550–1560 fragment interacts with eIF4E, while HA-eIF4GI 614–1560 does not.

phosphopeptide 7 was almost absent, even in the presence of rapamycin. Endogenous eIF4GI immunoprecipitated from the same lysates retained rapamycin- (Figure 7E and F) and LY294002-sensitivity (data not shown). (In this experiment, phosphopeptides 2, 3, 4 and 7 displayed acute sensitivity to rapamycin treatment, whereas peptide 1, which is the least responsive to serum, did not.) These data indicate that eIF4E binding *per se* is not responsible for conferring LY294002- and rapamycin-sensitivity upon eIF4GI.

The protease sensitivity of a 98 aa N-terminal fragment of the yeast eIF4GI homolog (TIF4631) is reduced upon binding to yeast eIF4E, as it progresses from a less structured to a more structured state (Hershey *et al.*, 1999). Thus, it remained possible that eIF4E binding to human eIF4GI could also cause a conformational change involving a much larger region of the N-terminus than is present in the aa 550–1560 fragment. To address this possibility, and to localize further the eIF4GI N-terminal region conferring rapamycin sensitivity to the C-terminal phospho-region, a much longer truncation mutant (aa 157–1560, the original eIF4GI clone; Yan *et al.*, 1992) was also expressed in 293T cells. As with the shorter fragments, phosphorylation of this fragment displayed rapamycin resistance (data not shown). Endogenous eIF4GI immunoprecipitated from the same cell lysates retained rapamycin sensitivity (data not shown). Thus, intramolecular interactions between the extreme N-terminus (aa 1–156) and the C-terminal phospho-region, or between a second

protein that interacts with the eIF4GI N-terminus (and whose binding site is not present in the truncation mutants), appear to confer rapamycin- and LY294002-sensitivity. Finally, these data also indicate that eIF4GI C-terminal phospho-region kinases are not rapamycin sensitive themselves, but that, instead, a conformational change that alters the accessibility of several key residues is effected by serum, and is inhibited by rapamycin, wortmannin and LY294002. A similar phenomenon has been reported for p70 S6 kinase 1 (Dennis *et al.*, 1996; Mahalingam and Templeton, 1996).

Discussion

Here, we have identified a set of serum-stimulated phosphorylation sites (Ser1108, Ser1148 and Ser1192) within the highly phosphorylated C-terminal one-third of the eIF4GI protein. Phosphorylation of these sites is demonstrated to be responsive to various extracellular stimuli, and is shown to be sensitive to specific inhibitors of the PI3K–FRAP/mTOR signaling pathway(s). Interestingly, the extreme N-terminus of eIF4GI appears to modulate the phosphorylation state of the C-terminal phospho-region. Taken together, our data suggest a two-step model for the phosphorylation of eIF4GI. The eIF4GI protein in serum-starved (or kinase inhibitor-treated) cells appears to be in a repressed state, either due to intramolecular interactions between the N- and C-termini, or due to interactions with unknown eIF4G binding partners. In step one, the repressed eIF4GI molecule is derepressed by the PI3K–FRAP/mTOR pathway(s), either by direct phosphorylation of the N-terminus of the protein, or via modulation of an eIF4GI binding partner. This event renders the C-terminal serine residues accessible to other kinases. Phosphorylation of these residues results in a ‘fully active’ eIF4GI. However, how phosphorylation may affect the activity of eIF4GI in translation initiation is unknown.

This work also outlines a technique to evaluate how any extracellular stimulus or cellular stress modulates eIF4GI phosphorylation, and provides the basis for further studies to assess how phosphorylation may modulate eIF4GI activity. In addition, this study points out previously unsuspected differences between members of the human eIF4G family, in that the corresponding region of two closely related proteins, eIF4GII and p97, is not phosphorylated. This observation is intriguing, in that it suggests that the various members of the eIF4G family may be differentially regulated by intracellular signaling pathways.

