Phosphorylation of human hnRNP protein A1 abrogates *in vitro* strand annealing activity

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ABSTRACT

In HeLa cells metabolically labeled *in vivo* with [³²P] orthophosphate in the presence of okadaic acid the concentration of phosphorylated A1 protein was increased significantly as compared to controls. Purified recombinant hnRNP protein A1 served as an excellent substrate *in vitro* for the catalytic subunit of cAMP-dependent protein kinase (PKA) and for casein kinase II (CKII). Thin layer electrophoresis of A1 acid hydrolysates showed the protein to be phosphorylated exclusively on serine residue by both kinases. V8 phosphopeptide maps revealed that the target site(s) of *in vitro* phosphorylation are located in the C-terminal region of A1. Phosphoamino acid sequence analysis and site directed mutagenesis identified Ser 199 as the sole phosphoamino acid in the protein phosphorylated by PKA. Phosphorylation introduced by PKA resulted in the suppression of the ability of protein A1 to promote strand annealing *in vitro*, without any detectable effect on its nucleic acid binding capacity. This finding indicates that phosphorylation of a single serine residue in the C-terminal domain may significantly alter the properties of protein A1.

INTRODUCTION

Protein A1, one of the major components of eukaryotic hnRNP complex, is a basic protein of 34 kDa which is organized in two well defined domains (1, 2). Recent studies on the purified recombinant protein (3, 4) have revealed that the N-terminal domain of ~195 aminoacids, also named UP1 (2), consists of two homologous sub-domains each of them containing two short consensus sequences common to many RNA binding proteins (5, 6) (Fig.1A). The C-terminal region of A1 of ~125 aminoacids has an unusually high glycine content (40%); previous results have suggested that this domain is responsible for the cooperativity of binding of intact A1 to single-stranded nucleic acid templates (4) and more recent results indicate that it can also establish direct interactions with single-stranded nucleic acid (7).

Recently, it was discovered that protein A1 is able to promote interstrand reannealing of DNA as well as RNA (8–10) and that this activity resides mainly in its C-terminal fragment (9).

Many hnRNP core proteins were shown to be modified posttranslationally and a large part of this modification seems to arise by phosphorylation (11, 12). Alonso et al.(13), using an exogenous protein kinase, showed that a subset of proteins of the large hnRNP complex, (> 100 S) in the 30 to 40 kDa range, is phosphorylated *in vitro*. Although few data have been published on the phosphorylation of A1, it was reported that a particular conformeric form of this protein, designated A1x (12), serves as phosphate acceptor *in vivo*. According to the same authors, both A1 as well as A1x appear to be phosphorylated *in vitro* by an endogenous unidentified protein kinase associated with the 40 S hnRNP complex. Phosphorylation is a well documented posttranslational modification with regulatory functions that affects the properties of a wide range of proteins. With regard to hnRNP protein A1, a detailed description of the *in vitro* phosphorylation parameters is a prerequisite to any study on the possible *in vivo* significance of such modification. In this study we have characterized the phosphorylation of recombinant A1 by the catalytic subunit of cAMP dependent protein kinase (PKA) and by casein kinase II (CKII) and we show that phosphorylation by PKA suppresses the ability of A1 to promote strand annealing *in vitro*. Furthermore, the site of phosphorylation was found to be a serine residue in the glycine-rich C-terminal domain of A1 exactly at the border with the N-terminal domain. Finally, we show preliminary data indicating that protein A1 is indeed a phosphoprotein in HeLa cells.

MATERIALS AND METHODS

Enzymes and proteins

The catalytic subunit of cAMP-dependent protein kinase (PKA) of bovine hearts was purchased from Sigma. Casein kinase II (CKII) was a gift of Dr F.Meggio (University of Padova).

L-tosylamido 2-phenylethyl chloromethyl ketone treated trypsin and *Staph.aureus* V8 protease were from Worthington. The recombinant hnRNP protein A1 and its N-terminal fragment were

produced and purified as described (4). The C-terminal fragment of A1 was obtained by limited V8 proteolysis of recombinant A1 protein and subsequent purification according to a published protocol (9).

