Influence of Electric Charge Variation at Residues 209 and 159 on the Interaction of eIF4E with the mRNA 5′ Terminus†

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ABSTRACT: Eukaryotic translation initiation factor 4E (eIF4E) is essential for efficient protein synthesis in cap-dependent translation. The protein specifically binds the cap structure at the mRNA 5′ terminus and facilitates the assembly of the mRNA with other initiation factors and the 40S ribosomal subunit. Phosphorylation of eIF4E is implicated in the regulation of the initiation step of translation. However, the molecular mechanism of this regulation still remains unclear. To address this problem, we have determined the binding affinities of eIF4E specifically mutated at position 209 or 159 for a series of novel mono- and dinucleotide cap analogues by a fluorometric time-synchronized titration method. A 1.5−3-fold reduction in the affinity of cap for the S209E mutant and a 1−2-fold increase in the affinity of cap for the S209K mutant, depending on the negative charge of phosphate chains, indicate that phosphorylation at Ser209 creates electrostatic repulsion between the protein and the negatively charged cap structure. The inhibition of the ability to bind cap analogues by the K159A mutant and its phosphorylated counterpart shows significant participation of Lys159 in the binding of the capped mRNA. Both structural modifications, phosphorylation and the replacement of lysine with alanine, result in an increase in the negative Gibbs free energy of association that is proportional to the length of the cap phosphate chain and additive, i.e., equal to the sum of the individual destabilizing changes of $\Delta G°$. The possible implication of these results for the mechanism of control of eIF4E by phosphorylation, especially for the "clamping model", is discussed.

Regulation of protein synthesis plays a significant role in controlling cell growth, translation initiation being an important step of this control. In most cases, translation begins with eukaryotic initiation factor 4E (eIF4E)[1] recognizing the 7-methylG(5′)ppp(5′)N (where N is any nucleotide) cap structure at the 5′ terminus of the mRNA (*1*). eIF4E forms an eIF4F complex together with two other proteins: eIF4A, an RNA helicase, and eIF4G, a scaffolding protein that binds several other factors necessary for recruiting the ribosome. The N terminus of eIF4G, apart from interacting with eIF4E, binds the poly(A)-binding protein, whereas its C terminus contains binding sites for eIF4A, eIF3, and the Mnk1 or Mnk2 kinase (*2*−*4*). The interaction of these factors with the 40S small ribosomal subunit is required for assembly of the 48S ribosomal preinitiation complex. The control of accessibility of eIF4E to form the eIF4F complex occurs through reversible interaction with one of the eIF4E-binding proteins, 4E-BPs (*5*). These small (10−20 kDa) acidic proteins compete with eIF4G for a common binding site in eIF4E, thereby preventing formation of the active eIF4F complex, and consequently inhibiting protein synthesis. The binding of the 4E-BPs to eIF4E is regulated by phosphorylation; i.e., hyperphosphorylation of 4E-BPs in response to stimulation of cells by hormones and growth factors abrogates interaction of these binding proteins with eIF4E (*6*).

For many years, phosphorylation was postulated to control the activity of eIF4E, and thus to influence the overall rate of protein synthesis. Mammalian eIF4E is phosphorylated at a single residue, serine 209, by the Mnk1 and Mnk2 kinases (*7*−*9*). Approximately 80−85% of the eIF4E in the 48S preinitiation complex is in the phosphorylated form, whereas the unbound protein is phosphorylated only to 50% (*10*). Most studies have reported a correlation between eIF4E phosphorylation and the rate of protein synthesis, implying that phosphorylation has a positive effect on cap-dependent

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[1] Abbreviations: CBD, chitin binding domain; DMF, dimethylformamide; IPTG, isopropyl β-D-thiogalactopyranoside; eIF4E, eukaryotic initiation factor 4E; eIF4Ewt, wild-type eIF4E; 4E-BP, 4E binding protein; MESNA, sodium salt of 2-mercaptoethanesulfonic acid; *Mxe* GryA intein, intein from the *Mycobacterium xenopi gryA* gene; m^7-GMP, 7-methylguanosine 5′-monophosphate; m^7GDP, 7-methylguanosine 5′-diphosphate; m^7GTP, 7-methylguanosine 5′-triphosphate; m^7Gp_4, 7-methylguanosine 5′-tetraphosphate; m^7Gp_5, 7-methylguanosine 5′-pentaphosphate; m^7GpppG, P^1-7-methylguanosine 5′-P^3-guanosine 5′-triphosphate; TCEP, tris(2-carboxyethyl)phosphine; TEAB, triethylammonium bicarbonate; TST, time-synchronized titration.

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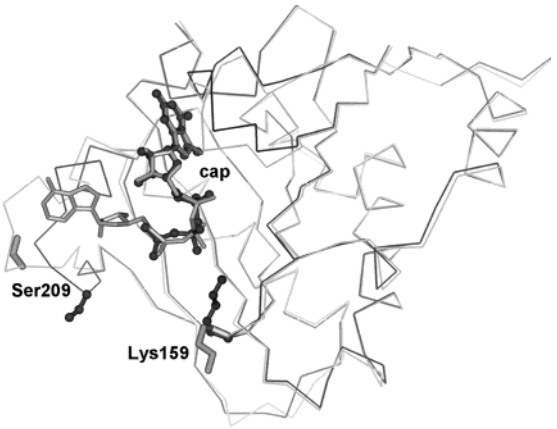

FIGURE 1: Superposition of C_α traces of truncated murine eIF4E-(28−217) in complex with m^7GpppG (*20*) (black) (PBB entry 1L8B) and full-length human eIF4E in complex with m^7GpppA (*21*) (gray) (PBD entry 1IPB). The cap analogues and the side chains of serine 209 and lysine 159, which influence the affinity of eIF4E for the capped mRNA, are represented with stick (human eIF4E) and ball-and-stick (murine eIF4E) models, respectively. The second nucleoside in m^7GpppG could not be detected by crystallography.

