Quantitative Analysis of Influenza Virus RNP Interaction with RNA Cap Structures and Comparison to Human Cap Binding Protein eIF4E

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ABSTRACT: Influenza virus polymerase uses capped RNA primers for transcription initiation in infected cells. This unique mechanism involves the specific binding of the polymerase to capped mRNA precursors in the nucleus of infected cells. These host RNAs are then cleaved by a polymerase associated endonuclease at a position 10−15 nucleotides downstream of the cap structure. The resulting capped RNA oligonucleotides function as primers for transcription initiation. The viral cap binding site has previously been mapped to the PB2 subunit of the trimeric influenza polymerase complex. We have established a quantitative assay system for the analysis of cap interaction with PB2 as part of the native, viral ribonucleoprotein complex (RNP) using a specific UV cross-linking approach. Cap binding was not affected by the RNase pretreatment of the capped RNA substrate and cap binding was not inhibited by excess uncapped RNA, indicating that under the assay conditions, the majority of the binding energy was contributed by the interaction with the cap structure. Binding to 7-methyl-GTP was found to involve synergistic interaction with 7-methyl guanosine and triphosphate binding subsites. A similar mode of interaction with 7-methyl-GTP was found for human cap binding protein eIF4E. However, the potency of 7-methyl-GTP for cap binding inhibition was 200-fold stronger with eIF4E and had a higher contribution from the triphosphate moiety as compared to influenza RNP. Due to this difference in cap subsite interaction, it was possible to identify novel cap analogues, which selectively interact with influenza virus, but not human cap binding protein.

The formation of functional influenza virus mRNA molecules in infected cells requires the addition of cap structures to the mRNA 5′-ends. Influenza virus uses a unique pathway to generate capped, viral mRNA molecules. The viral RNA polymerase binds to cellular, capped RNA molecules in the nucleus of infected cells and a polymerase associated endonuclease activity cleaves off capped RNA oligonucleotides of 9−15 nt length. These capped oligonucleotides are then used as primers for the initiation of viral transcription. The viral mRNA molecules therefore carry a cap structure and a short, host derived sequence at their 5′ ends, ensuring efficient nuclear export, translation initiation, and viral protein production in the cytoplasm (*1*).

This endonuclease-mediated mRNA capping mechanism is exclusively used by viruses from the Orthomyxoviridae and Bunyaviridae families, which include a number of important human and animal pathogens. This viral mechanism therefore constitutes an interesting target for antiviral therapy. Selective inhibitors of influenza virus endonuclease and polymerase have been described previously (*2*, *3*). However, no specific inhibitors of influenza virus cap binding have been reported so far.

The influenza virus polymerase carries the enzymatic functions to produce capped, viral RNA. It consists of three polypeptide subunits, PA, PB1, and PB2. A specific cap binding site has been identified on the PB2 subunit (*4*−*7*), whereas the PB1 subunit carries both a metal-dependent endonuclease and a RNA-dependent RNA polymerase active site (*7*−*9*). Previously, crystal structures from two unrelated cap binding proteins have been determined in complex with cap structure analogues (*10*−*13*). VP39 is a Vaccinia virus encoded cap binding protein, which also carries a methyltransferase enzyme active site. Eukaryotic cap binding protein eIF4E is part of the translation initiation complex eIF4F and initiates ribosome assembly at mRNA 5′-ends. Although phylogenetically and functionally unrelated, these two cap binding proteins show significant structural similarities in the architecture of their cap binding sites. In particular, the 7-methyl guanine moiety of the cap structure is bound between two aromatic side chains of the protein in a parallel stacked structure. In addition, essential amino acids are involved in hydrogen bond formation to the guanine N1 and N2 functional groups. A weak sequence similarity between influenza cap binding protein PB2 and eukaryotic cap binding protein eIF4E has been pointed out previously and peptides from this region were identified after cross-linking of PB2 to cap labeled RNA (*6*, *7*, *14*). It is therefore conceivable that cap binding to PB2 involves similar interactions, in particular stacking of 7-methyl-guanine between two aromatic side chains from PB2.

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We have established a quantitative assay system to further elucidate PB2 binding to eukaryotic cap structures. Using a range of cap structure analogues, we demonstrate strong synergistic requirement of both the electron-deficient aromatic ring system and the phosphate groups on the natural cap structure. In an effort to characterize influenza PB2 specific features of the cap binding site, we identified a novel compound, which binds to PB2 more strongly than 7-methyl-GTP, even in the absence of a negatively charged component. Consistent with the higher importance of negative charges for eIF4E binding, this new compound does not inhibit cap binding of human eIF4E, and therefore represents the first example of a PB2 specific cap binding inhibitor.

