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Structural insights into the allosteric effects of 4EBP1 on the eukaryotic translation initiation factor eIF4E

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Abstract

The eukaryotic translation initiation factor eIF4E plays key roles in cap dependent translation and mRNA export. These functions rely on binding the 7-methylguanosine moiety (5'cap) to the 5'-end of all mRNAs. eIF4E is regulated by proteins such as eIF4G and eIF4E binding proteins (4EBPs) that bind the dorsal surface of eIF4E, distal to the cap binding site, and modulate cap binding activity. Both proteins increase the affinity of eIF4E for 5'cap. Our understanding of the allosteric effects and structural underpinnings of 4EBP1 or eIF4G binding can be advanced by structural data on cap-free eIF4E bound to one of these proteins. Here, we report the crystal structure of apo-eIF4E and cap-free eIF4E in complex with a 4EBP1 peptide. We also monitored 4EBP1 binding to cap free eIF4E in solution using NMR. Together, these studies suggest that 4EBP1 transforms eIF4E into a cap receptive state. NMR methods were also used to compare the allosteric routes activated by 4EBP1, eIF4G, and the arenavirus Z protein, a negative regulator of cap binding. We observed chemical shift perturbation at the dorsal binding site leading to alterations in the core of the protein which were ultimately communicated to the unoccupied cap binding site of eIF4E. There were notable similarities between the routes taken by 4EBP1 and eIF4G and differences from the negative regulator Z. Thus, binding of 4EBP1 or eIF4G allosterically drives alterations throughout the protein that increase the affinity of eIF4E for the 5'cap.

Keywords

eIF4E; eIF4E binding protein (4EBP1); m⁷G cap binding; X-ray Crystallography; NMR Spectroscopy

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Accession Numbers

Atomic coordinates and structure factors have been deposited in the Protein Data Bank for human Apo-eIF4E (3TF2) and cap free 4EBP1-eIF4E (3U7X).

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Introduction

The eukaryotic translation initiation factor (eIF4E) modulates the expression of genes involved in cell growth, proliferation, and apoptosis via two major pathways: cap dependent translation and mRNA export. In both nuclear and cytoplasmic compartments, eIF4E associates with the 7-methylguanosine moiety (5'cap) on the 5' end of mRNA which is a prerequisite for its function. In the nucleus, eIF4E plays an important role in the export of a subset of growth promoting mRNAs containing the eIF4E-sensitivity element. While in the cytoplasm, eIF4E (along with eIF4G and eIF4A) forms the 5'-cap binding complex, eIF4F, which acts as a scaffold to recruit other factors to promote cap dependent translation. eIF4G binds to the dorsal surface of eIF4E, which is located at the opposite face of the 5'cap binding site. The same dorsal surface can be accessed by multiple factors, such as RING proteins (promyelocytic leukemia protein (PML) and arenaviral Z), select homeodomains (HOXA9, EMX2, PRH), and the family of translational inhibiting eIF4E-binding proteins (4EBPs) [8]. These dorsal surface binding proteins can act through two mechanisms. For example, 4EBP can sterically block the interaction with eIF4G thereby preventing recruitment of other translation factors and consequently downregulating translation [8, 9]. Whereas other proteins, such as PML and arenaviral Z, can also cause allosteric changes in eIF4E leading to reduction in cap affinity or for the case of eIF4G and 4EBP1, an increase in the same activity.

Many structural studies have elucidated the basic features of cap recognition. In general, eIF4E maintains a conserved fold comprising eight anti-parallel β -strands and three α -helices. The structure adopts the shape of a glove with the 5'cap binding site situated on the concave surface of the β -strands. Three α -helices on the convex face that serve as a protein binding interface. According to the structure of eIF4E in complex with either m⁷GDP, m⁷GTP or m⁷GpppA, the core binding site contains three principal regions that interact with the 5'cap. The indolyl moieties from two tryptophan residues W56 and W102, located within loop regions at the extremities of the 'glove' stacks against the guanine base within the 5'cap. A second region containing charged residues R112, R157, K159 and K162 which make contacts with the phosphates in the cap structure further stabilizing the complex. Finally, the A203-T211 loop located at the C-terminal end of the protein interacts with the second nucleotide from the m⁷GpppA cap analog. Together these contacts contribute to the overall conformational stability of the cap binding site.