Contrary to previous reports suggesting that treatment of cells with mitogenic stimuli leads to a net increase in eIF4G phosphorylation (Morley and Traugh, 1989, 1990; Donaldson *et al.*, 1991), we did not observe a change in the quantity of ^{32}P incorporated into eIF4GI after starvation, serum stimulation (Figure 1A) or kinase inhibitor treatment (B. Raught and A.-C. Gingras, unpublished observation). In these earlier reports, however, eIF4G (most likely to be a mixture of eIF4GI and eIF4GII) was isolated via $m^7\text{GTP}$ affinity purification as an eIF4E–eIF4G complex. Subsequent to these reports, the eIF4E-binding proteins (4E-BPs) were discovered, and found to compete with the eIF4Gs for an overlapping binding site on eIF4E (Lin *et al.*, 1994; Pause *et al.*, 1994; Haghghat

et al., 1995; Mader *et al.*, 1995). In quiescent or starved 293 cells, a relatively high proportion of eIF4E is bound to 4E-BPs, and a low amount of eIF4G protein is recovered after cap column purification of eIF4E (A.-C.Gingras and B.Raught, unpublished observation). Mitogenic stimulation leads to an increase in phosphorylation of the 4E-BPs, and a consequent decrease in their affinity for eIF4E. The liberated eIF4E is then free to interact with eIF4Gs, resulting in a significant increase in the amount of eIF4G recovered by cap column purification. Thus, the earlier observed increase in eIF4G phosphorylation in response to mitogen treatment probably reflected an augmentation in the quantity of eIF4G bound to eIF4E. We cannot, however, exclude the possibility that following other types of treatments, or in other cell types, the quantity of phosphate incorporated into eIF4GI changes.

It is clear that while both the human eIF4GI and eIF4GII proteins can function in translation initiation (Gradi *et al.*, 1998a), these molecules differ in several respects. For instance, the eIF4GII protein is present at ~1/4 of the level of eIF4GI in HeLa cells (Svitkin *et al.*, 1999), and the cleavage kinetics of eIF4GII in virus-infected cells differs markedly from that of eIF4GI (Gradi *et al.*, 1998b; Svitkin *et al.*, 1999). This study also suggests that the newly defined eIF4GI phospho-region is not phosphorylated in eIF4GII. Thus, it appears that these proteins have undergone some degree of divergent evolution, and that the activity of the eIF4G proteins may be regulated quite differently. These observations may have important implications regarding the regulation of translation initiation in response to different types of intracellular signals, or in the regulation of different mRNA populations.

We previously reported that Mnk1 can phosphorylate eIF4GI *in vitro* (Pyronnet *et al.*, 1999). After further study, it does not appear that this observation is relevant *in vivo*. Mnk1 is activated via the MAPK pathway, which is stimulated in response to serum treatment in 293 cells (von Manteuffel *et al.*, 1996). However, truncation of the Mnk1 binding site in eIF4GI does not negatively impact upon the phosphorylation state of the serum-stimulated phosphopeptides (e.g. Figure 2E), and mapping of a recombinant eIF4GI fragment phosphorylated *in vitro* by Mnk1 revealed that it is phosphorylated only on serum-repressed phosphopeptides 7 and 8 (B.Raught and A.-C.Gingras, unpublished observation). Thus, it appears unlikely that this kinase plays a role in mediating eIF4GI phosphorylation.

A signaling pathway that may be dedicated to translational control (Brown and Schreiber, 1996) consists of PI3K, Akt/PKB and FRAP/mTOR. These kinases signal to several factors involved in the regulation of translation, including the p70 S6 kinases (Jefferies and Thomas, 1996; Gout *et al.*, 1998; Shima *et al.*, 1998), eukaryotic elongation factor 2 (Redpath *et al.*, 1996) and the 4E-BPs (Gingras *et al.*, 1999b). eIF4GI may now be added to this list. However, even though the serum-stimulated eIF4GI phosphorylation sites identified here are rapamycin sensitive, they are not directly phosphorylated by FRAP/mTOR or p70 S6 kinases 1 and 2 in an *in vitro* kinase assay (A.-C.Gingras and B.Raught, unpublished data). *In vivo* evidence also argues against a role for rapamycin-sensitive kinases such as the p70 S6 kinases and FRAP/mTOR in the phosphorylation of the serum-stimulated eIF4GI sites:

the phosphorylation state of N-terminal truncation mutants is rapamycin- and LY294002-insensitive. Thus, an N-terminal region of eIF4GI appears both to repress phosphorylation of peptides 1–4 in the serum-starved state, and confer LY294002- and rapamycin-sensitivity to the C-terminal phospho-region.