EXPERIMENTAL PROCEDURES

Materials. Influenza virus A/PR/8/34 ribonucleoprotein (RNP) was prepared from purified influenza virus particles on glycerol gradients as described (*15*). The RNP fraction was concentrated by centrifugation through 30% glycerol in 10 mM Tris-HCl, pH 8, 50 mM NaCl, 5 mM β-mercaptoethanol onto a 60% glycerol cushion in the same buffer. RNP concentration was determined by OD 260 measurement as described (*15*).

The coding sequence for human eIF4E was amplified by PCR from human liver cDNA and inserted into BamHI/Eco RI cleaved vector plasmid pGEX4T-3, generating plasmid pGEX4T-3HueIF4E. The coding sequence was verified by sequence analysis of pGEX4T-3HueIF4E. Human eIF4E protein was expressed in *Escherichia coli* BL21-CodonPlus(DE3)-RIL cells (Stratagene), after induction with 0.5 mM IPTG for 4 h at 30 °C. Cells were disrupted by sonication and crude extract applied to a 7-methyl-GDP column (Amersham-Pharmacia) equilibrated in buffer A (20 mM HEPES, pH 7.5, 100 mM KCl, 0.2 mM EDTA, 1 mM DTT, 10% glycerol). Bound protein was eluted by increasing the KCl concentration to 2 M. The eluted protein was dialyzed against buffer A and stored at −80 °C. Protein concentration was determined by Bradford analysis using BSA as standard.

Capped RNA oligonucleotides were chemically synthesized and ^{32}P-labeled within the cap structure as described (*15*). Briefly, the CPG-bound oligoribonucleotide was 5′-triphosphorylated using 2-chloro-4-H-1,3,2-benzodioxaphosphorin-4-one and bis-(tri-*n*-butylammonium)-pyrophosphate followed by iodine oxidation. The 5′-triphosphorylated RNA was then capped, methylated, and radioactively labeled in a reaction using vaccinia virus capping enzyme, guanylyltransferase (Life Technologies). The sequences used were G11, 5′-m7GpppGmGAAUACUCAAG-3′ and G20, 5′-m7GpppGmGAAUACUCAAGCUAUGCAUC-3′. G11 RNA was also generated with a cap 0 structure, m7GpppGGAAUACUCAAG. To differentiate between the two types of capped G11 RNA, G11 containing a 2′-methoxy group is also referred to as G11M, G11 containing a 2′-hydroxy group is also referred to as G11H.

RO0794238 was prepared by benzylation of 9-(4-hydroxybutyl)guanine with benzyl bromide in DMSO following previously published procedures (*16*).

Cap Binding UV Cross-Linking Assay. In the standard assay 1−5 nM cap labeled RNA (specific activity, 10 000−20 000 cpm/fmol) was incubated for 5 min at room temperature with a final concentration of 0.4 mg/mL RNase A in 125 mM Tris-HCl, pH 8, 50 mM KCl, 0.2 mg/mL *E. coli* tRNA (Sigma) in a total volume of 5 μL. These incubation conditions were sufficient for complete digestion of RNA. The cap binding reaction was initiated by adding 10 μL of 20% DMSO and 475 ng of influenza RNP (4.24 nM) or 88 pg of human eIF4E (3.52 nM). The binding reaction was incubated at room temperature for 10 min to equilibrate, and then transferred to ice and cross-linked by UV irradiation at 254 nm for 10 min at 2−5 cm from the light source of a Stratalinker UV Cross-linker (Stratagene). The cross-linked proteins were denatured by addition of 8 μL of 4× SDS sample buffer (200 mM Tris-HCl, pH 7.5, 8% SDS, 20% glycerol, 3 M β-mercaptoethanol) and heating to 98 °C for 3 min, then analyzed by gel electrophoresis on 10% bis tris NuPAGE gels (Invitrogen). Gels were fixed in a solution of 10:10:80 ethanol/acetic acid/water, dried, and exposed in a phosphor imager cassette. Radioactive bands were quantified using the Storm phosphor imaging system and ImageQuant 5.1 software (Amersham Pharmacia). Data analysis was performed using Excel 97 and SigmaPlot 5.0 software.

RESULTS

This study was designed to establish a system for the quantitative evaluation of influenza virus PB2 interaction with capped RNA and cap analogues. It should then allow the assessment of cap binding inhibition by potential competitive inhibitors of PB2-cap interaction. The system was validated by parallel analysis of the cap binding activity of human translation initiation factor eIF4E, because details of cap interaction by this protein have been reported previously by several groups using a number of different biochemical techniques. Influenza virus RNP contain the cap binding protein PB2 in a functional complex with the associated polymerase subunits and the 5′- and 3′-ends of the viral RNA, which are modulating cofactors of cap binding, endonuclease and polymerase activities (*1*).

Previously, we have optimized the yield of purified RNP from influenza virus particles (*15*). Further concentration of RNP, as required in this study, was achieved by an additional centrifugation onto a glycerol cushion as described in Experimental Procedures. Figure 1 shows the protein content of the concentrated RNP samples obtained by this procedure, as well as the preparation of affinity purified, recombinant eIF4E used in this study.