translation (*10*−*12*). Genetic studies in *Drosophila* showed that introduction of a nonphosphorylatable mutant of eIF4E-(S211A) has negative consequences for the growth and viability of the fruitfly (*13*). On the contrary, some reports have presented evidence that eIF4E phosphorylation does not enhance and even inhibits global protein synthesis. Overexpression of the Mnk1 kinase in cardiomyocytes stimulates phosphorylation of eIF4E without any effect on translation (*14*). In addition, McKendrick *et al*. (*15*) showed that in a reticulocyte lysate translation system phosphorylation of eIF4E is not required for effective protein synthesis. Recent biophysical studies have reported that phosphorylated eIF4E exhibits a lower binding affinity for the cap structure (*16*, *17*), contrary to an earlier report (*18*) that showed a higher affinity of the phosphorylated eIF4E form for capped mRNA. The three-dimensional structures of mouse and human eIF4E bound to mono- and dinucleotide cap analogues (*19*−*22*) have been determined exclusively for the unphosphorylated form of eIF4E (Figure 1). The protein adopts the shape of a cupped hand with the cap analogue located in the narrow cap-binding slot on the concave surface of eIF4E. Two elements of the cap structure, the positively charged 7-methylguanine moiety and the negatively charged phosphate chain, are of primary importance for specific interaction of the cap with eIF4E. 7-Methylguanine is stabilized by a sandwich-type stacking between the side chains of two tryptophan residues (Trp56 and Trp102), and additionally by three hydrogen bonds. The phosphate groups of the cap structure form direct or water-mediated hydrogen bonds and salt bridges with the NH group of Trp102 and with the Trp166 indole ring as well as with the side chains of the lysine and arginine residues. The C-terminal loop of eIF4E containing the phosphorylated serine 209 is located on the trajectory of the mRNA chain. In the crystal complex of human eIF4E with m^7GpppA, adenine is bound via a hydrogen bond to Thr205, and via a water-mediated hydrogen bond to Thr211. Additionally, binding of the second base of the cap with the protein is stabilized by van der Waals contacts with Ser207 and its neighboring residues (*21*, *22*). Fluorescence titrations (*20*) and stopped-flow studies (*23*) showed that electrostatic interactions play a major role in recognition of the cap structure by eIF4E. On the basis of fluorescence titration experiments, Niedzwiecka *et al*. (*20*) proposed a two-step mechanism of binding of the cap by unphosphorylated eIF4E in which the negative charge of the cap phosphate chain acts as a molecular anchor, enabling formation of further contacts, sandwich stacking and Watson−Crick-type hydrogen binding, between the protein and the cap.

The crystallographic (*19*−*21*) and fluorescence (*20*, *23*) studies of the interaction between selected cap analogues and unphosphorylated eIF4E and recent reports on the interactions involving phosphorylated eIF4E (*16*, *17*) are still insufficient to unequivocally validate one of the models proposed for the role of eIF4E phosphorylation in regulation of the translation initiation step, i.e., the "clamping model" of Marcotrigiano *et al*. (*19*) and one of the two mechanisms proposed by Scheper *et al*. (*16*). To investigate this problem, the work presented here describes the binding affinity of specific mutants of eIF4E at position 209 for a series of mono- and dinucleotide cap analogues by means of fluorescence titration measurements. The selection of the cap analogues was based on the number of phosphate groups they contain. Their affinities for the mutated protein made it possible to establish the electrostatic character of the interaction between the phosphoserine and the capped mRNA. Additionally, replacement of Lys159 with alanine in eIF4E enabled verification of the clamping model (*19*, *24*). We also describe the synthesis of two novel cap analogues: the 5′-tetra- and 5′-pentaphosphate of 7-methylguanosine (m^7Gp_4 and m^7Gp_5, respectively) which together with the new eIF4E mutants provide new data on the eIF4E−cap interaction.

MATERIALS AND METHODS

Synthesis of Cap Analogues

Column Chromatography. Final products and intermediate nucleotides were isolated from appropriate reaction mixtures by column chromatography on DEAE-Sephadex (A-25, HCO_3^- form) using an appropriate linear gradient of triethylammonium bicarbonate (TEAB, pH 7.5) at 4 °C. Eluates were collected, and product peaks (as monitored by UV absorption at 260 nm) were pooled and evaporated (bath temperature not exceeding 30 °C, ethanol added repeatedly to remove the TEAB buffer). Thus, the products were obtained as triethylammonium (TEA) salts.

HPLC. Monitoring the purity of intermediates and products was performed by analytical HPLC using a Spectra-Physics SP8800 apparatus with a Supelcosil LC-18-T (25 cm, with a 2 cm guard) reverse phase column and a Supelcosil LC-SAX (25 cm, with a 2 cm guard) ion exchange column. Mobile phases were a linear methanol gradient from 0 to 25% in 0.05 M ammonium acetate (pH 5.9) over the course of 15 min with flow rate of 1.3 mL/min for the reverse phase column and a linear gradient of from 0.001 to 0.1 M KH_2PO_4 (pH 4.0) over the course of 20 min with a flow rate of 1.0 mL/min for the ion exchange column. All compounds used for biophysical studies were >95% pure in the reverse phase as well as in the ion exchange systems.

Guanosine 5′-Tetraphosphate (Gp_4). The GDP Na^+ salt (254 mg, 0.5 mmol) was changed into the TEA salt on a

Dowex 50WX1 resin and then used in the activation reaction. The GDP TEA salt, imidazole (170 mg, 2.5 mmol), and 2,2′-dithiodipyridine (220 mg, 1 mmol) were mixed with 6 mL of anhydrous DMF and 70 μL of TEA. Triphenylphosphine (262 mg, 1 mmol) was added, and the mixture was stirred overnight at room temperature and poured into a flask containing 250 mg of anhydrous sodium perchlorate dissolved in 30 mL of acetone. After the solution had been cooled (~2 h) at 4 °C, the precipitate that formed was filtered, washed twice with 10 mL of cold acetone, and dried in a vacuum desiccator over P_4O_{10}. Guanosine 5′-diphosphate P^2-imidazolide (256 mg, 0.43 mmol) was dissolved in 2 mL of anhydrous DMF, and 2.15 mmol of pyrophosphate (as TEA salt) was added. The mixture was stirred for 5 min, after which 157 mg of $ZnCl_2$ (4.30 mmol) was added. The mixture was stirred at room temperature for 2 h (completeness of the reaction followed by an HPLC, with an ion exchange column), poured into a beaker containing 1.5 g of EDTA dissolved in 200 mL of water, and neutralized with 1 M $NaHCO_3$. Chromatographic isolation on DEAE-Sephadex using a linear TEAB gradient from 0 to 1.2 M yielded the pure product as a TEA salt. Fractions containing the product were pooled and coevaporated four times with ethanol. Guanosine 5′-tetraphosphate (Gp_4, 423 mg, 0.42 mmol) was obtained as a TEA salt in 82% yield (after two steps).

7-Methylguanosine 5′-Tetraphosphate (m^7Gp_4). The TEA salt of Gp_4 (201 mg, 0.2 mmol) was dissolved in 6 mL of DMSO, and methyl iodide (25 μL, 0.4 mmol) was added. The solution was stirred for 4 h at room temperature. Cold water (80 mL) was added, and the solution was extracted three times with 100 mL of diethyl ether. After neutralization with 1 M $NaHCO_3$, the aqueous phase was chromatographed on a DEAE-Sephadex column and eluted with a 0 to 1.2 M linear TEAB gradient (yield of 139 mg as the TEA salt, 68%).

Guanosine 5′-Pentaphosphate (Gp_5). Synthesis of Gp_5 was carried out as described for guanosine 5′-tetraphosphate. The guanosine 5′-triphosphate (GTP, 305.5 mg, 0.5 mmol) Na^+ salt was used instead of GDP to obtain guanosine 5′-triphosphate P^3-imidazolide. The latter compound (249 mg, 0.39 mmol) used in the reaction with pyrophosphate yielded after chromatographic isolation (0 to 1.3 M linear TEAB gradient) Gp_5 (yield of 490 mg as the TEA salt, 0.38 mmol, 68%).