Structural details of the molecular recognition of 4EBP1 and eIF4G peptides are well understood for the cap bound eIF4E. Peptides from eIF4G and 4EBP1 contain a common eIF4E binding motif (YXXXXL Φ ; where X is any residue and Φ any hydrophobic) and in both cases interact to the same area on the dorsal surface in eIF4E. Notably, the peptides binding site on eIF4E is approximately 35Å away from the cap binding pocket with no direct contact between the peptide and the 5'cap. Fragments corresponding to the eIF4E binding region from eIF4G and 4EBP1 lack secondary structure in the free state, but undergo a disorder-to-order transition upon binding to eIF4E. The binding segment of approximately fifteen residues form a reversed L-shaped structure consisting of an extended chain region and a short α -helix which interacts with the N-terminal region and dorsal helices (α 1 and α 2) of eIF4E through a mixture of hydrophobic and hydrophilic interactions. Given that the peptides manifest a comparable affinity for eIF4E to that of full length 4EBP1 or eIF4G, the major determinants of specificity must be within the conserved binding motif [9]. Functionally, peptides derived from eIF4G or 4EBP1 are sufficient to impair translation of reporter mRNA by obstructing the association of dorsal binding partners.

Several biophysical studies describe a cooperative link between the association of 4EBP1 or eIF4G binding on the dorsal surface of mammalian eIF4E and 5'cap binding affinity. For

instance, interactions with a 100 residue eIF4G fragment or full length 4EBP1 can enhance the association of eIF4E to the 5'cap. Although there are some discrepancies in the literature, eIF4G or 4EBP peptides were shown to enhance 5'cap affinity of eIF4E by approximately two fold, suggesting that the peptides have an allosteric effect on eIF4E. Negative regulators of cap binding activity, such as the promyelocytic leukemia (PML) and the arenaviral Z proteins, can also bind the dorsal surface and alter the conformation of eIF4E. This leads to a reduction in 5'cap affinity by about two orders of magnitude and consequently downregulation of eIF4E's mRNA export and *in vitro* translation activity [7,16]. Overall, investigating how eIF4E regulatory factors influence cap affinity has been an important aspect in understanding the control of eIF4E dependent mRNA export and translation.

To understand the mechanism of increased cap affinity induced by 4EBP1 or eIF4G, multiple crystallographic studies of cap bound eIF4E structures in the presence or absence of the peptides were completed, but no complexes were reported without the cap. These analyses showed no significant structural changes in eIF4E-cap complexes upon binding to either 4EBP1 or eIF4G peptides. To fully comprehend the mechanism induced by peptide binding, it is essential to elucidate the set of structures in the absence of the 5' cap. To this end, we solved the first crystal structures of cap free eIF4E in the absence and in complex with a 4EBP1 peptide. In addition, to resolve the allosteric effects of peptide binding, we carried out solution studies monitoring the interaction of 4EBP1 with cap free eIF4E using NMR spectroscopy.

