Phosphorylation of a chloroplast RNA-binding protein changes its affinity to RNA

Irena Lisitsky and Gadi Schuster*

Department of Biology, Technion–Israel Institute of Technology, Haifa 32000, Israel

ABSTRACT

An RNA-binding protein of 28 kDa (28RNP) was previously isolated from spinach chloroplasts and found to be required for 3' end-processing of chloroplast mRNAs. The amino acid sequence of 28RNP revealed two ~80 amino-acid RNA-binding domains, as well as an acidic- and glycine-rich amino terminal domain. Upon analysis of the RNA-binding properties of the 'native' 28RNP in comparison to the recombinant bacterial expressed protein, differences were detected in the affinity to some chloroplastic 3' end RNAs. It was suggested that post-translational modification can modulate the affinity of the 28RNP in the chloroplast to different RNAs. In order to determine if phosphorylation accounts for this post-translational modification, we examined if the 28RNP is a phosphoprotein and if it can serve as a substrate for protein kinases. It was found that the 28RNP was phosphorylated when intact chloroplasts were metabolically labeled with [^{32}P] orthophosphate, and that recombinant 28RNP served as an excellent substrate *in vitro* for protein kinase isolated from spinach chloroplasts or recombinant α subunit of maize casein kinase II. The 28RNP was apparently phosphorylated at one site located in the acidic domain at the N-terminus of the protein. Site-directed mutagenesis of the serines in that region revealed that the phosphorylation of the protein was eliminated when serine number 22 from the N-terminus was changed to tryptophan. RNA-binding analysis of the phosphorylated 28RNP revealed that the affinity of the phosphorylated protein was reduced ~3–4-fold in comparison to the non-phosphorylated protein. Therefore, phosphorylation of the 28RNP modulates its affinity to RNA and may play a significant role in its biological function in the chloroplast.

INTRODUCTION

The expression of chloroplast genes is regulated by a variety of mechanisms, one of which is the modulation of RNA stability during chloroplast development (1–5). Most chloroplast RNAs contain stem–loop structures, essential for the stability of mRNAs *in vivo* and *in vitro*, which may function as 3' end-processing

signals (5–9). When the proteins involved in the 3' end-processing of chloroplast RNAs were analyzed, a 28 kDa RNA-binding protein (28RNP) was identified as the major polypeptide that co-migrated through all the biochemical purification steps of the *in vitro* 3' RNA end-processing activity. Immunodepletion of the 28RNP from soluble chloroplast protein extract or the addition of extra recombinant protein interfered with the *in vitro* 3' end RNA processing activity, suggesting possible involvement in that process (10). Similar to many other chloroplast proteins, the 28RNP is encoded in the nucleus, translated in the cytoplasm as a precursor protein and imported into the chloroplast. The deduced amino acid sequence disclosed two similar 80 amino acid RNA-binding domains, as well as an amino terminal acidic domain (10). In addition, cDNAs of similar RNA-binding proteins were cloned from tobacco (11–13), maize (14) and Arabidopsis (15,16).

The RNA-binding properties of the 28RNP were analyzed using UV cross-linking, saturation binding and competition methods. It was found that recombinant *Escherichia coli* expressed protein binds with apparently the same affinity to every chloroplastic 3' end RNA analyzed, as well as to RNAs derived from the 5' or the coding region (17). When the RNA-binding properties of the different structural domains were investigated, it was found that the acidic domain does not bind RNA, rather, each of the RNA-binding domains binds RNA, although with different affinities. Interestingly, the acidic domain has a positive effect on the binding of the full-length protein to RNA, since the mature protein binds RNA with better affinity than the truncated protein lacking the acidic domain (18). This result indicated that the acidic domain influences the conformational structure of the protein which modulates the affinity to RNA (18). The 28RNP binds strongly to poly G and poly U but poorly to poly C and poly A, and a stretch of two to three G residues was found to be sufficient to mediate binding of the recombinant 28RNP (17,19). In addition, it was found that the 28RNP is not associated with either thylakoid-bound or soluble polysomes, in which most of the chloroplast ribosomal and messenger RNAs are localized, and that there are differences in the RNA-binding affinities for some chloroplastic 3' end RNAs between the recombinant and the native 28RNP (17). It was therefore suggested that post-translational modification of the 28RNP can account for these differences in affinities to RNA.

