METHOD

Interaction of RNA with phage display selected peptides analyzed by capillary electrophoresis mobility shift assay

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ABSTRACT

A sensitive capillary electrophoresis mobility shift assay (CEMSA) to analyze RNA/peptide interactions has been developed. Capillary electrophoresis (CE) has been adapted for investigating the interaction between variously methylated 17-nt analogs of the yeast tRNA^{Phe} anticodon stem and loop domain (ASL^{Phe}) and 15-amino-acid peptides selected from a random phage display library (RPL). A peptide-concentration-dependent formation of RNA/peptide complex was clearly visible during CEMSA. In the presence of peptide, the UV-monitored CE peak for ASL^{Phe} with three of the five naturally occurring modifications (2'-O-methylcytidine (Cm₃₂), 2'-O-methylguanine (Gm₃₄) and 5-methylcytidine (m⁵C₄₀) shifted from 18.16 to 20.90 min. The mobility shift was observed only for methylated RNA. The negative effects of diffusion, electroosmotic flow and adhesion of molecules to the capillary internal wall were suppressed by using a buffer containing a sieving polymer and a polyacrylamide-coated capillary. Under these conditions, well-shaped peaks and resolution of RNA free and bound to peptide were achieved. Peptide t^F2, the most populated ligand in the RPL, specifically bound triply methylated ASL^{Phe} in a methylated nucleoside-dependent manner. CE was found to be an efficient and sensitive method for the qualitative analysis of RNA–peptide interaction and should be generally applicable to the study of RNA–peptide (protein) interactions.

Keywords: anticodon stem and loop; capillary electrophoresis; methylated nucleoside-dependent RNA–peptide interaction; phage display selected peptides; tRNA

INTRODUCTION

An understanding of how proteins recognize their cognate RNAs would help elucidate the influence of proteins on RNA function (Nagai, 1993; Moras & Poterszman, 1995; Varani & Nagai, 1998; Draper, 1999). Several RNA-binding motifs have been identified from the primary sequence of RNA-binding proteins (Haynes, 1992; Burd & Dreyfuss, 1994; Shamoo et al., 1995; Weiss & Narayana, 1998; Adinolfi et al., 1999; Cusack, 1999).

However, the structural diversity and dynamics of RNA molecules complicates the study of RNA identity elements as recognition determinants for protein interaction. RNA's modified nucleosides, when important to protein recognition, compound the difficulty of deducing the basic rules of the chemical and structural interactions of RNA with protein.

A random phage display library (RPL) is a combinatorial biochemical tool used for selecting peptide ligands from a large pool (10⁷–10⁹) of random sequences encoded within a phage coat gene (Smith, 1985; Scott & Smith, 1990; Houghten et al., 1991; Birnbaum & Mosbach, 1992; Shusta et al., 1999). Using an RPL, we have developed an experimental model system to study the interaction of modified RNAs with peptides (Agris et al., 1999; Mucha et al., 2001). To understand the function of modified nucleosides as identity elements for protein recognition, a triply methylated 17-nt anticodon stem and loop domain of yeast tRNA^{Phe}, ASL^{Phe}.

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Abbreviations: ASL, anticodon stem-loop domain; ASL^{Phe}-Cm₃₂, Gm₃₄, m⁵C₄₀, triply methylated anticodon stem-loop domain of yeast tRNA^{Phe} containing three of five natural modifications Cm₃₂, Gm₃₄, and m⁵C₄₀; CE, capillary electrophoresis; CEMSA, capillary electrophoresis mobility shift assay; EOF, electroosmotic flow; LPA, linear polyacrylamide; RPL, random phage display library; SGE, slab gel electrophoresis; yW, wybutosine.

Cm₃₂, Gm₃₄, m⁵C₄₀ (Fig. 1) was employed for random sequence peptide selection from an RPL (Agris et al., 1999). The altered ellipticity of the RNAs circular dichroism spectra (Agris et al., 1999) and the fluorescence quenching of the peptides' inherent tryptophans (Mucha et al., 2001) were used to analyze binding. Previously obtained quantitative results of fluorescence quenching showed that the methylated nucleosides as well as hairpin structure of the anticodon domain were critical for RNA-peptide interaction (Mucha et al., 2001).