Results and Discussion

Comparison of Apo-eIF4E and cap free eIF4E-4EBP1 peptide complex

The crystal structures of human apo-eIF4E and cap free eIF4E-4EBP1 peptide complex were solved at a resolution of 2.1 Å (Table 1). Although we previously solved the apo-eIF4E solution structure by NMR, a comparison with the crystal structure should confirm that differences between structures in the crystal lattice and solution did not confound our interpretation of conformational affects induced by 4EBP1. Globally, both the apo-eIF4E and eIF4E-4EBP1 cap-free crystal structures display the characteristic fold (Figure 1A and 1B) seen in the family of published crystal and solution structures for human eIF4E (2GPQ, 1IPC, 1IPB, 2V8W, 2W97 and 1WKW). The apo-eIF4E and cap-free eIF4E-4EBP1 crystal structures were unexpectedly similar to each other (Figure 1C; RMSD 0.585 Å). Seemingly, binding of the 4EBP1 peptide does not appear to affect the cap-binding pocket of eIF4E. This was surprising given our NMR data (details described below) which shows regions outside the peptide binding site, including the unoccupied 5'cap binding pocket, are perturbed upon binding of 4EBP1 to apo-eIF4E. Our previous NMR data also indicate that the W56 and W102 loops of apo-eIF4E are dynamic in solution. Consistent with this, the same regions were also mobile in the crystal apo-eIF4E structure as reflected by their high B-factor values. Studies have shown that the conformation of flexible loops observed in the crystal lattice may be the result of conformational selection triggered by crystal packing. We therefore analyzed the effects of crystal packing within the apo-eIF4E crystal structures.

Affects of crystal packing on the apo-eIF4E crystal structure

While the global fold was preserved between solution and crystal apo-eIF4E structures (RMSD 2.4 Å), important differences are observed in part of the 5'cap binding site. The W56-loop region in the crystal falls into a similar position within the ensemble of apo-eIF4E solution structures (Figure 2A). On the other hand, the W102-loop, which is in an extended conformation in the solution structure, is shifted closer to the cap binding pocket in the crystal structure. Further analysis of the apo-eIF4E crystal lattice shows four molecules in the asymmetric unit containing two similar dimers (Figure 2B). Each pair displays the dorsal

surface of the protein to be in close proximity with each other. Specifically residues V69, E70, W73, and N77 form contacts (2.7–4.3 Å) with residues L131, L135, E132, R186, and E185 on the other eIF4E molecule (Figure 2B). For comparison, the 4EBP1 peptide contacts a similar set of residues on the dorsal surface of eIF4E including V69, W73, L131, E132, L135, but the interactions are not as extensive and less close in space than the apo-apo eIF4E contacts (Figure 2B). Thus, it is possible that the crystal contacts on the dorsal surface of apo-eIF4E mediate similar conformational changes to those caused by 4EBP1 association (see below). Further, the motions observed for the apo-eIF4E have higher B-factors for the W56 and W102 loops indicating that the static snapshot presented by the crystal structure is likely not fully reflective of the dynamic nature of these loops. It is also possible that the solution apo-eIF4E structure exists in an equilibrium state with the W102 residue rotated into the cap binding site at a low frequency, and this state has been captured in the crystal structure. These features likely underlie the differences we observe between the apo-eIF4E crystal and NMR structures leading us to use the solution NMR apo-eIF4E structure for comparison below.