The RNP complex contains a majority fraction of RNA binding nucleoprotein (NP), and one set of polymerase subunits (PB1, PB2, PA) per genomic RNA segment, all of which are visible on the Coomassie stained gel. Small amounts of M1 protein aggregates tend to comigrate with RNP fractions on glycerol gradients. M1-free RNP fractions can be obtained by careful selection of gradient fractions (Figure 1, right panel). In addition, glycerol gradient RNP comigrating M1 aggregates have been shown previously not to interfere with polymerase function (*15*).

Specific Cap Interaction of PB2 and eIF4e. Figure 2 shows a flowchart of the cap binding assay, which involved the equilibration of cap binding protein with either intact, cap-labeled 20-mer RNA oligonucleotide (G20), or labeled cap structure, obtained by complete RNAse A digestion of G20. The extent of cap binding was then measured by cross-

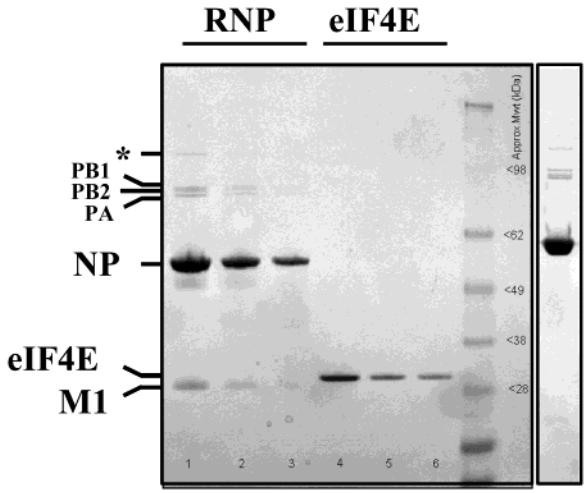

FIGURE 1: Purification of influenza RNP and human eIF4E proteins. Proteins were purified as described in Experimental Procedures and analyzed by Coomassie staining on denaturing acrylamide gels. Lanes 1−3 show a serial dilution of RNP. The protein components are indicated on the left and consist of PB1, PB2, PA (polymerase subunits), and NP (nucleoprotein), as previously determined using mass spectroscopy and peptide mapping (*15*). A small amount of M1 protein copurifies with RNP, but can be removed by careful gradient sampling (right panel). Lanes 4−6 show a serial 2-fold dilution of human eIF4E. The star on the left panel indicates an additional band of NP dimer, which has previously been identified by peptide analysis (*11*).

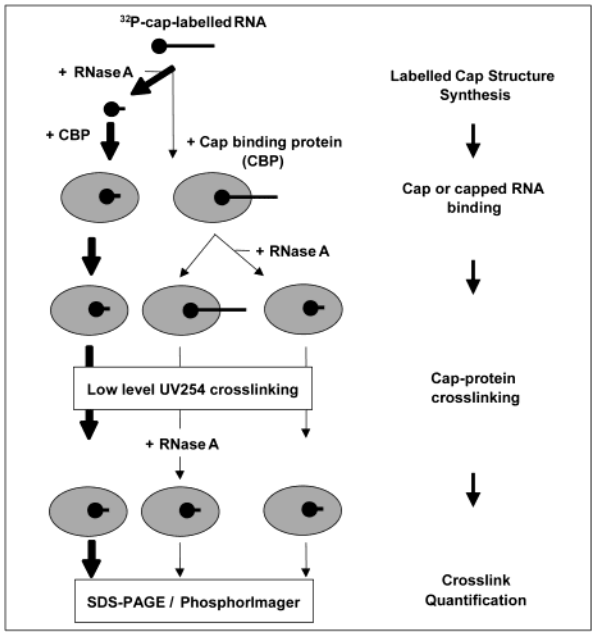

FIGURE 2: Cap binding assay flow scheme. Bold arrows show the standard assay scheme used in cap binding inhibition experiments. From top to bottom, cap-labeled RNA was either preincubated with RNase A to complete digestion before incubation with cap binding protein, or was incubated directly with cap binding protein (first level). Protein incubated with full-length, capped RNA could then be incubated with RNase A after binding (second level), or directly progressed to UV irradiation. Cap-bound protein was then irradiated with UV light to introduce low concentration covalent protein−cap cross-links (third level). Not previously RNAse A treated complexes were RNAse A treated before loading onto SDS polyacrylamide gels for protein analysis (fourth level). Protein gels were fixed by staining with Coomassie Blue, dried, and exposed to phosphorimager screens for band analysis.

linking the labeled cap structures or the cap-labeled RNA to the cap binding protein, using UV irradiation at 254 nm wavelength. The cross-linked protein samples were digested with RNaseA and analyzed by denaturing protein gel electrophoresis and phosphor imager quantification. The left column of the flow scheme indicated by bold arrows represents the standard cap binding assay used in this work, starting with labeled cap structure binding to cap binding protein.