Phosphorylation is a well-documented post-translational modification having regulatory functions affecting the properties

* To whom correspondence should be addressed

of a diverse range of proteins. In addition, many hnRNP core proteins were shown to be modified post-translationally, primarily by phosphorylation (20). One of these proteins is the human hnRNP A1 protein, a major component of the eukaryotic hnRNP complex, composed of two domains, one of which contains two RNA-binding domain consensus sequences similar to those of the 28RNP. Phosphorylation of the A1 protein on serine residue not located in the RNA-binding domains had no detectable effect on its nucleic binding capacity, but suppressed the ability of that protein to promote strand annealing *in vitro* (21). Changes in the nucleic acid binding properties of some plant chloroplasts and nuclear proteins as a result of their phosphorylation have been reported (22,23). It was recently reported that the 28RNP is an effective phosphate acceptor for a casein kinase II-like protein isolated from spinach chloroplasts (24). In addition, it was shown using circular dichroism spectra that nucleic acid binding induced conformational changes of the protein (24). A detailed description of phosphorylation parameters and their effect on RNA-binding properties is a prerequisite to any study on possible *in vivo* significance of such a modification.

In this study, we characterized the phosphorylation of the recombinant 28RNP using several protein kinase preparations. It was found that the 28RNP was phosphorylated when intact chloroplasts were incubated with [^{32}P]orthophosphate, and that recombinant 28RNP can apparently be phosphorylated on one site located in the acidic domain at the N-terminus of the protein. Phosphorylation of the 28RNP reduced the affinity to 3' end chloroplast RNA in comparison to the non-phosphorylated protein. Therefore, the differences between affinities of the recombinant and native 28RNPs to several RNAs, could be attributed to the phosphorylation state of the native protein. Modulation of the affinity of the 28RNP to RNA by phosphorylation may be significant to its biological function in the chloroplast.

MATERIALS AND METHODS

Recombinant 28RNP

Expression of the 28RNP cDNA in bacteria cells, purification of the recombinant protein as well as its different domains and oligonucleotide site-directed mutagenesis were performed as previously described (17,18).

Isolation of recombinant maize casein kinase II

The cDNA encoding subunit α of maize casein kinase II in the expression vector PT7-7 (25) was generously provided by Dr O.-G. Issinger. In order to purify the recombinant kinase, BL21(DE3) cells were induced with 0.4 mM IPTG for 4 h. Cells were collected by centrifugation, resuspended in buffer A (20 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.5 mM EGTA, 7 mM 2-mercaptoethanol, 50 mM NaCl, 0.2 mM PMSF), broken by passing through a French press cell operating at 20 000 p.s.i. and centrifuged for 3 h at 150 000 g. The supernatant was applied on a 1 ml heparin column (HiTrap, Pharmacia) that had been washed with buffer A. Proteins bound to the column were eluted with a gradient of KCl, and the casein kinase II α subunit was eluted at 0.55 M KCl. Analysis by SDS–PAGE showed this kinase preparation to be apparently homogenous. Fractions containing the purified kinase were pooled, dialyzed against buffer E (20 mM HEPES, pH 7.9, 60 mM KCl, 12.5 mM MgCl$_2$, 0.1 mM

EDTA, 2 mM DTT and 5% glycerol) and concentrated by centricons (Amicon).