7-Methylguanosine 5′-Pentaphosphate (m^7Gp_5). Synthesis of m^7Gp_5 was carried out as described for m^7Gp_4 using 258 mg (0.2 mmol) of Gp_5 instead of Gp_4. Chromatographic isolation (DEAE-Sephadex column, 0 to 1.3 M linear TEAB gradient) yielded 0.144 mmol of m^7Gp_5 (yield of 189 mg as the TEA salt, 72%).

The final products were converted to their Na^+ salts by ion exchange chromatography (Dowex 50WX8 resin, Na^+ form). The eluates were concentrated; residues were precipitated with ethanol, and centrifugation gave amorphous white powders that were dried in a vacuum desiccator over P_4O_{10}.

Syntheses of other cap analogues were preformed as described previously: m^7GMP, m^7GDP, and m^7GTP (*25*), m^7GpppG (*26*), m^7Gp_4G, $m^7Gppp m^7G$, and $m^7Gp_4m^7G$ (*27*), and m^7Gp_5G and $m^7Gp_5m^7G$ (*28*).

NMR Spectroscopy and Conformation Analysis

^1H NMR and ^{31}P NMR spectra were recorded on a Varian UNITYplus 500 MHz instrument in ^2H_2O at 20 °C and at a concentration of 1.5 mg/0.7 mL. Conformations of the sugar moieties were derived from the vicinal $^1H-^1H$ coupling constants (*29*), and conformations of the α-phosphate groups about the C5′−O5′ bond were derived from the vicinal $^1H-^{31}P$ coupling constants (*30*).

Mutagenesis, Expression, and Purification of eIF4Es

The QuikChange PCR-based site-directed mutagenesis kit (Stratagene) was used to obtain the point mutations in the truncated murine eIF4E(28−217). As the initial template, the cDNA for eIF4E(28−217) in a pET-3b vector (Novagen) was used; it possessed two mutations, Trp73 replaced with phenylalanine and Ser209 replaced with glutamic acid. The oligonucleotide primers were designed to introduce tryptophan at position 73 first, and subsequently alanine, lysine, and serine at position 209. The vector for the mutant possessing alanine instead of Lys159 was obtained using as a template the cDNA for eIF4E(28−217) in a pET-3b vector. Site-directed mutagenesis of eIF4E was performed according to the instructions provided by Stratagene. All inserts thus obtained were automatically sequenced to confirm the presence of the mutations.

The pET-3b vectors containing the mutated eIF4E(28−217) (S209A, S209K, S209E, and K159A) were transformed into the BL21(DE3) *Escherichia coli* strain (Novagen) and expressed as inclusion bodies as described previously for the wild-type protein (*31*). The proteins were purified from the inclusion bodies using 6 M guanidinium hydrochloride, refolded by one-step dialysis against 50 mM Hepes/KOH (pH 7.2), 100 mM KCl, 0.5 M EDTA, and 2 mM DTT, and purified by ion exchange chromatography on a Mono SP column, without contact with cap analogues at any step of the purification. The amount of protein obtained from the inclusion bodies [determined by the Bradford method (Bio-Rad)] varied from 80 to 120 mg from 1 L of culture. However, ~40−60% of the protein was lost during dialysis and purification by FPLC. The masses of the proteins were confirmed by mass spectroscopy: predicted mass of 22 113.1 Da and observed mass of 22 112.8 ± 1.0 Da for eIF4Ewt, predicted mass of 22 155.1 Da and observed mass of 22 153.8 ± 1.0 Da for S209E, predicted mass of 22 154.2 Da and observed mass of 22 153.8 ± 1.0 Da for S209K; predicted mass of 22 097.1 Da and observed mass of 22 096.5 ± 1.0 Da for S209A, and predicted mass of 22 056.0 Da and observed mass of 22 055.3 ± 1.0 Da for K159A.

Preparation of Phosphorylated Proteins

eIF4E phosphorylated at Ser209 (S209phos) and its variant with alanine instead of lysine at position 159 (K159A/S209phos) were obtained using the Intein-Mediated Protein Ligation (IPL) method (*32*, *33*). The protein was produced as two separate fragments in which Thr205 was replaced with cysteine to make the renewed ligation of both fragments feasible. The first one, the eIF4E(205−217) fragment containing phosphorylated serine, was synthesized chemically on a Wang resin by means of the Fmoc strategy as described

previously (*17*). The second fragment of the protein, eIF4E-(28−204), was expressed in *E. coli* fused to *Mxe* GryA intein, generating a C-terminal thioester on the eIF4E fragment as a result of incubation with ethanethiol. The ligation reaction was performed in 6 M guanidinium hydrochloride, and the ligated protein was refolded by one-step dialysis against 50 mM Hepes/KOH (pH 7.2), 100 mM KCl, 0.5 M EDTA, and 2 mM DTT and purified by ion exchange chromatography. The detailed procedure for the preparation of the phosphorylated protein was described previously (*17*).

Fluorescence Measurements and Data Analysis

Fluorescence measurements were run on an LS-50B spectrofluorometer (Perkin-Elmer Co., Hellma, Germany) in a quartz semi-micro cuvette with optical lengths of 4 and 10 mm for absorption and emission, respectively. Fluorescence time-synchronized titrations (*20*) were performed in 50 mM Hepes/KOH (pH 7.2), 100 mM KCl, 0.5 mM EDTA, and 1 mM DTT at 20 °C by adding 1 μL aliquots of the cap analogue solutions to 1.4 mL of a 0.1 or 0.05 μM eIF4E protein solution. The protein concentration was determined spectrophotometrically ($\epsilon_{280} = 53\,400\ M^{-1}\ cm^{-1}$). Fluorescence intensities (excitation at 280 nm and detection at 337 nm) were corrected for sample dilution (less than 4%) and for the inner filter effect. Equilibrium association constants (K_{as}) were determined by fitting the theoretical curve for the fluorescence intensity (F) on the total concentration of cap analogue ($[L_o]$) to the titration data points. The amount of active protein and the emission of free cap analogue were fit exactly as free parameters of the equilibrium equation (*20*). The final K_{as} was calculated as a weighted average of 3−10 independent titrations, with the weights taken as the reciprocals of the numerical standard deviations squared. Numerical least-squares nonlinear regression analysis was performed using ORIGIN version 6.0 (Microcal Software Inc.).

The Gibbs binding free energy was calculated from the K_{as} value according to the standard equation $\Delta G° = -RT \ln K_{as}$.