Preliminary experiments were performed to determine the optimal UV irradiation dose, protein and RNA concentration, to obtain statistically low cross-linking efficiency and direct correlation with protein and RNA concentration. Figure 3a demonstrates the specificity of cap cross-linking in this system using influenza RNP. The protein concentrations within the linear range of the binding assay were too low for the influenza polymerase subunits to be visible on Coomassie stained gels. To visualize the relative size of cross-linked proteins, additional RNP protein was added to the first lane of the Coomassie stained panel in Figure 3a after the cross-linking reaction. The analysis of the autoradiograph of RNP cross-linked to cap showed a single influenza polymerase band bound to the cap structure, as expected from previous identification of PB2 as the influenza cap binding protein (Figure 3a, left panel).

The assay was specific for cap binding, because uncapped RNA did not bind to PB2 (Figure 3a, lane 3). The influenza NP protein was not cross-linked to the cap, although it constitutes the major protein in the RNP preparation and has RNA binding properties. However, a second unidentified band was cross-linked to a lesser extent under the incubation conditions and migrated at a molecular weight position consistent with a PB2 dimer (PB2:PB2). A distinctive protein band of NP dimer could also be observed on the Coomassie stained gel, but was not cross-linked to cap. The NP dimer remained intact on denaturing acrylamide gels without UV irradiation and has been observed before. This suggested that a small amount of RNP dimers might be present in the RNP preparation.

Figure 3b shows the cross-linking of cap to eIF4E under the same conditions. Additional protein was added to lane 1 before loading to visualize the protein by coomassie staining. As expected, uncapped RNA did not cross-link to eIF4E (Figure 3b, lane 3). The cross-linked eIF4E band migrates slightly higher, due to the acquisition of additional mass after cross-linking of cap.

The cap specificity of the assay was further tested by analysis of competition with capped and uncapped RNA molecules. Figure 3c shows that cap binding by PB2 could be inhibited by increasing concentrations of capped RNA added to the binding reaction, but not by the addition of uncapped RNA. Interestingly, the presence of a 2′-methoxy group on the second cap nucleotide (cap I structure) appeared to contribute to slightly increased binding affinity to PB2 as compared to singly methylated cap structure (cap O structure) (Figure 3c, compare G11M with G11H inhibitory potencies).

Cap Binding of eIF4e Measured by Quantitative UV Cross-Linking. Cap binding was determined under conditions of increasing concentrations of eIF4E. The binding curve obtained was consistent with a single cap binding site on eIF4E and an apparent K_d value of 132 nM (Figure 4a).

This value is very similar to literature values reported for eIF4E interaction with cap using fluorescence quenching or surface plasmon resonance (SPR) technologies (*17*−*20*). To