Isolation of protein kinase from spinach chloroplast

Intact chloroplasts were isolated on percoll gradients (26), and were osmotically disrupted and diluted in buffer E. Membranes were pelleted at 150 000 g for 3 h and the supernatant was loaded on a heparin column, eluted and concentrated as for the maize recombinant kinase preparation, except that buffer E was used instead of buffer A.

Chloroplast protein extract

Chloroplast protein extract was prepared from young leaves of hydroponically-grown spinach (*Spinacia oleracea* cv. Viroflay) plants as previously described (25). For the isolation of the 0.5 M KCl ssDNA fraction described in Figure 1, the extract was applied on a single-stranded DNA–cellulose (Sigma Inc.) column, and the proteins eluted from the column with buffer E containing 0.5 M KCl were analyzed.

In vitro phosphorylation assay

Phosphorylation of the recombinant 28RNP or its different domains was performed in a buffer containing 20 μM [γ-^{32}P]ATP (500 c.p.m./pmol) in a total volume of 20 μl. A typical reaction contained 200 ng recombinant 28RNP and ~100 ng recombinant casein kinase II or another kinase preparation. Analysis of the phosphorylated proteins was performed by fractionation on SDS–PAGE and autoradiography. The protein bands of the recombinant 28RNP were cut out and counted for quantitative radioactivity. When the recombinant 28RNP was phosphorylated for use in an RNA-binding assay, the reaction mixture was scaled up 10-fold and phosphorylation time increased to 2 h. Aliquots were analyzed to determine if saturation of phosphorylation (mol phosphate per mol protein) was observed, and the reaction was terminated by extensive dialysis against buffer E. Control non-phosphorylated 28RNP was treated in the same way, but without the addition of ATP or the kinase preparation.

In vivo labeling of intact chloroplast with [^{32}P]orthophosphate

Intact chloroplasts were isolated from young spinach leaves on percoll gradients as previously described (26). Chloroplasts were washed and resuspended in a buffer containing 1 mM NaF and 400 μCi [^{32}P]orthophosphate in the light for 30 min at 25°C with gentle mixing. Following incubation, the chloroplasts were osmotically disrupted by adding 10 vol cold buffer E containing 1 mM NaF, MgCl$_2$ was added to the final concentration of 5 mM and membranes were precipitated at 10 000 g for 10 min. The supernatant was applied on a single-stranded DNA–cellulose column (Sigma Inc.). The phosphorylated 28RNP was eluted from the column with the same buffer containing 0.5 and 1.0 M KCl. The phosphorylated proteins in each fraction were identified using SDS–PAGE and autoradiography.

Synthetic RNA

Synthetic RNA corresponding to the 3' end of the chloroplastic *psbA* gene was transcribed as described before using 2.5 μM [α-^{32}P]UTP and 25 μM non-radioactive UTP (10).

UV-crosslinking

UV-crosslinking of protein(s) to [α-^{32}P]UTP-labeled RNAs was carried out as previously described (10). Essentially, 30 fmol RNA (240 000 c.p.m.) or the amount indicated in the figure legends, was incubated in 15.5 μl with protein(s) in a buffer containing 10 mM HEPES, pH 7.9, 30 mM KCl, 6 mM MgCl$_2$, 0.05 mM EDTA, 1 mM DTT and 2.5% glycerol. Following UV irradiation in a UV crosslinker (Hoeffer Inc.) at 1.8 J, the RNA was digested by 1 μg RNase A at 37°C for 1 h and the proteins were fractionated by SDS–PAGE. The transfer-labeled from the RNA to the proteins was detected using autoradiography, and quantitation was performed by scanning the autoradiogram with a laser-scanner densitometer.