RESULTS

Synthesis of Novel Mononucleotide Cap Analogues. Syntheses of m^7Gp_4 and m^7Gp_5 were performed using the strategy depicted in Figure 2. The TEA salts of GDP or GTP were converted into appropriate imidazolides in which the imidazole ring is connected to the terminal phosphorus atom in the phosphate chain (*28*). The coupling reaction of imidazolide GDP or GTP with the TEA salt of pyrophosphate in DMF yielded the tetra- or pentaphosphate of guanosine, respectively, with excellent yields (*34*); zinc chloride (10 molar equiv) served as the catalyst. Methylation of the guanosine oligophosphates with methyl iodide in DMSO gave the appropriate 7-methylguanosine oligophosphates as the main products.

Solution Conformations of the Free Cap Analogues. The solution conformations of the new synthesized cap analogues were determined from ^1H NMR measurements (Table 1), and are essentially typical for 7-substituted guanosines (*28*). Elongation of the phosphate chain only slightly alters the conformer populations compared to those of m^7GTP, i.e., ~10% decrease in the amount of the N (C3′-endo) and +sc (gauche orientation of C5′ to O4, and C3′), while the population of the ap conformer (trans orientation of C4′ and Pα) remains the same.

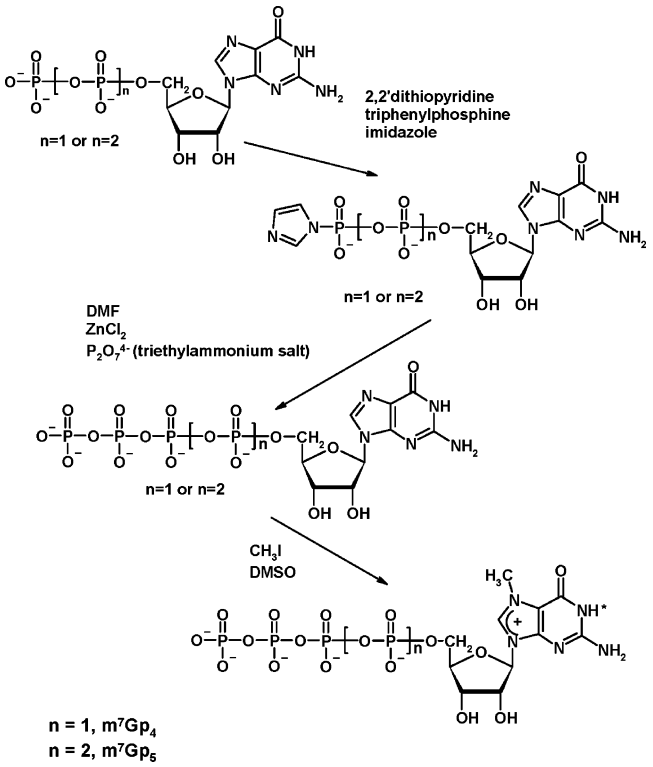

FIGURE 2: Synthesis of 7-methylguanosine 5′-tetra- and 5′-pentaphosphate (m^7Gp_4 and m^7Gp_5, respectively). Protons that partially dissociate at pH 7.2 are marked with an asterisk.

Table 1: NMR Analysis of the New Synthesized 7-Methylguanosine Polyphosphates in ^2H_2O[a]

δ	m^7Gp_4	m^7Gp_5	$J(i,j)$	m^7Gp_4	m^7Gp_5
H8	*b*	*b*			
H1′	6.074	6.077	1′,2′	4.0	4.4
H2′	4.739	4.750	2′,3′	5.2	5.0
H3′	4.605	4.595	3′,4′	4.5	4.6
H4′	4.421	4.426	4′,5′	2.5[c]	2.8
H5′	4.313	4.328	4′,5″	2.5[c]	2.4
H5″	4.290	4.291	5′,5″	11.6	11.9
CH_3	4.137	4.141	5′,P	5.0[c]	4.0
Pα	−8.78	−9.47	5″,P	5.0[c]	5.4
Pβ	−23.12	−23.44	4′,P	1.5[c]	1.5
Pγ	−23.34	−23.44			
Pδ	−12.02	−23.44			
Pε	−	−11.97			

conformation	m^7Gp_4	m^7Gp_5
%N	53	51
%+sc	85	82
%ap	75	78

[a] ^1H chemical shifts in parts per million (±0.01) vs internal sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]propionate, ^{31}P chemical shifts in parts per million (±0.01) vs external H_3PO_4, $^1H-^1H$ and $^1H-^{31}P$ coupling constants in hertz (±0.2), and conformer populations of the sugar ring in the N ⇔ S dynamic equlibrium, of the exocyclic group around C4′−C5′ (+sc) and C5′−O5′ (%ap) bonds (±5%). [b] Deuterated. [c] Approximate value due to signal overlap.

Positive Effect of the Negative Charge of Cap Analogues on Their Affinity for Wild-Type eIF4E. To extend our previous finding that elongation of the phosphate chain of mononucleotide cap analogues (m^7GMP, m^7GDP, and m^7GTP) results in a systematic increase in their binding affinity for

Table 2: Equilibrium Association Constants (K_{as}) for the Complexes of Mutated Murine eIF4E(28−217) with Mono- and Dinucleotide Cap Analogues Possessing Different Numbers of Phosphate Groups

cap analogue	K_{as} (μM^{-1})							
	wt	wt[a]	S209A	S209K	S209E	S209phos[a]	K159A	K159A/S209phos[a]
m^7GMP	1.5 ± 0.1	1.5 ± 0.1	1.15 ± 0.2	1.3 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.06 ± 0.1	0.75 ± 0.12
m^7GDP	28.9 ± 1.5	29.4 ± 0.5	32.7 ± 1.2	36.9 ± 1.3	23.3 ± 1.7	15.1 ± 0.5	21.6 ± 1.6	7.7 ± 0.7
m^7GTP	119.7 ± 5.7	117.5 ± 5.2	140.2 ± 7.8	207 ± 10	86.0 ± 2.7	42.6 ± 1.8	38.8 ± 2.3	9.97 ± 0.49
m^7Gp_4	734 ± 56	694 ± 97	650 ± 39	1140 ± 133	371 ± 31	140.3 ± 4.0	102.6 ± 6.9	19.4 ± 0.8
m^7Gp_5	1625 ± 195	1549 ± 260	1509 ± 159	2966 ± 487	561 ± 53	202.4 ± 9.3	113.4 ± 4.3	17.6 ± 0.4
m^7Gp_3G	12.5 ± 0.3	10.5 ± 0.5	11.6 ± 0.3	14.1 ± 0.4	9.1 ± 0.5	5.8 ± 0.3	7.0 ± 0.3	
m^7Gp_4G	110.9 ± 6.0	97.5 ± 4.1	107.3 ± 3.9	177.4 ± 8.5	60.9 ± 2.2	27.3 ± 1.1	33.7 ± 1.4	
m^7Gp_5G	543 ± 55	411 ± 19	502 ± 31	903 ± 87	240.7 ± 10.4	94.0 ± 3.8	80.9 ± 3.0	
$m^7Gp_3m^7G$[b]	2.0 ± 0.1	2.4 ± 0.1	2.3 ± 0.1	2.6 ± 0.2	2.3 ± 0.2	1.42 ± 0.05	1.95 ± 0.05	
$m^7Gp_4m^7G$[b]	24.7 ± 0.8	25.4 ± 1.2	24.2 ± 1.3	39.8 ± 1.9	21.6 ± 0.7	10.1 ± 0.5	13.3 ± 0.6	
$m^7Gp_5m^7G$[b]	166 ± 12	177 ± 15	172 ± 12	293 ± 24	61.3 ± 5.1	36.8 ± 1.5	39.3 ± 1.8	

[a] Proteins obtained via the IPL method. [b] For symmetrical cap analogues ($m^7Gp_nm^7G$), microscopic association constants [$K_{as}^{(micro)}$] are presented. The observed K_{as} values must be divided by 2 because of entropic effects (*20*).