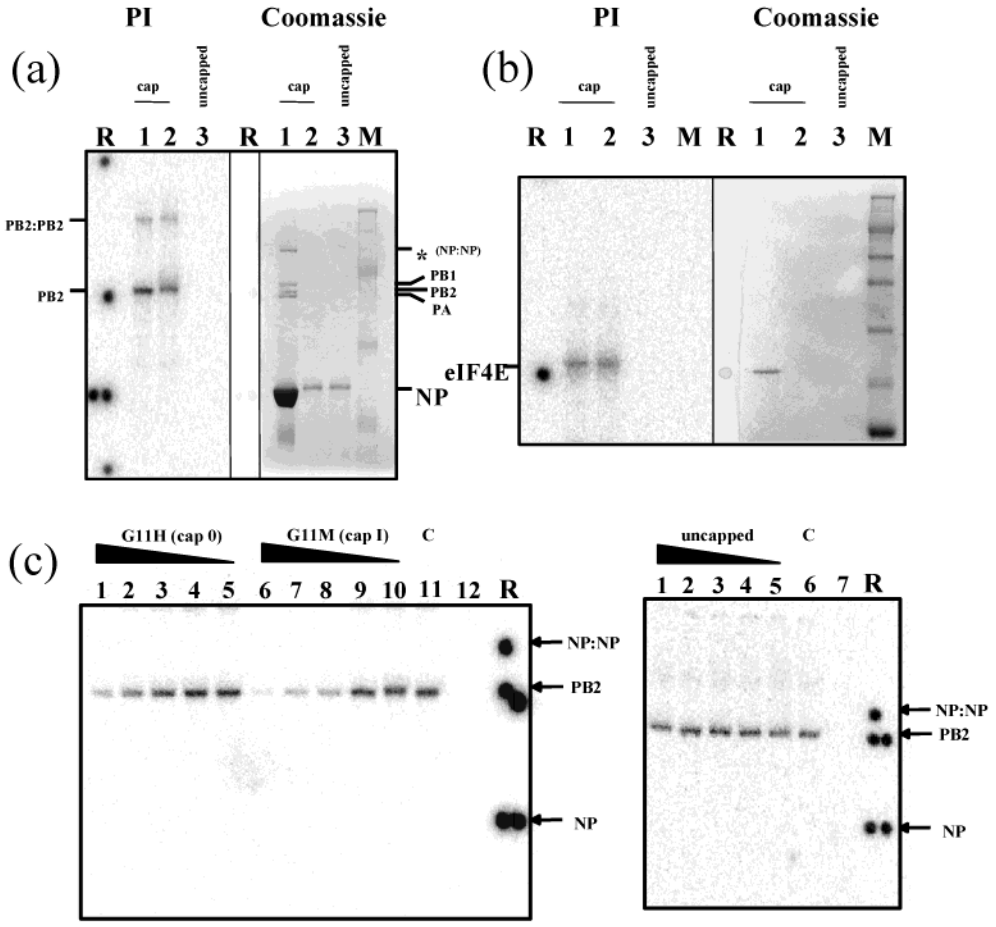

FIGURE 3: The UV cross-linking assay is specific for capped RNA. Covalently labeled proteins were identified by phosphorimager analysis of SDS−PAGE gels. Cross-linking reactions were performed with capped RNA (lanes 1 and 2) or uncapped RNA (lane 3). Bands identified on Coomassie stained gels were marked with radioactive dots as size standards (a) Cap cross-linking to influenza RNP covalently labels one polymerase subunit, PB2. Additional RNP protein was added to lane 1 before electrophoresis to identify the RNP protein components. The star on the right panel indicates an additional band of NP dimer, which has previously been identified by peptide analysis (*11*). (b) Cap cross-linking to human eIF4E. Additional protein was added to lane 1 before gel electrophoresis to identify the position of the protein by Coomassie staining before phosphorimager analysis. (c) Cap cross-linking to PB2 was performed in the presence of increasing amounts of capped RNA (left panel), or uncapped RNA (right panel). Size marker dots are shown on the right of the gels. C, control reactions performed in the absence of competitor RNA. Left panel, lanes 1−5, and 6−10 contained 360, 100, 50, 10, and 0 μM competitor RNA, respectively. Lane 12 contained a control cross-linking reaction performed with uncapped RNA. Right panel, lanes 1−5 contained 1000, 100, 10, 1, and 0.1 μM competitor RNA, respectively. Lane 7 contained a control cross-linking reaction performed with uncapped RNA.

further evaluate the cap binding assay, we measured the binding of other cap analogues to eIF4E as competitive inhibitors of labeled cap cross-linking. Figure 4b shows the effect of increasing concentrations of 7-methyl-GTP, 7-methyl-GDP, 7-methyl-GMP, and guanosine on the binding of labeled cap structure. The IC_{50} values determined from these dose response curves are consistent with an important role of cap structure phosphates for cap binding to human eIF4E protein (Table 1), as has been described previously (*19*). These results confirmed the suitability of the assay for the quantitative analysis of cap binding activity.

Cap Binding of Influenza Virus PB2 Protein Measured by Quantitative UV Cross-Linking. The cap binding assay also showed a protein-dependent increase in cross-linking efficiency with influenza RNP (Figure 5a). Interestingly, similar cap binding curves were obtained when cap labeled 20-mer RNA, cap labeled 11-mer RNA or completely RNase A digested, cap labeled RNA was used in the UV cross-linking reaction. RNase A treatment either before RNP binding, after RNP binding or after UV cross-linking gave identical results, indicating that under the reaction conditions, the assay was measuring cap interaction without interference from RNA binding. The assay also showed dose dependent, competitive inhibition by cap analogues (Figure 5b). 7-Methyl-GTP was the strongest inhibitor among the natural cap analogues and slightly more potent than m7GpppG (Table 1).

Design of a Influenza PB2 Selective Cap Binding Inhibitor. Preliminary structure−activity relationship analysis of cap analogue competitive inhibitors showed a strong contribution of phosphate groups on cap binding affinity to PB2. However, the relative loss of affinity due to the removal of phosphate groups on cap analogues was significantly lower for PB2 as compared to human eIF4E, suggesting that the contribution of phosphate interaction to cap binding affinity was significantly higher for eIF4E as compared to PB2. Consistent with this, pyrophosphate or triphosphate did not interfere with cap binding to PB2 at concentrations up to 4 mM, whereas significant inhibition was observed with eIF4E (Table 1).