RESULTS

In vivo phosphorylation of the 28RNP

Analysis of the RNA-binding properties of the 28RNP revealed that there were differences in the affinities of the "native protein that was isolated from spinach chloroplasts" and the recombinant bacteria-made protein, to several chloroplast 3' end RNAs (17). In order to determine if these differences could be obtained due to post-translational modifications of the 'native' 28RNP modulating its affinity to RNAs, we first investigated the possibility that the 28RNP is a phosphoprotein. To this end, intact chloroplasts were incubated with [^{32}P]orthophosphate in the light and in the presence of the phosphatase inhibitor NaF. Following incubation, the chloroplasts were fractionated into the non-soluble membrane fraction and the 28RNP was purified from the soluble protein fraction using single-stranded DNA (ssDNA) chromatography (Fig. 1) and identified by probing with specific antibodies. Analysis of the phosphoproteins using autoradiography revealed the phosphorylation of LHCII, D1 and other thylakoid photosystem II proteins in the membrane fraction as described before (27). The 28RNP, isolated by ssDNA chromatography, was phosphorylated under these experimental conditions (Fig. 1). This result indicated that the 28RNP is a phosphoprotein in the chloroplast, thereby raising the possibility that phosphorylation can modulate the RNA-binding affinity of this protein. Additional analysis of the 28RNP phosphorylated and non-phosphorylated forms by a two dimensional gel electrophoresis system revealed that ~50% of the 28RNP population is phosphorylated in the chloroplasts of young leaves (data not shown).

In vitro phosphorylation of the 28RNP

The 28RNP, either purified from spinach leaves or recombinant produced in bacteria, could be phosphorylated when incubated in the presence of [γ-^{32}P]ATP and several preparations of protein kinases (Fig. 2). We first analyzed the 'native' protein purified from soluble chloroplast protein extract using ssDNA column chromatography. The fraction bound to the ssDNA column and eluted at 0.5 M KCl included several proteins (Fig. 1, lane A3), the most abundant of which are the 28RNP and 24RNP (10; Abrahamson, S., Schuster, G. and Gruissem, W., submitted), as well as the 100RNP exonuclease and the 33 kDa associated with it (Hayes, R., Kudla, J., Schuster, G., Gabay, L., Maliga, P. and Gruissem, W., submitted). It was found that the 28RNP was phosphorylated when that fraction was incubated with
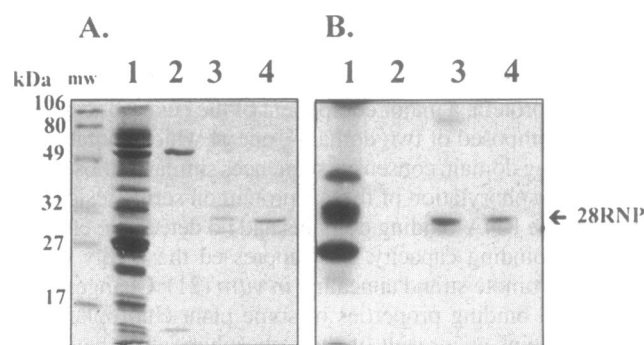

Figure 1. *In vivo* phosphorylation of the 28RNP. Isolated intact chloroplasts were incubated in the light for 30 min in the presence of [^{32}P]orthophosphate and 1 mM NaF. Following incubation, the chloroplasts were re-isolated, disrupted osmotically and fractionated into soluble and membrane fractions. The 28RNP was purified from the soluble fraction using ssDNA chromatography and subjected to: (**A**) SDS–PAGE and (**B**) autoradiography. Lane 1 is the membrane proteins fraction. Lane 2 shows proteins from the soluble fractions that did not bind to the ssDNA column. Lanes 3 and 4 are proteins eluted from the ssDNA column at 0.5 and 1.0 M, respectively. The location of the 28RNP, as identified with specific antibodies, is indicated to the right. mw refers to molecular weight standards as shown in kilodaltons in the margins.
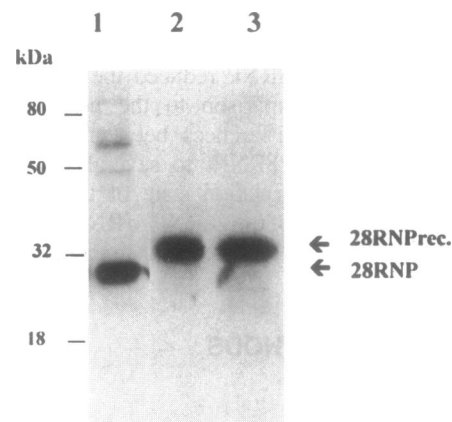