Methods based on changes in electrophoretic mobility, mobility shift assays (MSA), are commonly used to characterize RNA-ligand interactions. Slab gel electrophoresis (SGE) under native conditions is one of the conventional methods for studying RNA-peptide interaction. However, SGE displays significant limitations, especially when macromolecular interactions are of moderate or weak affinities. Compared to SGE, capillary electrophoresis (CE) allows for rapid (typically within minutes), efficient (usually 10⁵ theoretical plates) and completely automated separation of minute quantities (nanoliter injection volumes and as little as attomole amounts) of analytes (Karger et al., 1995).

CE studies of DNA electrophoretic behavior have been described (Karger et al., 1995, 1996; Baba, 1996; Righetti & Gelfi, 1997, 1998, 1999; Odin et al., 1999; Carrilho, 2000). There are some previous CE studies

analyzing DNA interactions with small ligands and larger macromolecules such as peptides and proteins (Stebbins et al., 1996; Xian et al., 1996; Xue et al., 1997; Foulds & Etzkorn, 1998, 1999, 2001; Li & Martin, 1998; Wan & Le, 1999). A few examples of the CE characterization of RNA molecules have been published, as well (Katsivela & Hofle, 1995; Skeidsvoll & Ueland, 1996; Guetens et al., 2000). Surprisingly, only one article describes RNA-protein interaction by CE (Zhang et al., 1998). However, the authors did not observe complex formation directly but inferred tRNA^{Trp}-tryptophanyl-tRNA synthetase interaction by monitoring changes of the tRNA peak height and shape. Here, we report the development of capillary electrophoresis mobility shift assay (CEMSA) conditions for the direct and sensitive observation of modified and unmodified RNAs and their interactions with peptides. To suppress the negative effects of molecular adhesion to the inner capillary wall and electroosmotic flow (EOF), a phenomenon of the bulk flow of separation buffer caused by the formation of a double electric layer on the inner capillary surface in an applied voltage, both a linear polyacrylamide (LPA)-coated capillary and a soluble sieving matrix-containing separation buffer were applied. Under these conditions, a qualitative analysis of a methylated nucleoside-dependent RNA-peptide complex formation was readily achieved.

RESULTS

In the development of a CE-based assay of RNA-peptide interaction, obtaining unambiguous evidence of RNA-peptide complex formation is of primary concern. Therefore, we chose an existing model composed of a methylated nucleoside-dependent, peptide recognition of RNA. A triply methylated yeast tRNA^{Phe} anticodon stem and loop domain (ASL^{Phe}) containing three of the five naturally occurring modified nucleosides, Cm₃₂, Gm₃₄, and m⁵C₄₀, had been employed to select tRNA-binding peptides from an RPL (Fig. 1; Agris et al., 1999). Three 15-amino-acid peptides of the highest affinity for the triply methylated ASL^{Phe} were selected from 200 phage clones from the RPL:

FIGURE 1. Primary sequence and secondary structure of ASL analogs unmodified ASL^{Phe} (A), ASL^{Phe}-Cm₃₂, Gm₃₄, m⁵C₄₀ (B), ASL^{Phe}-m¹G₃₇, Ψ₃₉, m⁵C₄₀ (C), and native yeast tRNA^{Phe} stem and loop (D).

- t^F2: Ser¹-Ile-Ser-Pro-Trp⁵-Gly-Phe-Ser-Gly-Leu¹⁰-
Leu-Arg-Trp-Ser-Tyr¹⁵;
t^F3: Ala¹-Ala-Gly-Gly-Phe⁵-Phe-Arg-Trp-Ile-Phe¹⁰-
Ser-Pro-Val-Tyr-Val¹⁵;
t^F33: Leu¹-Arg-Tyr-Thr-Leu⁵-Glu-Thr-Arg-Trp-Ala¹⁰-
Val-Leu-Ser-Val-Tyr¹⁵;
t^F0: Ser¹-Trp-His-Gln-Trp⁵-Ile-Trp-Pro-Ser-Gly¹⁰-
Gln-Pro-Leu-Ser-Glu¹⁵.