It was also interesting to note that the loss of positive charge on the guanine moiety had a relatively minor effect

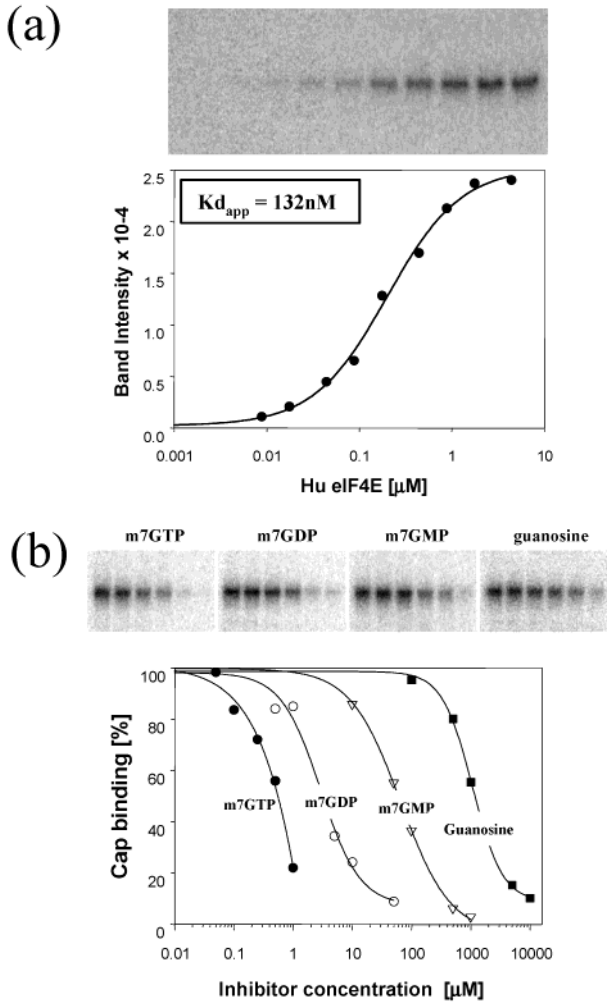

FIGURE 4: Quantitative analysis of cap binding to eIF4E and inhibition of cap binding by cap analogues. (a) Increasing concentrations of eIF4E were cross-linked to labeled cap structure as described in experimental procedures. The resulting dose response curve was fitted to a standard, single site quadratic binding equation to determine the apparent K_d value. (b) 5 nM labeled cap structure were cross-linked to 88 pg of eIF4E in the presence of increasing concentrations of cap analogues as indicated. IC_{50} values were determined from hyperbolic dose response curves fitted to the band intensity data from phosphorimager analysis. The calculated IC_{50} values are shown in Table 1.

on PB2 cap binding affinity, with an approximate 5-fold drop in binding affinity observed between m7GTP and GTP. In addition, the substitution of the ribose with an acyclic moiety on the guanosine backbone increased binding affinity to PB2 about 2-fold (Table 1). These results suggested a clear strategy for the design of PB2 selective cap binding inhibitors. The absence of negative charge would increase selectivity of compounds with regards to eIF4E, and the replacement of the ribose with an acyclic structure might increase both potency and selectivity toward influenza PB2 interaction.

On the basis of these observations, compound RO0794238 was designed from a guanosine backbone containing an aliphatic ribose replacement and no negative charges (Figure 6). RO0794238 selectively prevented cap binding to influenza PB2 as compared to human eIF4E. In addition, PB2 cap binding inhibition was increased relative to m7GTP, indicating that negative charges were not required to achieve high affinity interaction with PB2.

Table 1: Inhibition of Cap Binding by Cap Analogues

compound	eIF4E IC_{50} [μM]	relative loss of affinity compared to m7GTP	RNP IC_{50} [μM]	relative loss of affinity compared to m7GTP
guanosine	1550[a]	3100	2205	19.5
m7Guanosine	>1000	>2000	2176	19.3
m7GMP	26	52	1700	15
m7GDP	3.5	7	400	3.5
m7GTP	0.5	1	113	1
m7GpppG	0.8	1.6	127	1.1
PPi			>5000	
GTP			550	
acycloguanosine	>1000		1025	
tripolyphosphate	400		>4000	
RO0794238	>1000		45	

[a] IC_{50} values were determined from dose response curves as described in Experimental Procedures and constitute the concentration of compound required to reduce the cross linked band volume by 50%.

DISCUSSION

The cap-dependent endonuclease function is a unique feature of influenza virus replication, which is essential for viral replication and potentially useful as a target for antiviral therapy. Cap binding to the viral PB2 protein constitutes the first step in viral mRNA synthesis. Recent structural and functional studies on two unrelated cap binding proteins, the translation initiation factor eIF4E and vaccinia virus cap methyl transferase protein VP39, have demonstrated a number of conserved principles of protein cap interaction, which might apply more generally to cap binding proteins. High levels of structural similarity might be expected to decrease the probability of success for the design of highly selective viral cap binding inhibitors. However, very little is known about the cap binding site structure of influenza PB2 and limited comparative data exist with regards to structural requirements of cap interaction by PB2. This study was designed to compare cap binding structure activity relationships between human cap binding protein eIF4E and influenza PB2, a component of the viral ribonucleoprotein, RNP.