Figure 2. *In vitro* phosphorylation of the 28RNP. Lane 1, the proteins of chloroplast protein soluble extract bound to a ssDNA column were incubated for 30 min in buffer containing [γ-^{32}P]ATP as described in Materials and Methods. Lane 2, recombinant 28RNP (28RNPrec.) (200 ng) was phosphorylated by a protein kinase preparation from spinach chloroplast as described in Materials and Methods. Lane 3, recombinant 28RNP phosphorylated with recombinant casein kinase II from maize. Note that the molecular weight of the recombinant protein is higher than the native one due to the addition of several amino acids of the expression vector.

[γ-^{32}P]ATP (Fig. 2, lane 1). This result indicated that the 28RNP is an effective phosphate acceptor *in vitro* and that a protein kinase is able to phosphorylate the 28RNP in the ssDNA 0.5 M KCl fraction. However, the activity of this kinase was very low in comparison with the other kinase preparations described below. We then isolated a protein kinase activity from spinach chloroplast extract using heparin affinity chromatography (see Materials and Methods). The result presented in Figure 2, lane 2 showed that this kinase preparation phosphorylated recombinant 28RNP. The most pronounced protein of that kinase preparation was a polypeptide of 36 kDa, resembling the α subunit of casein kinase II (25,28). Nevertheless, even with this kinase preparation,
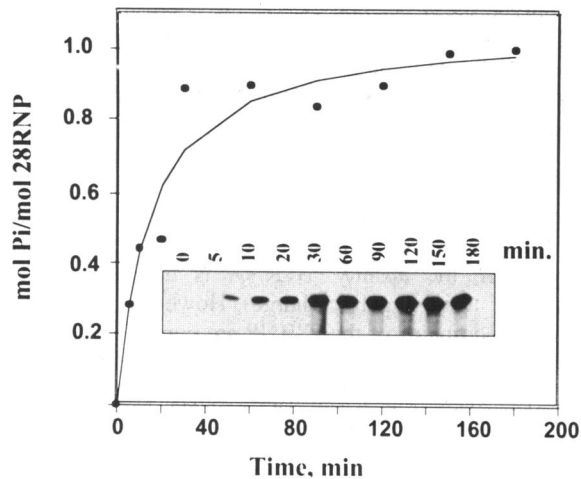
Figure 3. Time course of phosphorylation of the recombinant 28RNP with recombinant maize casein kinase II. 28RNP was incubated under standard reaction conditions in the presence of maize casein kinase II, and at the indicated times, samples were withdrawn for quantitation of phosphoprotein using SDS–PAGE as described in Materials and Methods. The autoradiograph of the corresponding experiment is shown in the figure's insert.

Figure 4. The 28RNP is phosphorylated on the acidic domain. (**A**) Schematic structure of the 28RNP. AD represents the acidic domain, and R1 and R2 the first and second RNA-binding domains, respectively. (**B**) Stained SDS–PAGE of the different 28RNP domains expressed and purified from *E.coli*. (**C**) Autoradiograph of an experiment where the different 28RNP domains were phosphorylated *in vitro* by recombinant maize casein kinase II in the presence of [γ-^{32}P]ATP.