Table 3: Binding Free Energies ($\Delta G°$) for the Complexes of Murine eIF4E(28−217) with Mono- and Dinucleoside Cap Analogues with Different Numbers of Phosphate Groups

cap analogue	$\Delta G°$ (kcal/mol)			
	wt	S209phos[a]	K159A	K159A/S209phos[a]
m^7GMP	−8.279 ± 0.039	−8.099 ± 0.053	−8.077± 0.055	−7.872 ± 0.096
m^7GDP	−10.002 ± 0.030	−9.624 ± 0.019	−9.832 ± 0.043	−9.234 ± 0.053
m^7GTP	−10.829 ± 0.028	−10.228 ± 0.025	−10.173 ± 0.035	−9.383 ± 0.028
m^7Gp_4	−11.885 ± 0.044	−10.921 ± 0.017	−10.739 ± 0.039	−9.769 ± 0.023
m^7Gp_5	−12.348 ± 0.070	−11.135 ± 0.027	−10.798 ± 0.022	−9.712 ± 0.014
m^7Gp_3G	−9.514 ± 0.014	−9.067 ± 0.030	−9.175 ± 0.022	
m^7Gp_4G	−10.785 ± 0.032	−9.969 ± 0.023	−10.091 ± 0.025	
m^7Gp_5G	−11.709 ± 0.059	−10.688 ± 0.024	−10.601 ± 0.022	
$m^7Gp_3m^7G$[b]	−8.447 ± 0.029	−8.247 ± 0.021	−8.432 ± 0.015	
$m^7Gp_4m^7G$[b]	−9.910 ± 0.019	−9.390 ± 0.029	−9.552 ± 0.027	
$m^7Gp_5m^7G$[b]	−11.019 ± 0.042	−10.142 ± 0.024	−10.181 ± 0.027	

[a] Proteins obtained via the IPL method. [b] For symmetrical cap analogues ($m^7Gp_nm^7G$), microscopic association constants [$K_{as}^{(micro)}$] are presented. The observed K_{as} values must be divided by 2 because of entropic effects (*20*).

eIF4E (*20*), we determined the association constants for the complexes of eIF4Ewt with the newly synthesized cap analogues that possess phosphate chains of increasing lengths (Table 2).

The α- and *β*-phosphates of the cap exert the strongest influence on the binding to eIF4Ewt. The subsequent addition of phosphate groups (m^7GTP and m^7Gp_4) results in a similar, ca. 6-fold increase in the affinity of the cap analogue for eIF4E. The corresponding energetic effect ($\Delta\Delta G°$) is ≅0.9 kcal/mol (Table 3), typical for a hydrogen bond, suggesting that the *δ*-phosphate most probably directly interacts with the protein by forming a hydrogen bond, as for the *γ*-phosphate (*20*). In the case of m^7Gp_5, an only 2-fold increase of the association constant ($K_{as} = 1625 \pm 195\ \mu M^{-1}$) in comparison with that of m^7Gp_4 ($K_{as} = 734 \pm 56\ \mu M^{-1}$) is observed. The resulting change in the standard Gibbs free energy of binding of m^7Gp_5 ($\Delta\Delta G° \cong 0.4$ kcal/mol) is not sufficient for the *ϵ*-phosphate to be involved in intermolecular contacts with eIF4E inside the cap-binding slot. On the contrary, addition of the second guanosine to m^7Gp_4 reduces the affinity of m^7Gp_4G for eIF4Ewt to the level observed for m^7GTP ($K_{as} = 110.9 \pm 6.0\ \mu M^{-1}$). Similar destabilization was observed in the case of m^7Gp_5G, whose binding affinity for eIF4E is similar to that of m^7Gp_4 (Table 2). Introduction of a methyl group to the second guanine of the dinucleotide cap analogues further reduces the affinity of these ligands for the protein in comparison with their unmethylated counterparts. The decreased affinity of eIF4E for the dinucleotide cap analogues resulting from the addition of the second nucleoside to the mononucleotide cap analogues can be explained by the fact that the second guanosine does not bind directly to eIF4E (*20*), and destabilizes the intermolecular contacts of 7-methylguanine in the eIF4E cap-binding center. Additionally, the second base partially reduces the negative charge of the phosphate chain.

The Electric Charge at Position 209 of eIF4E Influences Its Affinity for Cap Analogues. It was demonstrated previously that phosphorylation of eIF4E at Ser209 inhibits its ability to bind mRNA 5′ cap analogues and that this effect depends predominantly on the resulting negative charge of the cap analogues (*16*, *17*). To establish in more detail the influence of the negative charge of the phosphoserine on the character of the eIF4E−cap interactions, we examined the binding affinities of a series of cap analogues for eIF4E mutated at position 209. To accurately compare the tested proteins, all of them were purified on an ion exchange column (MonoSP) and stored and titrated with the same solutions of the cap analogues under the same conditions (ionic strength and pH).

The K_{as} values for the complexes of mutated eIF4Es with the cap analogues are presented in Table 2. All mutants exhibit higher binding affinities for cap analogues with the extended phosphate chain. However, the increase differs considerably within the family of mutated proteins. The replacement of Ser209 with a neutral amino acid, alanine, has no effect on binding of the cap analogues (Figure 3).