First we devised an assay system that was adaptable for use with a variety of cap binding proteins in comparative studies. The UV cross-linking principle has been successfully used before in qualitative cap binding assays with different proteins, but in this study we established the use of this assay type in a quantitative format to measure cap interaction by human eIF4E and influenza RNP. In particular, with this assay format we were able to overcome the physicochemical restrictions imposed by the nature and stability of a large protein complex like RNP (average molecular weight = 5600 kDa). The assay format should therefore be suitable for the study of other multiprotein complexes without major modifications. Cap binding by RNP and eIF4E as measured by this assay was highly cap specific, as shown by the absence of binding to uncapped RNA, the absence of competition by uncapped RNA, competition by capped RNA and cap analogues and similar binding curves with capped RNA and cap structures obtained from complete RNaseA digestion of capped RNA. High cap specificity was also apparent from the fact that only one of the polymerase subunits was linked to the cap structure on the RNP complex, and nucleoprotein (NP) was not labeled, despite being the major protein of the

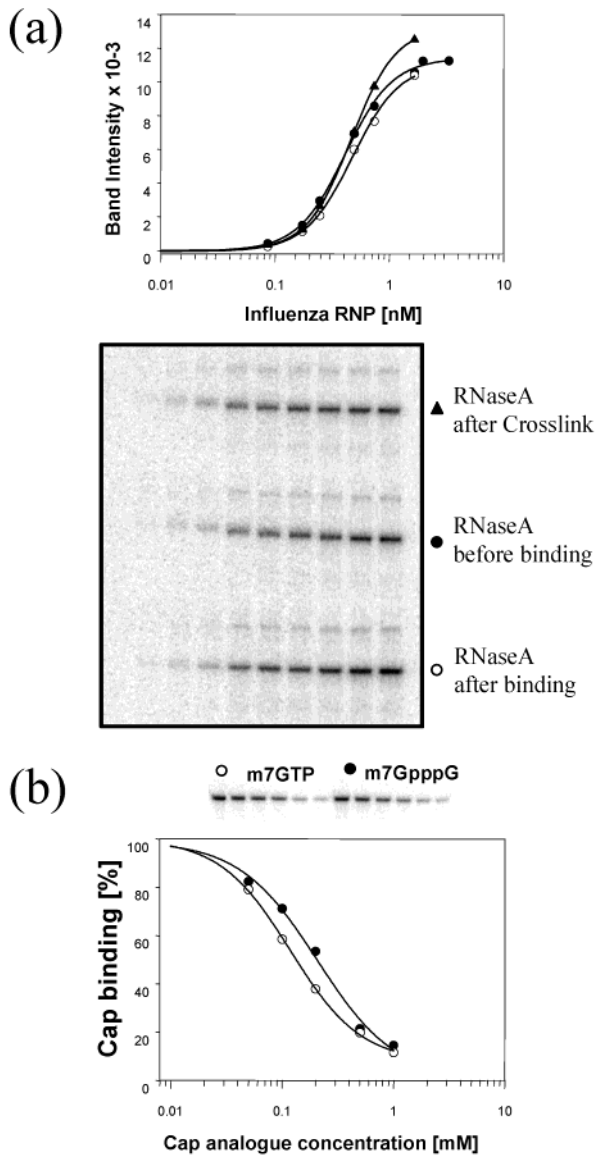

FIGURE 5: Quantitative analysis of cap binding to influenza RNP and inhibition of cap binding by cap analogues. (a) Increasing concentrations of RNP were cross-linked to labeled cap under different conditions as indicated. Identical dose response curves were obtained independent of the stage of RNase A treatment of the capped RNA. (b) 5 nM labeled cap structure were cross-linked to 475 ng RNP in the presence of increasing concentrations of cap analogues as indicated. IC_{50} values were determined from gels showing reduced PB2 cross-link band intensity and hyperbolic dose response curves fitted to the band intensity data from phosphorimager analysis (shown in Table 1).

complex. The fact that cap binding by eIF4E in this assay was consistent with a 1:1 stoichiometry interaction and the similarity of the apparent K_d value obtained to values previously observed with other assay formats demonstrated the suitability of this assay format for quantitative cap binding studies. This was further confirmed by the analysis of cap binding inhibition by cap analogues. The characteristic increase in affinity of eIF4E for cap analogues, m7GMP ≪ m7GDP < m7GTP, correlated with the increasing number of phosphate residues on these compounds, as has been described before (*18*, *19*, *21*).