Figure 5. Changing serine number 22 eliminated phosphorylation of the 28RNP. The serine and threonine residues of the acidic domain of the 28RNP were subjected to site directed deletions or mutagenesis as shown in the figure. Each mutagenized 28RNP cDNA was expressed in *E.coli*, purified and phosphorylated *in vitro*, using recombinant maize casein kinase II. Proteins were analyzed using SDS–PAGE and autoradiography. The three putative casein kinase II phosphorylation sites are underlined.

we were unable to phosphorylate the recombinant 28RNP to >0.2 mol Pi/mol 28RNP. The addition of RNA, ssDNA or poly-lysine, known to stimulate casein kinase II activity (28) did not significantly increase that ratio (data not shown). After testing several preparations of protein kinases, either commercially obtained or isolated from different sources, we found that by using the recombinant α subunit of casein kinase II from maize (25), the 28RNP could be phosphorylated to a ratio of one mol Pi per mol protein (Fig. 2, lane 3; Fig. 3). This was the maximal ratio we could obtain; possibly indicating that the 28RNP was phosphorylated at one site. We next determined if the phosphorylation site is located at one of the two RNA-binding domains or at the N-terminus acidic domain. When each of these domains, expressed and purified from *E.coli*, was phosphorylated *in vitro* using the maize casein kinase II, the acidic domain was the only one to be phosphorylated (Fig. 4). Site directed mutagenesis of the serine residues in that domain revealed that changing serine 22 to tryptophan eliminated phosphorylation of the 28RNP, whereas changing other serine residues in the acidic domain had no effect (Fig. 5). The same results were obtained when the protein kinase that was isolated from spinach chloroplasts was incubated with different domains of the 28RNP and the serine 22 to tryptophan mutant (not shown). These results indicated that the 28RNP is most likely phosphorylated at one amino acid, serine number 22, located at its amino terminal acidic domain.

Phosphorylation of the 28RNP changes the affinity to RNA

We have previously shown that there were differences in the affinities of the recombinant and 'native' 28RNP to several chloroplast RNAs, and suggested that these differences could be attributed to post-translational modifications of the 'native' protein not performed with the bacterial expressed one (17). In order to determine if the phosphorylation phenomenon described above could account for these differences, we performed RNA
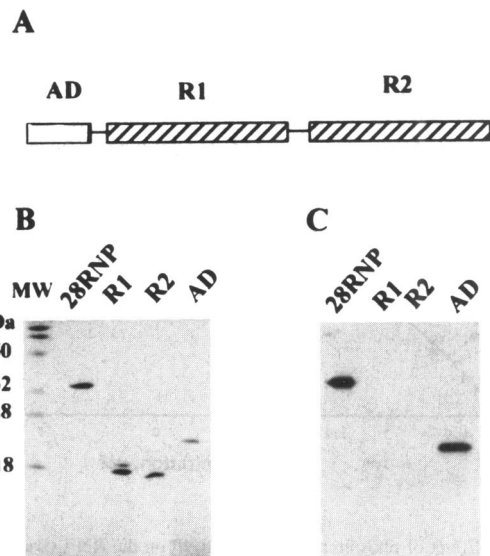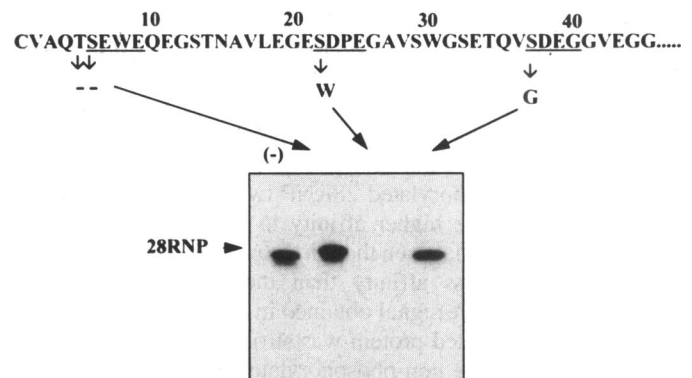
saturation UV crosslinking assays whereby increasing amounts of RNA were crosslinked to the phosphorylated and non-phosphorylated recombinant 28RNP. The binding constant (K_d) was defined as the concentration of RNA giving half saturation of binding (29). The results presented in Figure 6 showed that phosphorylation of the 28RNP reduced the affinity to *psbA* 3' end RNA. In agreement with our previous studies (17,18), a K_d value of 0.4 nM was obtained for the non-phosphorylated 28RNP. However, the K_d value changed to 1.4 nM when recombinant 28RNP phosphorylated to the ratio of one mol phosphate per mol
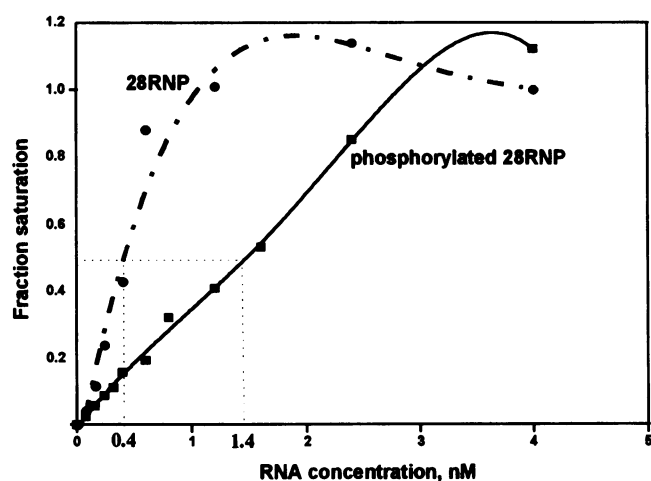
Figure 6. Effect of phosphorylation of 28RNP on the RNA-binding activity. Different amounts of ^{32}P-labeled *psbA* 3' end RNA were incubated with phosphorylated or non-phosphorylated recombinant 28RNP and subjected to UV crosslinking analysis in the presence of a 200-fold excess of the non-competitive inhibitor poly C. Quantitation of the binding was carried out by scanning autoradiographs from three different experiments. The binding affinity, K_d, is defined as the concentration of RNA giving half-saturation of binding.