Comparison of 4EBP1-eIF4E cap free and cap bound structures

The 4EBP1-eIF4E crystal structure is highly similar to the ternary 4EBP1-eIF4E-cap complex (e.g. 1WKW; RMSD of 0.890 Å; Figure 2C). The 4EBP1 peptide binds to the dorsal surface of cap free eIF4E with identical interactions as those previously observed in the human ternary eIF4E-4EBP1-cap complex structures (1WKW, 2V8W, 1EJ4). The N-terminus remains flexible, based on the absence of electron density, in the eIF4E-4EBP1 structure as is observed in eIF4E-cap-4EBP1 ternary complexes. In terms of resolving the allosteric effects of 4EBP1, we were most interested in examining features around the cap binding site. Comparisons of our cap free eIF4E-4EBP1 and cap bound eIF4E complexes (with or without 4EBP1) (e.g. 1WKW, 2V8W, 1IPC, 1IPB), show differences in the cap binding site. First, the W56-loop is further away from the cap binding site in the cap free eIF4E-4EBP1 complex relative to the cap bound forms and is more flexible than other parts of the protein (B-factor; 60–100 Å²) (Figure 2C). The prior and preceding strands of the W56-loop (β1 and β2) show no changes in position suggesting that the loop moves as a hinge relative to the structural core when it binds the 5'cap. This is consistent with solution studies of cap binding to the apo-eIF4E protein. Second, the backbone conformation of the W102 loop (K95-G111) is similar to those of the cap bound structures reported with the exception that the side chain of E103 is repositioned and the aromatic W102 is at an angle oblique to the plane of the 5'cap rather than parallel in the cap bound structure (Figure 2C). The phosphate binding region of the cap pocket (N155-K162) remains in a similar conformation between structures, however the K159 side chain which contacts the phosphates in the cap bound structure is only partially resolved in the eIF4E-4EBP1 cap free structure suggesting that it is mobile (data not shown). Finally, the C-terminal region (A203-T211), changes from a short-helix in the cap free eIF4E-4EBP1-bound structure to an extended loop which interacts with the second base of m⁷GpppA in eIF4E-dinucleotide complexes, relative to mononucleotide complexes (Figure 2C). Given the potential crystal contact issues with the apo-eIF4E crystal structure; we also examined the crystal contacts in the eIF4E-4EBP1 cap-free complex. There are two molecules in the asymmetric unit and the contacts are observed between the W102 of both molecules. Thus, one cannot rule out the possibility that the W102 side-chain is rotated into the cap binding site due to these crystal contacts. In summary, our comparative analysis between the cap-free and cap-bound structures show the changes induced by the binding of the cap analogue to eIF4E.

Conformational changes induced by the 4EBP1 peptide in cap free eIF4E

A comparison of the solution apo-eIF4E structure and the eIF4E-4EBP1 cap free crystal structure revealed important similarities and differences. The W56 loop is in a similar

position and highly flexible in both structures. The W56 hinge appears not to be altered relative to the cap free eIF4E-4EBP1 structure indicating that this part of the binding site is not altered by 4EBP1 peptide, but is considerably altered by cap binding. A major difference is seen where W102 is extended away from the cap binding site in apo-eIF4E and rotated into the site in the eIF4E-4EBP1 cap free form. This suggests that 4EBP1 binding can cause a major alteration in the cap binding site of eIF4E, principally leading to rotation of W102 into the ligand binding pocket and possibly leading the formation of a cap receptive state in eIF4E. However, the W102 loop is highly flexible as evidenced by heteronuclear NOE experiments and with higher than average B-factors in this region.

Solution NMR studies of BP1 eIF4E cap free complex

We extended our studies to assess the alterations induced by 4EBP1 in solution. Chemical shift perturbations were monitored using ^1H - ^{15}N - HSQC analysis of ^{15}N labelled eIF4E titrated with unlabelled 4EBP1 peptide. Striking changes were seen throughout the ^1H - ^{15}N - HSQC spectrum of eIF4E upon addition of the 4EBP1 peptide (Figure 3A). The amide chemical shift for each residue in eIF4E was determined before and after saturation with peptide and the difference plotted as a function of residue number (Figure 3B). Analysis of residues at the dorsal surface correlates with the contact points found in the eIF4E-4EBP1 crystal structures. Specifically, significant shifts were seen in the N-terminal loop and strand $\beta 1$ (V28-A44), helix $\alpha 1$ (V69-N77), and helix $\alpha 2$ (L131-S141) (Figure 3B). Surprisingly, especially given the similarity of the crystal structures at the cap binding site, we observe small but significant chemical shift perturbations at the unoccupied cap binding site (Figure 4; W102, W166, R157, K159). Since there is no evidence for direct binding of the peptide at the cap binding site, these perturbations most likely reflect a change in the conformation driven by binding of the peptide at the distal dorsal surface. This is further supported, by the ability to define allosteric tracks from the 4EBP1 peptide binding site to the cap binding site (Figure 4A).