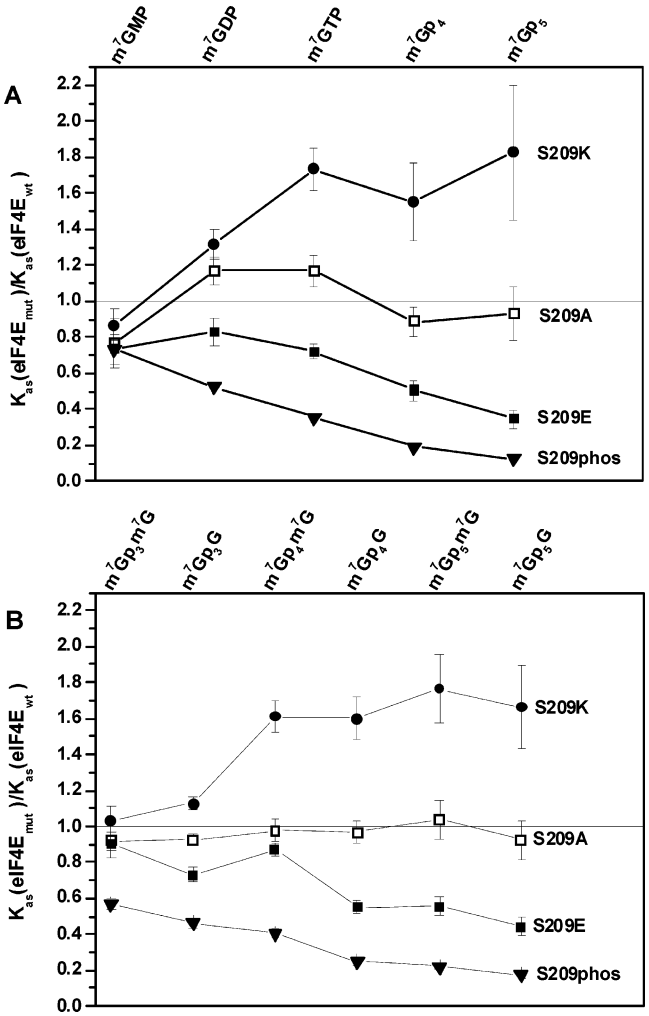

FIGURE 3: Effect of mutation at position 209 on the ability of eIF4E to bind (A) mononucleotide and (B) dinucleotide cap analogues. The ratios of K_{as}(eIF4E$_{mut}$) to K_{as}(eIF4E$_{wt}$) were determined from fluorescence titration measurements performed in 50 mM Hepes (pH 7.2), 100 mM KCl, 0.5 mM EDTA, and 1 mM DTT at 20 °C.

The K_{as} values for S209A are very similar to those of the wild-type protein (differences within an experimental error of ±20%). The introduction of a positive charge at position 209 of murine eIF4E by replacement of the serine with lysine causes an increase of the affinity of S209K for both mono- and dinucleotide cap analogues (Figure 3), depending on the length of the phosphate chain. The increase in K_{as} for m^{7}GMP, m^{7}GDP, m^{7}GpppG, and m^{7}Gpppm^{7}G is hardly noticeable (Table 2). For example, the K_{as} for the S209K−m^{7}GpppG complex is 14.1 ± 0.4 μM^{-1}, while the K_{as} for the eIF4Ewt−m^{7}GpppG complex is 12.5 ± 0.3 μM^{-1}. The cap analogues with longer phosphate chains (m^{7}GTP, m^{7}Gp$_4$, m^{7}Gp$_5$, m^{7}Gp$_4$G, and m^{7}Gp$_5$G) bind to S209K more efficiently, the appropriate values of K_{as} for the complexes with S209K being ~2-fold higher than those of unmodified eIF4E (Figure 3). The opposite effect was observed in the case of S209E. Introduction of one negative charge results in a systematic decrease in the binding affinity of the caps for S209E versus eIF4Ewt depending on the resultant charge of the ligand (Figure 3A,B). The strongest reduction was observed for m^{7}Gp$_5$, a K_{as} of 561 ± 53 μM^{-1}, for S209E versus a K_{as} of 1625 ± 195 μM^{-1} for eIF4Ewt.

The level of inhibition of the ability to bind the cap by eIF4E phosphorylated at Ser209, with two additional negative charges, is significantly greater than the reduction induced by replacement of Ser209 with glutamic acid, i.e., introduction of one additional negative charge. The pentaphosphate analogue m^{7}Gp$_5$ binds to the phosphorylated protein ~2.8-fold more weakly than to S209E, and 8-fold more weakly than to the unphosphorylated wild-type protein. It is worth noting that the replacement of threonine 205 with cysteine in the phosphorylated protein, necessary to obtain the latter by the IPL method, had no effect on the affinity for capped mRNAs. The binding affinities of the unphosphorylated eIF4E T205C, obtained by the IPL method, for the investigated cap analogues are very similar to those obtained for wild-type eIF4E (Table 2; see also ref *17*).

Phosphorylation of eIF4E and Mutation of Lysine 159 Similarly Reduce the Affinity of the Protein for the Cap but by Different Mechanisms. In eIF4E, Ser209 and Lys159 are located on the opposite sides of the trajectory of the mRNA chain (Figure 1). Examination of the crystal structure of the complex formed between the murine eIF4E(27−217) and m^{7}GDP led Marcotrigiano *et al.* (*19*) to propose that phosphorylated Ser209 and Lys159 might form a salt bridge to clamp the capped mRNA with eIF4E. On the other hand, more recent crystallographic studies of full-length human eIF4E in a complex with m^{7}GpppA showed that Ser209 is 12−19 Å from Lys159, possibly too far to enable formation of a salt bridge (*21*, *22*).

In this work, we tried to establish the character of the interaction of Lys159 with the capped mRNA chain and to verify the potential intermolecular contacts that can be created between phosphorylated Ser209 and Lys159. The results of fluorescence quenching measurements for the three modified eIF4Es, the one phosphorylated at Ser209 (S209phos), the one mutated at position 159 (K159A), and the one possessing both modifications (K159A/S209phos), are presented in Tables 2 and 3. The ability of both the K159A mutant and the phosphorylated eIF4E to bind the cap analogues is inhibited to different extents depending on the cap analogue. All investigated eIF4Es have very similar affinities for m^{7}GMP, whereas m^{7}GDP and m^{7}GpppG are bound ~2-fold more weakly by the phosphorylated protein in comparison with its unphosphorylated counterpart (Figure 4A). The change in the ability of the K159A protein to bind m^{7}GDP remains at the same level as for m^{7}GMP. The values of K_{as} for the complexes of m^{7}GTP with the K159A protein or with S209phos are very similar (K_{as} = 38.8 ± 2.3 and 42.6 ± 1.8 μM^{-1}, respectively). Introduction of one or two extra phosphates to m^{7}GTP (m^{7}Gp$_4$ and m^{7}Gp$_5$) causes a further reduction of the binding affinity in comparison with that of eIF4Ewt. However, this effect is stronger for eIF4E with mutated lysine. m^{7}Gp$_5$ binds to K159A ~14-fold more weakly than to the wild-type protein and only 2-fold more weakly in comparison with S209phos. It is remarkable that both m^{7}Gp$_4$ and m^{7}Gp$_5$ show a similar affinity for K159A, indicating that as opposed to the other mutants, the ϵ-phosphate does not contribute to the interactions between eIF4E and the capped mRNA if Lys159 is missing from the protein (Table 2). The energetic cost of the K159A mutation is 0.9 kcal/mol for the interaction with m^{7}Gp$_4$ and 1.5 kcal/mol for the interaction with m^{7}Gp$_5$. This suggests that Lys159 can form molecular contacts with the δ- and γ-phosphates of the cap phosphate chain.