Cap binding to PB2 was also found to be inhibited by cap analogues, and the apparent affinity increased with

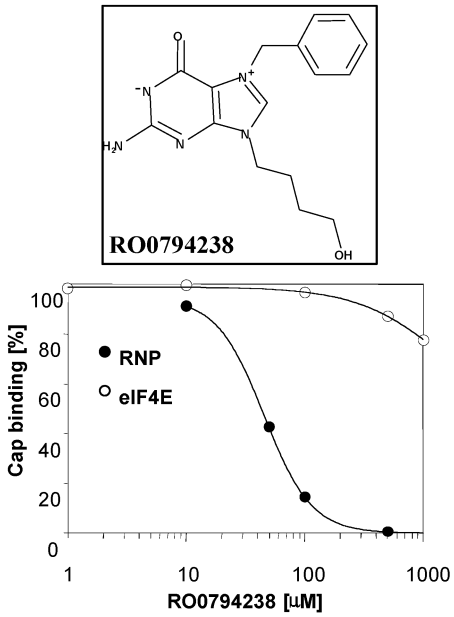

FIGURE 6: Selective inhibition of cap binding by RO0794238. The graph shows the analysis of reduced cap-cross-linked band intensities in the presence of increasing concentrations of RO0794238 under the same conditions as in Figures 4 and 5. Black circles, influenza RNP; white circles, eIF4E.

increasing numbers of phosphate groups, m7GMP ≪ m7GDP < m7GTP. A notable difference was that m7GTP was significantly less potent as a cap binding inhibitor with PB2, as compared to eIF4E, and 200-fold higher concentrations were required to reach half-maximal inhibition of cap interaction. 7-Methyl-guanosine was only a very weak inhibitor of cap binding, as was triphosphate in isolated form, using either PB2 or eIF4E. This suggested a strong synergistic contribution of two separate cap binding sub-pockets interacting with the m7G and the triphosphate moieties, respectively, consistent with structural information for eIF4E and VP39 (*10*−*13*).

Interestingly, the contribution of triphosphate to the overall binding affinity of m7GTP to PB2 appeared to be significantly lower than for eIF4E. The relative loss of affinity upon phosphate removal was lower for PB2, and triphosphate did not inhibit cap binding to PB2 at concentrations up to 4 mM, whereas cap binding by eIF4E was significantly more sensitive to triphosphate inhibition (IC_{50} = 400 μM). Previously, it has been shown that base substitutions to generate a delocalized positive charge on the base were critical for high affinity interaction of cap analogues with VP39 (*22*). Similarly, a significant (>4000-fold) drop of affinity has been described with eIF4E, when comparing methylated with unmethylated guanosine phosphate analogues (*19*). In comparison, there was only a 5-fold difference in PB2 cap binding inhibition potency between m7GTP and GTP. Therefore, the contribution of aromatic stacking interactions to cap binding could be significantly less for PB2 as compared to eIF4E and VP39, and the relative contribution of hydrogen bond interactions between guanine base and PB2 protein could be increased as compared to eIF4E and VP39. Overall, significant differences in cap binding interactions between the three cap binding proteins suggested that selective PB2 binding compounds were conceivable. In addition, the increased affinity of PB2 for acycloguanosine as compared to guanosine indicated that

replacing ribose with acyclic, aliphatic groups could be further exploited for the development of PB2 binding compounds.

On the basis of these considerations, a novel compound, RO0794238, was identified, which could inhibit cap binding to PB2 with higher potency than m7GTP, despite lacking any negative charges or phosphate groups. On the basis of the structure of RO0794238, it is reasonable to assume that this compound binds to the m7G sub-pocket of the cap binding site on PB2. Therefore, this compound must have compensated the loss of the synergistic contribution from the phosphate binding sub-pocket, which significantly contributes to high affinity binding of m7GTP as compared to m7G. A benzyl substitution replacing methyl on position 7 of the guanine base has been shown previously to increase affinity of monophosphorylated guanosine analogue for eIF4E, which also correlated with increased potency in translation inhibition, but there was no significant effect in the background of the triphosphate (*21*, *23*). A major improvement of both cap binding inhibition potency and selectivity toward eIF4E was achieved by the replacement of the ribose with an acyclic aliphatic group on N9 of guanine. Optimization of this group could further increase potency and selectivity in this series of compounds.

In summary, we have established a quantitative assay system for the analysis of protein−cap structure interaction. Using this system, we have defined a number of key differences in cap structure interaction between influenza virus cap binding protein and human translation initiation factor eIF4E. The assessment of simple, prototype inhibitors of cap binding has resulted in the identification of RO0798234 as a novel, selective inhibitor of influenza cap binding protein. The discovery of RO0798234 confirmed that structural differences between eIF4E and PB2 exist that can be exploited for the design of selective inhibitory compounds. RO0798234 also confirms that negative charges are not required to achieve high affinity binding to influenza cap binding protein. Considering the significant differences in cap interaction, it will be highly interesting to get additional structural information on the PB2 cap binding site.

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