How might phosphorylation modulate eIF4GI activity? Kinase inhibitor treatment does not appear to affect the binding of several known eIF4GI binding partners, including eIF3, eIF4A (B.Raught, A.-C.Gingras, S.Morino and H.Imataka, unpublished observations) and Mnk1 (S.Pyronnet, personal communication). These data are consistent with the fact that the phospho-region does not overlap with the binding site of any known eIF4GI binding protein. Secondary structure predictions suggest that this region is relatively unstructured (B.Raught and A.-C.Gingras, unpublished data). The phospho-region also contains a caspase cleavage site (M.Bushell and S.J.Morley, personal communication), suggesting that it is solvent exposed. We therefore posit that this region acts as a flexible 'hinge' between the middle and C-terminal domains of eIF4GI. In the absence of any evidence for quantitative changes in protein–protein interactions, we suggest that phosphorylation alters intramolecular interactions to cause short- or long-range changes in eIF4GI structure. How these changes modulate the ability of eIF4GI to initiate translation remains to be determined.

Materials and methods

Cell culture/inhibitors

Cell culture and [³²P]orthophosphate metabolic labeling were carried out as described (Gingras *et al.*, 1998). Wortmannin and LY294002 were purchased from Calbiochem and diluted in DMSO at 1000×. Rapamycin was purchased from the same source, dissolved in EtOH at 25 μM, and used at a final concentration of 25 nM. 293T cells were transfected using a modified calcium phosphate procedure (Chen and Okayama, 1988). Bovine insulin was purchased from Gibco-BRL and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) from Sigma.

Constructs

HA-tagged eIF4GI constructs were described previously (Imataka and Sonenberg, 1997). Fragments encompassing aa 1035–1206 of eIF4GI, aa 1057–1225 of eIF4GII and aa 395–547 of p97 were amplified by PCR, and cloned in-frame into *EcoRI*–*XhoI* sites of pcDNA3–GST (Imataka *et al.*, 1998). Point mutations of eIF4GI were generated using pcDNA3–GST–eIF4G(1035–1206) as a template, and mutants were cloned into *EcoRI*–*XhoI* sites of pcDNA3–GST. All inserts were sequenced.

Antibodies

Antiserum raised against the eIF4GI N-terminus was characterized previously (Craig *et al.*, 1998). Antiserum prepared against the eIF4GI C-terminus was derived from a New Zealand White rabbit injected with the peptide CRSRERPSQPEGLRKAASLTEDRDRGR, corresponding to aa 1154–1179 (Sheldon Biotechnologies, McGill University), conjugated to keyhole limpet hemocyanin. This antiserum does not cross-react with eIF4GII (A.Gradi and B.Raught, unpublished data).

Tryptic phosphopeptide mapping

To isolate labeled eIF4GI protein, cell culture plates were washed twice with phosphate-buffered saline, and lysed in 1 ml of lysis buffer (Gingras *et al.*, 1998) containing a 1:500 dilution of the Sigma protease inhibitor cocktail, 10 mM NaF, 0.25 mM sodium orthovanadate and 10 nM calyculin. Cells were lysed for 15 min at 4°C, and all subsequent steps were carried out at 4°C. Lysate was scraped from the plates and centrifuged for 15 min at 14 000 *g*. Supernatant was pre-cleared by adding 50 μl of protein A–Sepharose, and incubated with end-over-end mixing for 60 min. eIF4GI was immunoprecipitated with 25 μl of protein A–Sepharose beads coupled to 5 μl of antiserum for 3.5 h. Beads were

collected by centrifugation at 3000 g, washed twice in lysis buffer and twice in RIPA (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). Laemmli loading buffer was added to the washed beads, and samples were boiled and subjected to SDS-8% PAGE. Separated proteins were transferred to a 0.2 µm nitrocellulose membrane (Schleicher & Schuell), which was exposed to film, and the region of the membrane harboring labeled eIF4GI was excised and Cerenkov counted. HA-tagged eIF4GI proteins were immunoprecipitated with anti-HA11 (BabCO) coupled to protein G-Sepharose beads, and subjected to SDS-12% PAGE. Tryptic digests and TLE/TLC mapping were carried out as described (Gingras *et al.*, 1998) using Kodak (Figures 1, 2 and 6) or Merck (Figures 5 and 7) TLC plates. Phosphorylation intensity was quantified using the BAS 2000 phosphorimager (Fuji).

Mass spectrometry

Large-scale, keratin-free TLE/TLC mapping was conducted to obtain samples of sufficient mass to subject to mass spectrometry, as described (Gingras *et al.*, 1999a). Cellulose from regions harboring radioactivity was scraped from the plate backing and prepared for analysis by LC-MS/MS, as described (Watts *et al.*, 1994). The system used was as described (Gingras *et al.*, 1998; Gygi *et al.*, 1999).

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