Phosphorylation assays

Phosphorylation of recombinant A1 or its C-terminal fragment was performed in a buffer containing 50mM TRIS-Cl pH 7.5, 5mM MgCl₂ and 50 μ M [γ -³²P] ATP (4000 cpm/pmol) in a total volume of 10 μ l. A typical reaction contained approximately 100 μ g/ml of recombinant protein and was initiated by the addition of PKA (2.4 μ g/ml) or of CKII (1.4 μ g/ml) and incubated at 30°C for the indicated times. Reactions were stopped by the addition of 5 $\times$ SDS-PAGE sample buffer and immediate boiling or by addition of 0.4 mg/ml deoxycolate (sodium salt) and 10% cold trichloroacetic acid (TCA). Analysis of phosphorylated proteins was carried out as described below. For the time course experiment, the reaction conditions were the same, but the reaction was scaled up 10 fold. Aliquots were removed at various time points and immediately blocked by addition of cold deoxycolate-TCA mixture. After incubation at 0°C for 60 min samples were centrifuged, the pellets were washed with cold acetone, resuspended in SDS-PAGE sample buffer, boiled and subjected to SDS-PAGE electrophoresis (12.5% gels). Dried gels were exposed for autoradiography and protein bands of interest were cut out and counted to quantitate radioactivity. For Km determinations, phosphorylation assays were performed in the presence of 100 μ M [γ -³²P] ATP (1000 cpm/pmol) with A1 at varying concentrations in the range of 0.1 to 20 μ M and with PKA at 0.5 μ g/ml or with CKII at 0.3 μ g/ml. Reactions were initiated by the addition of labeled ATP and, after 15 min at 30°C, terminated by addition of 5 $\times$ SDS-PAGE sample buffer and boiling. Samples were analyzed by SDS-PAGE electrophoresis.

Analysis of phosphoamino acids

Recombinant A1 protein ³²P-labeled by PKA or by CKII was hydrolyzed in 6 M HCl for 3 h at 110°C and then analyzed by electrophoresis on thin layer cellulose plates in presence of phosphoamino acid standards as described (14). ³²P-labeled phosphoamino acids were localized by autoradiography and identified by comparison with the ninhydrin stained phosphoamino acid standards.

Sequence analysis of the phosphorylation site

A sample (20 nmol) of protein A1 ³²P-phosphorylated by PKA was proteolyzed with trypsin in the reaction conditions described by Swenson et al. (15). Tryptic digest was then resolved by reverse-phase high performance liquid chromatography on a Brownlee Labs Aquapore RP 300 column (10 mm $\times$ 25 cm) at a flow rate of 1.5 ml/min, with a linear gradient of acetonitrile (0–35% in 80 min) in 0.05% aqueous trifluoroacetic acid (TFA) at 22°C. The column eluent was monitored at 220 nm and the radioactivity present in the peak was quantitated by liquid scintillation counting. The tryptic ³²P-phosphopeptide was sequenced with a Beckman 890 M spinning-cup liquid-phase sequencer, using the fast-protein Quadrol program with precycled Polybrene. The phenylthiohydantoin amino acid derivatives were identified by RP-HPLC on a Beckman Ultrasphere ODS column (5 mm $\times$ 25 cm), as described by Pucci et al. (16) and analyzed for radioactivity.

Oligonucleotide-directed mutagenesis

Site-directed mutagenesis was performed by overlap extension using the polymerase chain reaction as described (17). The overlapping oligo pair used to introduce the mutation into the plasmid carrying wild type A1 cDNA results in the substitution of serine 199 by proline. The entire A1 coding region was sequenced to ensure that only the desired mutation was present (data not shown) and the mutated protein was produced in *E. coli* and purified as previously described (4).

Preparative *in vitro* phosphorylation of proteins

Recombinant protein A1 (0.5 mg) or its C-terminal peptide (0.1 mg) were phosphorylated as described above except that ³²P-labeled ATP was replaced by cold ATP (100 μ M). 50 μ g of PKA in case of A1 or 10 μ g of PKA in case of C-terminal peptide were added to the reaction mixture (1 ml). The reactions were incubated at 30°C for 30 min, stopped by addition of EDTA to 5 mM, and immediately placed on ice. Each reaction was applied to a FPLC cation column (Mono S HR 5/5, Pharmacia) preequilibrated with 20 mM potassium-phosphate buffer, pH 7.5, 1mM EDTA, 10 mM Na₂S₂O₅, 1 mM DTT, 0.5% Tween-20 (Buffer A). The column was washed with 5 ml of Buffer A+0.1 M NaCl and then eluted using a 5 ml linear gradient from 0.1 to 1 M NaCl in Buffer A. The major protein peak was collected and dialyzed against Buffer A.