Peptide t^F0 was designed as a negative control by comparison to known RNA-binding motifs. The most

populated of peptides in the RPL selected clones (42%), peptide t^F2, exhibited moderate affinity and high specificity for ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ (Mucha et al., 2001). The binding affinity of the triply methylated ASL^{Phe} for peptide t^F2 ($K_d = 1.3 \mu\text{M}$), determined by fluorescence quenching of the peptide's tryptophans, was comparable to that of the native yeast tRNA^{Phe} for peptide t^F2 ($K_d = 2.3 \mu\text{M}$; Mucha et al., 2001).

In contrast to monitoring the peptides' tryptophan fluorescence, our choice of experimental conditions and observation of the CEMSA at 254 nm allowed preferential monitoring of both free and bound RNA-containing peaks. Triply methylated ASL^{Phe} was visible as a single, sharp peak with a migration time of 18.16 min (Fig. 2). Observation of complex formation of the triply methylated ASL^{Phe} with peptide t^F2 was dependent on the peptide concentration. At a constant concentration of triply methylated ASL^{Phe} (2.1 μM), a new peak of increasing size and a slower and distinct migration time (20.90 min) was observed with increasing concentration of the peptide (Fig. 2A). The use of an LPA-coated capillary and sieving matrix containing the separation buffer resulted in sharp peaks of both unbound and complexed ASL^{Phe}. At equimolar ASL^{Phe}/t^F2 concentration of 2.1 μM , no shift of the RNA peak was ob-

served (data not shown). A small peak ($t_m = 20.90 \text{ min}$) of the complex became visible at the RNA/peptide molar ratio 1:7. With increasing concentration of peptide t^F2 (ASL^{Phe}/t^F2 molar ratio 1:15), complex formation increased. Free peptide migrates slowly in the opposite direction to RNA molecules and is not visible under CEMSA conditions (data not shown). Migration time of the complex was independent of the peptide concentration and only one peak in addition to the unbound RNA was observed. This result indicates that only one type of RNA/peptide complex had been formed. Previously obtained observations of complex formation by fluorescence spectroscopy showed that triply modified ASL^{Phe} bound peptide t^F2 at 1:1 stoichiometry (Mucha et al., 2001). At a high peptide t^F2 concentration (ASL^{Phe}/t^F2 ratio 1:30), complex had been efficiently formed, but some free ASL^{Phe} was still detectable by CEMSA (Fig. 2A). However, in the presence of denaturing agents, sodium dodecylsulfate (SDS) or urea, only the free RNA was observed in the CEMSA (Fig. 2B). Results from previous studies (Mucha et al., 2001) had indicated that hydrophobic effects dominated the RNA-peptide t^F2 interaction. Therefore, we used the highly hydrophilic, 18-amino-acid peptide systemin, a plant hormone (AVQSKPPSKRDPPKMQTD) as one of our negative

FIGURE 2. CEMSA of ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ interactions with RPL-selected peptide t^F2 under native and denaturing conditions. **A:** Triply modified ASL^{Phe} interaction with peptide t^F2. Free and peptide-bound RNA is observed in the presence of peptide at molar ratios of RNA to peptide of 1:7, 1:15, and 1:30. **B:** CEMSA of ASL^{Phe}-Cm₃₂,Gm₃₄,m⁵C₄₀ interaction with t^F2 in the presence of denaturing agents sodium dodecylsulfate (SDS) and urea. For analysis conditions, see Materials and Methods.

controls. No interaction was observed between systemin and the RNA molecules studied (data not shown). Taking these data together, we concluded that the peak at 20.90 min corresponded to RNA-peptide complex.