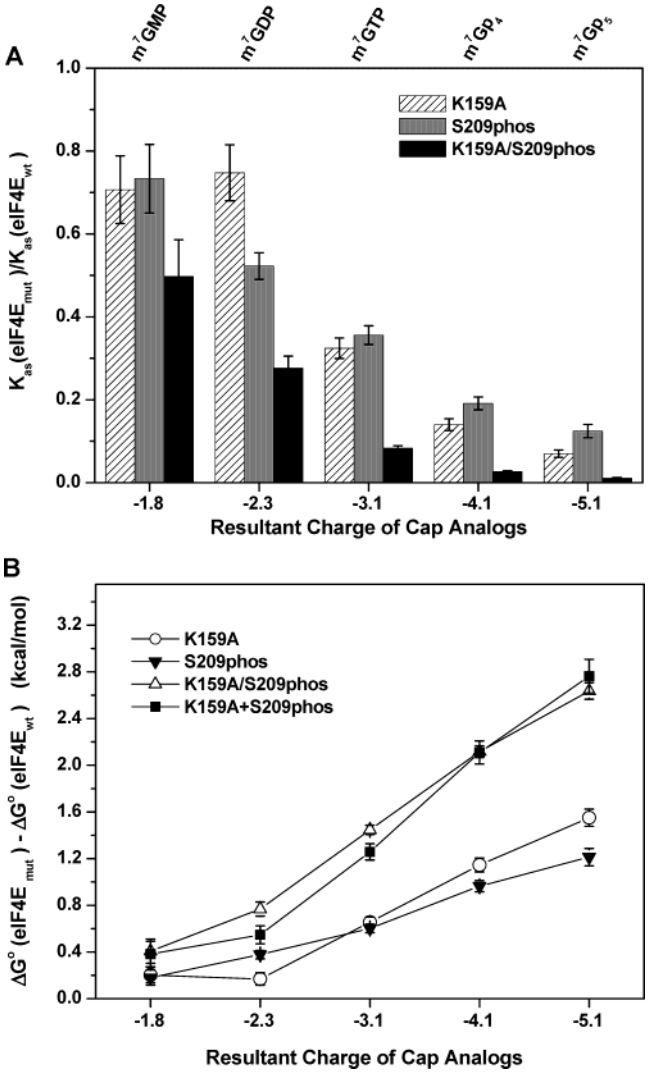

FIGURE 4: (A) Decrease in the affinity for eIF4E phosphorylated at Ser209 and mutated at Lys159 [K_{as}(eIF4E$_{mut}$)/K_{as}(eIF4E$_{wt}$)] vs the negative charge of cap analogues. The resultant charges of cap analogues at given pH values were estimated from experimental pK_{a1} values for dissociation of the N1 proton of 7-methylguanosine (*35*, *36*) and from the experimental pK_{a2} values for the dissociation of the second proton of the terminal phosphate group (*37*). (B) Changes in the standard Gibbs free energy ($\Delta\Delta G°$) on binding the mononucleotide cap analogues by modified eIF4E at 20 °C.

The double modification of eIF4E, phosphorylation of Ser209 and replacement of Lys159 with alanine, causes a further reduction of eIF4E binding affinity for the cap (Table 2 and Figure 4A). The decrease in K_{as} is more pronounced for the longer cap phosphate chain, e.g., ~12-fold for m^{7}GTP and ~100-fold for m^{7}Gp$_5$, and substantially exceeds the individual contribution from the K159A mutation and Ser209 phosphorylation. A comparison of standard Gibbs free energies of binding ($\Delta G°$) rather than of association constants reflects the effects of the structural modifications on the internuclear association (Table 3). The increase in $\Delta G°$ ($\Delta\Delta G°$) on the concomitant effects of Ser209 phosphorylation and K159A mutation ranges from 0.20 kcal/mol for m^{7}GMP to 2.64 kcal/mol for m^{7}Gp$_5$ (Figure 4B). The resulting $\Delta G°$ for each of the investigated cap analogues equals the sum of the $\Delta G°$ values for the single, separate modifications within experimental error. Contributions of the phosphorylation and removal of the positively charged lysine

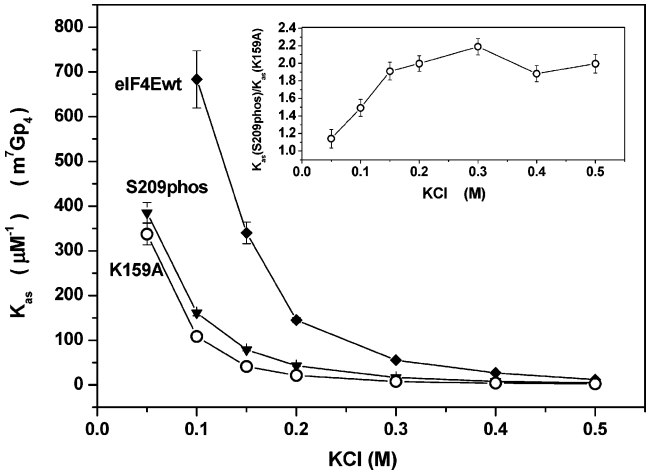

FIGURE 5: Efficiency of eIF4E−cap analogue binding vs K$^+$ concentration. The K_{as} values were determined for the complexes of eIF4Ewt, S209phos, and K159A with m^{7}Gp$_4$ at different KCl concentrations. The inset shows the changes in K_{as} ratios for phosphorylated and K159A eIF4Es vs KCl concentration.

are thus additive in destabilization of the protein−cap complexes.

The equilibrium binding constant depends on the environmental conditions, i.e., pH value of the solution, ionic strength, etc. To complete the information about the influence of the charge distribution due to protein mutation and/or phosphorylation on the character of the eIF4E−cap interaction, we examined the effect of salt on the affinity of eIF4E for the cap analogues. The representative dependence of K_{as} on KCl concentration for the complexes of m^{7}Gp$_4$ with eIF4Ewt, S209phos, and K159A is shown in Figure 5. With increasing KCl concentrations, the affinity of all three proteins for the cap analogues is reduced and the effect of phosphorylation is systematically canceled; e.g., at 0.1 M KCl, phosphorylated eIF4E binds m^{7}Gp$_4$ ~4-fold more weakly than the unphosphorylated protein does, while at 0.5 M KCl, the difference in K_{as} is only 2-fold. A similar effect was also observed for other cap analogues (not shown). The influence of KCl concentration on cap affinity for phosphorylated versus unphosphorylated eIF4Es is further evidence that phosphorylation of eIF4E decreases its binding affinity for the cap through electrostatic repulsion. Moreover, comparison of the dependence of K_{as} on KCl concentration in the case of K159A and S209phos shows some differences in the character of the interaction of both proteins with cap analogues (Figure 5). For example, at 50 mM KCl, the K_{as} values of the S209phos−m^{7}Gp$_4$ and K159A−m^{7}Gp$_4$ complexes are very similar (384.99 ± 22.74 and 337.18 ± 23.75 μM^{-1}, respectively). With increasing salt concentrations, differences appear; e.g., at 0.5 M KCl, the K159A mutant binds m^{7}Gp$_4$ ~2-fold more weakly than the phosphorylated protein (K_{as} = 4.93 ± 0.11 and 2.47 ± 0.12 μM^{-1}, respectively). At lower ionic strengths, the negative charge of the phosphoserine is more weakly screened by the ions, resulting in the inhibition of the ability of the protein to bind the cap compared with higher salt concentrations. In the case of the K159A mutant, the observed dependence of K_{as} on the ionic strength in comparison with S209phos as well as the wild-type protein shows that the positive charge of Lys159 not only contributes to unspecific electrostatic attraction between eIF4E and the capped mRNA but also

plays a part in the formation of more specific intermolecular contact(s) between the protein and capped mRNA. However, the values of the equilibrium association constant for the complexes of the K159A mutant and of the phosphorylated protein with cap analogues are very similar at the physiological concentration of K^+.