Assay of annealing activity

Reactions were performed as described by Kumar and Wilson (9), except that M13mp18 plus strand DNA and a 19 residue oligodeoxynucleotide complementary to position 6280–6298 as shown below were used as templates.

6280 6290 6300
 --- GGCATGCAAGCTTGGCACTGGCCGTCGTTTTAC ---
 GTTCGAACCGTGACCGCA

Briefly, the 19 mer oligonucleotide was 5'-end labeled with [³²P] and mixed with equimolar amount (0.2 nM) of M13mp18 plus strand DNA in a 10 μ l buffered reaction mixture containing protein A1 or its C-terminal fragment at the concentration indicated in the figure legends. After incubation at 25°C for 5 min, samples were run on agarose gel, the gel was dried and exposed for autoradiography.

Oligodeoxynucleotide binding assay

Binding assays were performed with the UV crosslinking protocol previously described (18). Purified proteins in the amounts indicated in figure legends and [³²P]-labeled oligonucleotide (0.2 μ M) were mixed in a 10 μ l buffered reaction mixture and incubated at 22°C for 10 min. After UV irradiation, samples were run on SDS-PAGE, the gel was then dried and autoradiographed.

In vivo labeling of HeLa cells with [³²P] orthophosphate

Cells were seeded at a density of 5 $\times$ 10⁶ per 90 mm dish and grown for 48 h in DMEM medium supplemented with 10% FCS. Cells were then washed with phosphate free DMEM containing 1% FCS and incubated for 4 h in 2.5 ml phosphate free DMEM+1% FCS and 1 mCi/ml [³²P]-orthophosphate. Where indicated, 20 μ l of okadaic acid (Calbiochem, stock solution at

100 $\mu\text{g/ml}$ in dimethylformamide) were added to the culture (1 μM final concentration) and incubation was continued for an additional 2.5 h. In the control culture 20 μl of dimethylformamide were added and incubation continued for the same length of time. Cells from both cultures were washed two times with ice cold PBS supplemented with 1 mM EDTA, 0.1 mM DTT, 1% Empigen BB and 100 $\mu\text{g/ml}$ Aprotinin (Buffer W) and then collected in 0.4 ml of Buffer W supplemented with 1 μM okadaic acid, 50 $\mu\text{g/ml}$ DNase I, 50 $\mu\text{g/ml}$ RNase A and 1 U/ml RNase T1. Cells were lysed by sonication (3 $\times$ 5 sec at 20 W), lysates were clarified by centrifugation at 3000 $\times$ g for 5 min and supernatants collected.

Immunoprecipitations

Supernatants from low speed centrifugation of cell lysates were incubated for 30 min at 4°C with the anti-A1 monoclonal antibody 4B10 (6, 19) attached to protein A-Sepharose as described by Buvoli et al.(20). Beads were then pelleted, washed and immunocomplexes eluted by boiling for 5 min in SDS-PAGE sample buffer and analyzed by SDS-PAGE. Dried gels were exposed for autoradiography.

RESULTS

Phosphorylation of A1 protein by PKA and by CKII

Purified catalytic subunit of cAMP dependent protein kinase (PKA) as well as purified casein kinase II (CKII) catalyzed the incorporation of phosphate into recombinant A1 protein in the presence of [γ - ^{32}P]ATP. The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. As shown in Fig. 1B lanes 1–2, the 34 kDa polypeptide of A1 was phosphorylated by PKA and by CKII. As expected the phosphorylation of A1 was dependent on the presence of kinases and MgCl_2 . The recombinant N-terminal peptide of A1 (residues 1–198)(4)(See Fig. 1A) was not labeled whereas the C-terminal peptide (residues 186–320) obtained by digestion of A1 with V8 protease (9) was readily labeled by both PKA and CKII (Fig. 1B lanes 3–4 and 5–6, respectively). This result was confirmed in an experiment in which A1 was first phosphorylated and then exposed to the V8 digestion. As shown in Fig. 1C (lanes 1 and 2) only the ~14 kDa C-terminal peptide was found labeled. Thus the most likely sites for PKA and CKII catalyzed *in vitro* phosphorylation of A1 are residues located at the carboxyl terminus of the protein (see Fig. 1A).