A less populated peptide of the RPL selected clones (5%), peptide t^F3, displayed a lower affinity ($K_d = 10 \mu\text{M}$) for the triply methylated ASL^{Phe} than the most populated peptide t^F2 (Mucha et al., 2001). At a ASL^{Phe}/t^F3

FIGURE 3. CEMSA of ASL^{Phe}-Cm₃₂Gm₃₄m⁵C₄₀ interaction with other RPL-selected and control peptides. **A:** CEMSA of ASL^{Phe}-Cm₃₂Gm₃₄m⁵C₄₀ interaction with peptide t^F3. With increasing concentrations of peptide, peptide-bound RNA is observed. In contrast to t^F2, aggregation occurs at high t^F3 peptide concentrations. **B:** CEMSA of ASL^{Phe}-Cm₃₂Gm₃₄m⁵C₄₀ interaction with peptide t^F33. **C:** CEMSA of ASL^{Phe}-Cm₃₂Gm₃₄m⁵C₄₀ interaction with peptide t^F0. For analysis conditions see Materials and Methods.

molar stoichiometry of 1:5, only a marginally observable peak, if any, was observed with a migration time longer ($t_m = 21.40$ min) than that of the unbound ASL^{Phe} (Fig. 3A). With increasing concentrations of peptide t^{F3} ($\text{ASL}^{\text{Phe}}/\text{t}^{\text{F3}}$ molar ratio 1:30), a peak of $\text{ASL}^{\text{Phe}}/\text{t}^{\text{F3}}$ complex became clearly visible, but unbound ASL^{Phe} still dominated. Under these conditions, intensity of the peak of the $\text{ASL}^{\text{Phe}}/\text{t}^{\text{F3}}$ complex was comparable to the intensity of the peak of the $\text{ASL}^{\text{Phe}}/\text{t}^{\text{F2}}$ complex obtained at $\text{ASL}^{\text{Phe}}/\text{t}^{\text{F2}}$ molar ratio 1:15. At an $\text{ASL}^{\text{Phe}}/\text{t}^{\text{F3}}$ molar ratio of 1:45, the peak of the complex dominates and only a small peak of unbound ASL^{Phe} remained. At a high excess of peptide t^{F3} ($\text{ASL}^{\text{Phe}}/\text{t}^{\text{F3}}$ molar ratio 1:65), aggregation occurred and no peaks were observed.

Peptide t^{F33} populated the RPL-selected clones at a low level (only 1%; Mucha et al., 2001). Although population of peptide t^{F33} was low, some affinity for triply methylated ASL^{Phe} was detected by CEMSA (Fig. 3B). At an $\text{ASL}^{\text{Phe}}/\text{t}^{\text{F33}}$ molar ratio of 1:15, a small, but visible, peak ($t_m = 21.03$ min) of $\text{ASL}^{\text{Phe}}/\text{t}^{\text{F33}}$ complex was observed. At high peptide t^{F33} concentration (25–40-fold), only slightly higher efficiency of the complex formation was visible and free ASL^{Phe} still dominated. As expected, the negative control peptide t^{F0} displayed no affinity (in excess range from 25- to 50-fold) for the triply methylated ASL^{Phe} (Fig. 3C). Only at a large excess (250-fold) of the peptide did three small peaks of unspecific interaction with migration times $t_m = 20.53$ – 22.31 min appear.

A series of variously methylated ASL^{Phe} analogs were examined for peptide binding and specificity. Unmethylated ASL^{Phe} analogs or analogs with methylated nucleosides different from those of $\text{ASL}^{\text{Phe}}\text{-Cm}_{32}, \text{Gm}_{34}, \text{m}^5\text{C}_{40}$ generally did not bind the peptides under the conditions of CEMSA. Unmethylated ASL^{Phe} and its DNA analog have had a significantly reduced affinity for peptide t^{F2} ($K_d \approx 70 \mu\text{M}$) as determined by fluorescence quenching (Mucha et al., 2001) and no affinity for this peptide was observed by CEMSA (data not shown). $\text{ASL}^{\text{Phe}}\text{-m}^1\text{G}_{37}, \Psi_{39}, \text{m}^5\text{C}_{40}$, an ASL analog that contains two new modifications (m^1G_{37} and Ψ_{39}), displayed a weak affinity for peptide t^{F2} manifested in CEMSA as three small peaks ($t_m = 20.01$ – 22.35 min; Fig. 4). These peaks were visible only at a high peptide concentration and probably reflected unspecific RNA/peptide interaction. This ASL analog has only one methylated nucleoside, m^5C_{40} , in common with the triply methylated $\text{ASL}^{\text{Phe}}\text{-Cm}_{32}, \text{Gm}_{34}, \text{m}^5\text{C}_{40}$. The influence of Ψ_{39} for peptide t^{F2} binding has not been studied, but the presence of m^1G_{37} strongly decreased ASL^{Phe} affinity for t^{F2} peptide (Mucha et al., 2001).