Differing allosteric routes for positive and negative regulators of cap binding

To visualize the extent of structural changes and understand the allosteric effects induced by 4EBP1, the chemical shift changes were mapped onto the apo-eIF4E structure (Figure 4A). For comparison, we also examined the effects on eIF4G peptide binding and the negative regulator Z (Figure 3B, 4). Similar to 4EBP1, significant changes can also be observed throughout the eIF4E structure upon addition of the eIF4G peptide (Figure 4B). The largest magnitude effects were on the dorsal surface, consistent with the known binding sites of 4EBP1 and eIF4G. However, modulations were also observed in many of the aromatic residues throughout the hydrophobic core. For instance, with 4EBP1, chemical shift changes can be observed in the core of the protein initiated by F72 in helix $\alpha 1$, followed by F66 ($\beta 2$), W43 and A44 ($\beta 1$), F94 ($\beta 3$), G151 ($\beta 5$), I165 and W166 ($\beta 6$) located behind the cap binding pocket, and ends at L114 ($\beta 4$) which is close to the C-terminal region (Figure 4A, B). These changes also influence the main chain of W102 (Figure 4B) and residues behind W102 (e.g. H200). Interestingly, eIF4G and 4EBP1 binding shift similar sets of residues; however, the direction and magnitude of these shifts were not always the same (Figure 4B). For 4EBP1 and eIF4G peptides, residues in the phosphate binding loop of eIF4E, including R157, K159, are observed to shift upon peptide binding. Consistent with our results, residues suggested to be involved in the allosteric transmission of dorsal surface ligand on eIF4E in yeast, including F48, D90, R157, and W166 were also found to be altered. In summary, eIF4G and 4EBP1 peptides, by virtue of binding highly overlapping sites on the dorsal surface, induce similar conformational alterations in the core of eIF4E that are transmitted to the cap binding site.

As mentioned, both the eIF4G and 4EBP1 peptides increase the affinity of eIF4E for the cap. We then compared allosteric tracks induced by these peptides with the arenavirus Z protein, a repressor of cap binding. Arenavirus Z (Z) is a ninety residue RING protein that associates with eIF4E on a surface overlapping but distinct from eIF4G and 4EBP1. The binding of Z is more centred on α -helix 1 and induces not only chemical shift perturbations, but also substantial line broadening in regions distal to its binding site. This underlies its ability to increase conformational exchange in the cap binding site of eIF4E, contributing to its ability to reduce cap binding. A comparison of the chemical shift perturbation pattern of Z with the eIF4G and 4EBP1 peptides indicated important findings. First, there was substantial overlap in the allosteric pathways that were activated in both cases including alterations in the phosphate binding region of the cap binding surface of eIF4E (K159, R157) and alterations in the main chain of W102, suggesting that similar changes observed in the crystal structure can also be observed in solution. Comparing the areas with chemical shift perturbation revealed that the region corresponding to β -sheet 3, residues L81-M101, was perturbed much more upon Z binding than either peptide eIF4G or 4EBP1 peptide (Figure 3B, 4A). Given that Z binding centres on binding helix α 1 which is adjacent to β 3 and ends at W102, it seems likely that this route is specific to Z and thus uses a unique communication route to the cap binding site. Unlike Z, both eIF4G and 4EBP1 induce chemical shift perturbation in region E171-A201 which corresponds to areas that bind the second nucleotide of the m⁷GpppA complex (Figure 4). Taken together, the distinct effects of eIF4G and 4EBP1 relative to Z on cap affinity appear to arise, at least in part, from the allosteric pathways activated by binding distinct regions on the dorsal surface.