of protein was used for the binding assay, indicating that the affinity of the phosphorylated 28RNP to RNA was reduced 3–4-fold in comparison with the non-phosphorylated protein. The small amounts of ATP or recombinant casein kinase II proteins existing in the binding solution cannot account for the differences described since the corresponding controls where the kinase preparation or the ATP were added separately to the non-phosphorylated 28RNP gave the same results. In addition, dephosphorylation of the phosphorylated 28RNP by alkaline phosphatase treatment restored the higher affinity to RNA (not shown). It should be also noted that even though the phosphorylated 28RNP binds RNA with less affinity than the non-phosphorylated protein, the radioactive signal obtained in the crosslinking assay with the phosphorylated protein was stronger in comparison to that obtained with the non-phosphorylated one using the same concentration of RNA (data not shown). This observation is similar to our previous one in that a protein composed of the two RNA-binding domains of the 28RNP binds RNA with less affinity than the full-length protein but confers a stronger crosslinking signal (18). It should also be noted that since we are not certain that saturation of binding has been attained (see Fig. 6), the affinity of the phosphorylated 28RNP could be even less and the K_d value thus >1.4 nM, therefore resulting in greater differences due to phosphorylation. Increasing RNA concentration leads to non-specific binding to the protein, even in the presence of excess poly C used to prevent non-specific binding. Taken together, it was concluded that phosphorylation of the 28RNP at its acidic domain changed the affinity of the protein to RNA.