DISCUSSION AND CONCLUSIONS

The unique advantages of CEMSA for the study of RNA–peptide interactions of moderate affinity have been demonstrated. One advantage to the method described is

FIGURE 4. CEMSA of $\text{ASL}^{\text{Phe}}\text{-m}^1\text{G}_{37}, \Psi_{39}, \text{m}^5\text{C}_{40}$ interaction with RPL-selected peptides. For analysis conditions see Materials and Methods.

that free RNA and RNA/peptide complex are observed while free peptide migrates slowly in the opposite direction and is not observed. Although quantitative results are not presented here, the ability to determine free RNA and bound RNA/peptide complex will allow comparison of affinities for variously modified RNAs and peptides of altered amino acid sequences. The sensitivity of the method provides observation of the RNA/peptide complex formation in the micromolar range of concentrations. Qualitative CEMSA results of RNA/peptide interaction were in good agreement with quantitative results obtained previously by fluorescence spectroscopy (Mucha et al., 2001).

Analysis of variously modified RNAs by CEMSA confirmed the importance of the three methylated nucleosides, Cm_{32} , Gm_{34} , and m^5C_{40} , as identity elements of the triply methylated ASL^{Phe} when recognized by peptide t^{F2} . Peptide t^{F2} selectively recognized the triply methylated ASL^{Phe} . Other RPL-selected peptides, t^{F3} and t^{F33} , recognized $\text{ASL}^{\text{Phe}}\text{-Cm}_{32}, \text{Gm}_{34}, \text{m}^5\text{C}_{40}$ with lower affinities. These results clearly demonstrate that capillary electrophoresis is a valuable tool in the study of RNA/peptide interactions. A mobility shift assessed by CE requires far less material than SGE, a method commonly used to study RNA/peptide interaction. Key features of CEMSA conditions that were applied include the use of LPA-coated capillary and soluble siev-

ing matrix in the separation buffer. LPA-coated capillary and sieving matrix containing separation buffer effectively minimized EOF and interaction of the macromolecules with the capillary wall. It allowed us to observe well-shaped and separated peaks of unbound and bound RNA. Presented CEMSA methodology should be generally applicable to study RNA/peptide (protein) interaction.

MATERIALS AND METHODS

Synthesis of ASLs analogs

The 17-nt ASLs were synthesized using the phosphoramidite method as described earlier (Agris et al., 1999). All investigated RNA molecules adopted hairpin structures under the experimental conditions (Mucha et al., 2001).

Peptide synthesis

Four 15-amino-acid peptides were synthesized by the solid phase method using standard Fmoc/TBTU protocol as described earlier (Agris et al., 1999; Mucha et al., 2001).

Capillary electrophoresis mobility shift assay

CEMSA experiments were performed on a Beckman P/ACE 2100 capillary electrophoresis system using BioCap LPA-coating capillary (Bio-Rad) 75 $\mu\text{m} \times 57$ cm (50 cm to the detector). The running buffer (Bio-Rad) was the commercially available 267 mM Tris/borate with a sieving matrix (physical gel) pH 8.3, adjusted manually with a solid boric acid to pH 7.1. CE was conducted with reverse polarity and constant voltage 15 kV at $20 \pm 0.1^\circ\text{C}$. Electrokinetic injection for 15 s was conducted at 10 kV. RNA was detected at 254 nm. RNA/peptide complex was incubated in 10 mM Tris-HCl, 70 mM NaCl, 20 mM EDTA, 5% glycerol, pH 7.5 buffer for 30 min at 0°C before injection. The RNA concentration was 2.1 μM .

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