Conclusions

In summary, these are the first structural studies to elucidate the basis for the allosteric regulation 4EBP1 or eIF4G on the eIF4E protein. Given that eIF4E-cap and 4EBP1-eIF4E-cap complex structure have been solved, it was important to determine the structure of the cap-free 4EBP1-eIF4E complex. Our first approach was to solve the crystal structure of 4EBP1-eIF4E cap free complex and compare this to the crystal structure of cap free eIF4E. Unfortunately, crystal contacts in the cap free form of eIF4E possibly drove allosteric changes in the cap binding site, confounding interpretation of our crystallographic data. However, the studies with apo-eIF4E crystals do highlight the effects of dorsal surface ligands, including itself, on the cap binding site. Relative to the solution apo-eIF4E structure, the 4EBP1-eIF4E cap free complex shows that W102 rotates partially into the cap binding site while the W56 loop remains flexible. However, again there are crystal contacts that could contribute to the W102 loop conformation. We extended our studies to assess if similar changes were induced by 4EBP1 in solution and to characterize the allosteric modes used. These studies revealed that 4EBP1 and eIF4G peptides activate very similar allosteric pathways in eIF4E to communicate changes in the phosphate binding loop, W102, and the C-terminal nucleotide binding region. It is possible that the changes observed would be amplified if larger or full length constructs were used. This would correlate with the higher affinity of eIF4E for the 5'cap in the presence of larger fragments as described in previous studies. In contrast, arenavirus Z binding on helix α ¹ of the dorsal surface is communicated down the β 3 -sheet to the cap binding site. This route appears to be less prevalent, in eIF4G or 4EBP1. Overall, the analysis of cap free eIF4E have given insight, at least in part, to the allosteric mechanisms driving ligand induced conformational changes in eIF4E that ultimately impact its biological activities. Clearly, alterations in protein dynamics by ligands will likely play a significant role in this process and is an important area of future work.

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Abbreviations

eIF	eukaryotic translation initiation factor
4EBP	eIF4E binding protein
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
PDB	Protein Data Bank
PML	promyelocytic leukemia, Arenavirus Z (Z)

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Highlights

- 1. Cap free eIF4E-4EBP1 complex and apo-eIF4E crystal structures solved at 2.1 Å.
- 2. 4EBP1 induces allosteric changes in eIF4E converging at the cap binding site.
- 3. Ligand specific allosteric routes on eIF4E underlie different effects on cap affinity.

Figure 1. Crystal structures of Apo-eIF4E and cap-free eIF4E in complex with the 4EBP1 peptide

Both structures were solved at a resolution of 2.1 Å. (A) Ribbon representation Apo-eIF4E (3TF2, blue) and (B) eIF4E in a cap-free state (3U7X, green) bound with a 20 residue 4EBP1 peptide (red) with the W56 loop not resolved in the latter structure. 4EBP1 makes contacts with helix $\alpha 1$ and $\alpha 2$ located at the dorsal surface of eIF4E. The cap binding pocket, highlighted by W56 and W102 (yellow, orange) is distal to the peptide binding site. (C) An overlay of both structures displays high similarity (RMSD 0.585 Å). The region corresponding to the eIF4E binding site from human 4EBP1 (GI: 34921508, 47-PGGTRIIYDRKFLMECRNSP-66) was synthesized by Fmoc *N*-(9-fluorenyl)-methoxycarbonyl solid-phase peptide synthesis (Sheldon Biotechnology Center) and purified by reverse-phase chromatography on a Vydac C18 column. The composition and purity of the peptide was verified by ion-spray quadrupole mass spectroscopy. All structure figures were generated using Pymol (The PyMOL Molecular Graphics System, Version 1.23pre, Schrödinger, LLC). Structural alignment and comparisons were completed by SUPERPOSE software.

Figure 2.