DISCUSSION

The results obtained with the mutants of eIF4E and the phosphorylated protein clearly show that electrostatic repulsion is the main factor that reduces the binding affinity of the phosphorylated protein for capped mRNAs. Our conclusion was drawn from the binding of a series of cap analogues with increasing negative charge to three eIF4E mutants possessing a positive, negative, and neutral charge at position 209, as well as to the phosphorylated protein (two negative charges). First, the replacement of Ser209 with the neutral amino acid, alanine, does not affect the affinity of eIF4E for the cap analogues. This suggests that the hydroxyl group of serine does not take part in intermolecular contacts, and hence, phosphorylation of serine 209 cannot rupture such a contact. The replacement of Ser209 with lysine raises the positive charge localized at the entry of the eIF4E cap-binding slot (Arg112, Arg157, Lys159, Lys162, and Lys206), leading to an increase in the binding affinity for the cap. Since a notable increase in the binding affinity has been observed for m^7GTP and for the cap analogues with longer phosphate chains, formation of a hydrogen bond or a salt bridge between the lysine and one of the cap phosphate groups cannot be ruled out. Previous studies of Scheper *et al*. (*16*) showed that either Asp or Glu poorly mimics the phosphorylated state of eIF4E, and no change in the binding of m^7GTP to these mutants was observed. On the contrary, genetic studies showed that the nonphosphorylatable mutant of *Drosophila* eIF4E, Ser211Asp, mimics the phosphorylated state of eIF4E (*13*). We have chosen glutamic acid that possesses a longer side chain than aspartic acid and can therefore better mimic phosphoserine. Replacement of serine with glutamic acid results in a systematic decrease in the K_{as} for the cap analogues with increasing negative charge. However, this effect is weaker than that for the phosphorylated protein. The pK_a value of free glutamic acid is 4.3, and the pK_{a2} of phosphoserine exposed to the solvent is ~5.9−6.1 (*38*, *39*). Hence, at pH 7.2 most eIF4E phosphoserines adopt the dianionic form. A double negative charge of the phosphoryl group causes stronger electrostatic repulsion between the cap and phosphoserine than in case of the single negative charge of the glutamic acid carboxyl group. The distances in the human eIF4E−m^7GpppA complex are 6.98 Å between Ser209 C^α and N1 of the adenine base and ~12−13 Å between Ser209 C^α and the γ-phosphate (*22*), sufficiently close for strong electrostatic repulsion between the protein and the negatively charged ligand. Moreover, using molecular dynamics simulations of phosphorylated eIF4E, Tomoo *et al.* (*22*) showed that the transition of phosphoserine from the monoanionic to the dianionic form closes the entrance to the cap-biding slot, shortening the distance between Ser209 and Lys159 from 19 to 10 Å. These data are in agreement with our results which show that glutamic acid at position 209 impairs the binding affinity of eIF4E for the capped mRNA less than phosphoserine.

Another important issue in the role of phosphorylated eIF4E is the hypothesis of a salt bridge between the phosphate group of Ser209 and the ϵ-amino group of Lys159, clamping the bound mRNA (*19*, *24*). The aforementioned distances between these two amino acids contradict this hypothesis. On the other hand, our data show that Lys159 is very important for interactions between the capped mRNA and eIF4E. Mutation to a neutral amino acid inhibits ~3-fold the ability of eIF4E to bind m^7GTP as compared to the wild-type protein. Lys159 probably forms a hydrogen bond with the δ-phosphate group of the cap analogues. Our data for the K159A/S209phos mutant show that the presence of Lys159 in the phosphorylated protein does not significantly enhance the affinity of phosphorylated eIF4E for the cap. Lower K_{as} values of K159A/S209phos−cap complexes in comparison with those of S209phos−cap and K159A−cap complexes seem to result from the additive effect of these two modifications. The above observations together with an analysis of the structural data (*20*−*22*) suggest that the clamping model (*17*, *24*) is problematic. The significant participation of lysine in binding of capped mRNA also disproves our previous assumption (*17*) in that the phosphate group at Ser209 pushes the cap phosphate chain toward Lys159 and the latter acts like a positively charged molecular "angle" to pull the negatively charged triphosphate chain of the capped mRNA out of the protein, thus facilitating dissociation of the eIF4E−cap complex. These findings showing that phosphorylation of eIF4E destabilizes the interactions between eIF4E and capped mRNA by electrostatic repulsion are far from clarifying the biological role of phosphorylated eIF4E. The electrostatic interactions linked to the eIF4E modifications discussed here at the step of complex formation obscure the role of phosphorylated Ser209 and Lys159 in the eIF4E−cap interaction within the complex in which phosphorylation occurs. Moreover, electrostatics is not a unique factor in determination of the affinity of the cap for eIF4E. Molecular dynamics simulations of Tomoo *et al.* (*22*) showed an important role for conformational changes of eIF4E in binding the cap, e.g., stabilization of the protein in the cap-binding pocket area. In particular, the eIF4E phosphorylation was postulated to stabilize the complex via narrowing of the entrance width in the case of the dianionic state of the eIF4E phosphate group. Two of three hydrogen bonds, which keep 7-methylguanine in the eIF4E-binding slot, are highly dynamic. They were shown to be periodically broken and re-formed in the course of the MD simulations. Hence, the dynamic aspects of the eIF4E−cap complexes should be taken into account in formulating a mechanical model of the regulation of eIF4E by phosphorylation. Reliable measurements of the dissociation rates of the complexes with the K159A mutated and/or phosphorylated eIF4E seem to be the first crucial step in building such a model. The data gathered by Scheper *et al.* (*16*), who took advantage of phosphorylated eIF4E and the K159M mutant of eIF4E, are still incomplete in this respect. Two models of the role of the eIF4E phosphorylation based on those results (*16*), i.e., facilitating the release of tethered eIF4F from mRNA that leads to enhanced unwinding/ribosome migration, or enhancing the release of initiation factors that allow other (untranslated) RNAs to become translated, need further supporting or excluding solid data.

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