DISCUSSION

In this work, we demonstrated that the spinach chloroplast 28RNP is phosphorylated *in vitro* at Ser 22 and that this

modification results in reducing the affinity of the protein to RNA derived from the 3' untranslated region of chloroplast gene. The phosphorylation occurs at the acidic amino terminal domain of the protein. This domain does not bind RNA by itself but has a major effect on the RNA-binding properties of a nascent RNA-binding domain. When the RNA-binding properties of each domain as well as different combinations of the three domains were analyzed, it was found that each RNA-binding domain by itself binds RNA and that, when connected to the acidic domain, the affinity to RNA is drastically reduced (probably due to its negative charge). However, when the two RNA-binding domains of the 28RNP are considered, the acidic domain has a positive effect on the binding to RNA since the mature full-length protein binds RNA with better affinity than the truncated protein lacking the acidic domain (18). Taken together, these results indicate that the acidic domain of the 28RNP that does not bind RNA by itself affects the affinity of the protein to its target RNA. This could be achieved for example, by conferring conformational changes altering the affinity to RNA. Conformational changes of the 28RNP when bound to poly G or DNA were indeed observed using CD spectroscopy (24). Moreover, we found that protein composed of the two RNA-binding domains gives a stronger signal than the full-length 28RNP in the UV crosslinking assay, despite its lower affinity for the same RNA (18). A similar situation was observed in the present work between the phosphorylated and non-phosphorylated 28RNP (data not shown). This phenomenon implies that phosphorylation of Ser 22 in the acidic domain actually induces a conformational change affecting the number of uridines that can be cross-linked to RNA and/or protected from ribonuclease digestion. It is tempting to speculate that phosphorylation/dephosphorylation of Ser 22 on the 28RNP is a mechanism in which the RNA-binding properties of the protein in the chloroplast is modulated. It should be noted that although a 3-fold difference in the affinity may appear to be insignificant, such a quantitative difference in affinity was recently shown to account for the ability of an other consensus sequence RNA-binding protein, hnRNP A1, to distinguish between its binding site and an unrelated RNA sequence (30).

Several other RNA-binding proteins were found to be phosphorylated, including putative chloroplast translational enhancers (22) and hnRNP proteins of the C and U groups and the hnRNP protein A1 (23). In the latter case, it was found that similar to the 28RNP, the phosphorylation site is not in the consensus sequence RNA-binding domain. On the other hand unlike the 28RNP, phosphorylation of the A1 protein had no apparent effect on the affinity to RNA and inactivated the strand annealing activity (21).

cDNAs for several chloroplast RNA-binding proteins similar in their domains structure and molecular weight from tobacco (11–13), maize (14) and Arabidopsis (15,16) have been described. Although these proteins share high homology in their RNA-binding domain sequences, possibly suggesting an evolutionary common ancestor and similar functions (11,31–34), their N terminus acidic domain sequences share no homology. In addition, from the amino acid sequences of the chloroplast RNA-binding proteins described above there are none with serine or threonine at position 22 or with casein kinase II conserved phosphorylation site like the 28RNP. Two other spinach chloroplast RNA-binding proteins, 24RNP and 33RNP (unpublished results), were not phosphorylated under the experimental conditions described in the present work. Therefore, the possibility that

the protein performing the same function of the 28RNP in other plants is phosphorylated, awaits experimental data.

Casein kinase II is a multifunctional and ubiquitous Ser/Thr protein kinase shown to phosphorylate DNA-binding proteins such as the Arabidopsis G-box binding factor GBF1 (23), Sp1 transcription factor (35), translation initiation factor 2 subunit (eIF-2a) (36) and other nucleic acid-binding proteins (28). Casein kinase II characteristics were recently reported for the protein kinase that phosphorylated the 28RNP (24). We tend to speculate that modulation of RNA 3' end-processing and stability in the chloroplast is modulated by the binding of the 28RNP to the 3' end RNA, which is controlled by the phosphorylation state of the protein. In that model, the activation/deactivation of the chloroplastic casein kinase II and/or the 28RNP phosphatase by light or the developmental program of the plant will determine RNA stability similar to the process that was recently reported for the regulation of translation of the D1 protein in the chloroplast of *Chlamydomonas reinhardtii* (22).

ACKNOWLEDGEMENTS

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