(A) Overlay of crystal and solution apo-eIF4E structures. On the left, ribbon overlay of solution (2GPQ; purple, W56 and W102 in green) and crystal (3TF2; blue, W56 and W102 in yellow) structures of apo-eIF4E (RMSD 2.3Å). The right shows overlay of apo-eIF4E NMR ensemble with crystal structure displaying aromatic residues (W56, W102) within the cap binding pocket. **(B) Analysis of residues involved in apo-eIF4E crystal contacts.** The far left shows a representative pair of apo-eIF4E crystal structures found in one asymmetric unit. The dorsal area is boxed and the following panel is an expanded view of the same region highlighting the helices of both structures that are in close proximity. The third panel from the left shows one apo-eIF4E crystal (red) and the residues that make apo-apo eIF4E dorsal crystal contacts. Residues with contacts to both helix $\alpha 2$ and $\alpha 3$ are shown in blue, to helix $\alpha 2$ alone in cyan, and to helix $\alpha 1$ in yellow. The far right panel is the cap free eIF4E-4EBP1 crystal structure (3U7X, green) displaying contact points between 4EBP1 peptide (red) and eIF4E. Key residues are labelled on eIF4E and are shown in yellow. **(C) Comparison of the cap free and M^7 GpppA cap bound 4EBP1-eIF4E crystal structures.** Ribbon overlay of cap free 4EBP1-eIF4E (green) and ternary 4EBP1-eIF4E- m^7 GpppA (salmon) crystal complexes. Cap analogue, m^7 GpppA, is shown in grey and known to make additional contacts relative to m^7 GTP or m^7 GDP ligands. Boxed in is the cap binding site and changes induced by the cap analogue are highlighted in panels two, three, and four, showing the W56-loop, W102 and E103, and the C-terminal region interacting with second base of the m^7 GpppA cap analogue, respectively.

Figure 3. Solution NMR experiments with eIF4E and 4EBP1 and comparative chemical shift analysis of eIF4E binding partner eIF4G and arenavirus Z

(A) ^1H - ^{15}N HSQC overlay of cap-free eIF4E (red) and eIF4E bound with 4EBP1 peptide (blue). 4EBP1 peptide was added at a 5:1 molar ratio to eIF4E. (B) ^1H - ^{15}N chemical shift perturbation analyses are shown for eIF4E with 4EBP1 (top) and eIF4G (middle) peptide and the RING protein Z (bottom). The change between apo-eIF4E and peptide bound forms were calculated and plotted as a function of residue number. Residue numbers correspond to the human eIF4E sequence (GI: 4503535). Cartoon diagram seen below chart represent β -sheets (blue) and α -helices (red). The W56, W102, and phosphate binding loops are also labeled. Labeled human eIF4E was expressed in *Escherichia coli* BL21 (DE3) and purified as previously described. NMR samples contained ~0.25–0.3 mM protein in 7% D_2O , 50mM sodium phosphate buffer (pH 7.3), 100 mM NaCl 250 μM 100 μM TCEP, and 1mM NaN_3 . NMR experiments were completed at 20°C on either a 600 or 800 MHz Varian INOVA spectrometer equipped with an HCN cold probe. Data were processed with NMRPipe and analyzed with Sparky.

Figure 4. Mapping of chemical shift changes on the apo-eIF4E structure and select residues from ^1H - ^{15}N HSQC experiments

(A) Chemical shifts induced by 4EBP1 peptide (left), eIF4G (middle), and arenavirus Z (right) on the dorsal surface of eIF4E are shown in blue, green, and red, respectively. Residues affected away from the dorsal surface are highlighted in orange and yellow representing relative strong and medium chemical shifts. In cyan are residues that show line broadening. Overall, contacts on the dorsal surface lead to alterations in the core of the protein which are ultimately communicated to the cap binding site of eIF4E. (B) ^1H - ^{15}N HSQC overlay of apo-eIF4E (red) bound with 4EBP1 (blue) and eIF4G (green) peptides. From left to right, each column displays residues tracing the allosteric tracks as shown in the above panel. These include residues from the core of the protein, phosphate binding loop, and C-terminal region.

Table 1
Crystallography data and refinement statistics

Crystallization and structure determination

The plasmid containing the full-length cDNA for human eIF4E was a gift from Dr N. Shimma, *Chugai Pharmaceutical Co.*, Japan). Human eIF4E was expressed in *Escherichia coli* BL21 (DE3) and purified as previously described. The protein was purified from the soluble fraction and solution conditions used were previously optimized to ensure the proper folding of eIF4E. For crystallization trials, eIF4E protein samples were then exchanged into 20mM HEPES, 100mM KCl, 1mM TCEP, pH 7.3 using an Amicon Ultra Centrifugal Filter device (10K MWCO, Ultra Centrifugal Filter Device, Millipore) and concentrated to 8–10mg/ml prior to plating (320–400 μ M). Apo-eIF4E protein and solution was mixed in a 1:1 ratio with 20% polyethylene glycol 3350 and 0.2M di-ammonium citrate. For the complex, 4EBP1 peptide (900 μ M–1mM) and 4mM ribavirin were mixed with eIF4E and incubated at 4°C for 1 hour. The sample was then mixed in a 1:1 ratio with 2M ammonium sulfate, 2% PEG400, 0.1M Na HEPES, pH 7.5. Even though the eIF4E–4EBP1 peptide complex was crystallized in the presence of ribavirin, we did not observe plausible electron density for the putative ribosyl and triazolyl moieties of ribavirin supporting that the eIF4E–4EBP1 is in its cap free state. The high affinity analogue is the triphosphorylated form of ribavirin, which would be more suitable for future studies. Both samples were placed at 16°C and crystallized using the sitting drop vapour diffusion method. Thin needle like crystals grew to mountable size within 10–14 days. Prior to data collection, crystals were flash frozen in liquid nitrogen. For each structure, 180 continuous 1° diffraction images were collected at APS beamline 19ID and reduced using the HKL3000 program suite. Initial molecular replacements were performed with the program PHASER and coordinates derived from PDB entry 1IPB. Subsets of reflections were selected in thin resolution shells using SFTOOLS (Dr. B. Hazes, University of Alberta) and used for calculation of the “free” residual (R_{free}). For both structures, model refinement and rebuilding were performed with REFMAC and COOT, respectively. Model geometry was monitored on the MOLPROBITY server. Model depositions in the PDB were prepared with PDB_EXTRACT.

	Apo-eIF4E	eIF4E - 4EBP1
PDB code	3TF2	3U7X
X-ray wavelength (Å)	0.9793	0.9792
Space group	P1	P21
a, b, c (Å)	39.24, 56.74, 100.31	86.50, 38.26, 93.53
α , β , γ (°)	103.61, 91.36, 110.14	90.00, 97.97, 90.00
Resolution (outer shell, Å)	40.00–2.10 (2.14–2.10)	50.00–2.10 (2.14–2.10)
Unique observed HKLs	44808 (2190)	35859 (1728)
Completeness (%)	97.8 (97.0)	99.7 (96.9)
Friedel Redundancy	1.9 (2.0)	3.6 (3.4)
R _{sym}	0.050 (0.374)	0.089 (0.640)
$\langle I \rangle / \langle \sigma \rangle$	15.9 (2.1)	16.4 (1.8)
Refinement resolution (Å)	30.00–2.10	30.00–2.10
Number of reflections (of which free set)	43329 (1342)	35692 (1503)
R _{work} /R _{free}	0.215/0.260	0.198/0.224
Number of atoms/mean B factor (Å ²)	5823/36.1	3427/50.3
Protein (eIF4E)	5654/36.2	3068/49.1
Peptide (4EBP1)	none	216/67.4
Water	169/32.9	124/47.3

	Apo-eIF4E	eIF4E - 4EBP1
RMSD bond lengths (Å)/angles (°)	0.013/1.3	0.014/1.3
Ramachandran Plot (%)		
Most favored regions (%)	94.1	91.7
Generously allowed	none	0.6
Disallowed	0.7 ^a	none

^aResidues Asp-143 in protein chains A through D with ϕ of approx. 60° and ψ of approx. -115°.