A typical time course of A1 phosphorylation is shown in Fig. 2: the amount of phosphate incorporated increased in a time dependent manner reaching a plateau at about 1 mol/mol of A1 protein with both PKA and CKII. The apparent K_m of PKA and CKII for A1 were 5.5 μM and 4 μM respectively and the V_{max} were 14.4 and 20 nmol/min/mg of kinase, respectively. Incubation of protein A1 with high concentrations of PKA or CKII (> 20 $\mu\text{g/ml}$) or sequential additions of smaller amounts of kinase led to a maximum phosphorylation of 1 mol of phosphate/mol of A1 suggesting the presence of a single site of phosphorylation (not shown). Since the consensus sequence for PKA and CKII is different (21), we tested the effect of sequential phosphorylation by the two enzymes. Surprisingly when A1 was phosphorylated by PKA to about 1 mol of phosphate/mol of protein there was no additional detectable phosphorylation upon addition of CKII. In the converse experiment where A1 was first

Figure 1. Phosphorylation of protein A1 and its fragments by PKA and CKII. Panel A: Schematic structure of hnRNP protein A1 and location of potential phosphorylation sites for PKA. The possible phosphate acceptor sites are shown by asterisks. V8 N-fragment (residues 1–185) and V8 C-fragment (residues 186–320) represent proteolytic peptides obtained by digestion of A1 with *Staph. aureus* V8 protease under the conditions described by Kumar and Wilson (9). N-TER (residues 1–198) is the recombinant N-terminal fragment of A1 expressed in *E. coli* and purified as described previously (4). Panel B: Recombinant A1 (lanes 1 and 2); recombinant N-terminal peptide (lanes 3 and 4) and the V8 generated C-terminal peptide (lanes 5 and 6), were incubated with PKA (lanes 1, 3 and 5) or with CKII (lanes 2, 4 and 6). Phosphopeptides were analyzed as described in Materials and Methods. The β subunit of casein kinase II was also autophosphorylated in these reactions. Panel C: Recombinant A1 was first phosphorylated and then subjected to V8 partial digestion. The resulting ^{32}P -labeled proteolytic fragments were separated on SDS-PAGE and visualized by autoradiography. Lanes 1 and 2 represent the autoradiogram of A1 pre-labeled with PKA and CKII, respectively, and then partially digested with V8. Lanes 3 and 4 are lanes 1 and 2, respectively, dye stained and dried, before autoradiography. Position of the molecular weight standards is shown in the margin.

phosphorylated with CKII and then incubated with PKA (see Fig. 3), we observed an apparent small increment of phosphorylation (from 0.75 to 1 mol of ^{32}P /mol of A1). However, on the bases of the results reported ahead, we believe that such increase is due to the completion of phosphorylation at the same site. These results suggest that phosphorylation of A1 by PKA and CKII involves the same site in the C-terminal domain of the protein.

Phosphoamino acid analysis and determination of the phosphorylated site of A1

Protein A1 ^{32}P -labelled by PKA or by CKII was subjected to acid hydrolysis and phosphoamino acids were then resolved by thin layer electrophoresis. In both cases A1 was found to be phosphorylated exclusively on serine (not shown). Since PKA and CKII appear to phosphorylate the same region of A1 (see above) we decided to concentrate our attention on PKA and to perform the next experiment with this enzyme, commercially available in purified form. In order to determine the site of PKA phosphorylation, the phosphorylated recombinant hnRNP A1 was

Figure 2. Time course of the phosphorylation of protein A1 by PKA and CKII. A1 was incubated under standard reaction conditions in the presence of PKA (□) or CKII (♦), and at the indicated times, samples were withdrawn for quantification of phosphoprotein by SDS-PAGE, as described in Materials and Methods.

Figure 3. Sequential phosphorylation of protein A1 by PKA and CKII. Protein A1 was incubated, at standard conditions, with 2.4 μ g/ml of PKA and after 150 min (arrow) CKII at 1.4 μ g/ml was added (□); or protein A1 was incubated with 1.4 μ g/ml of CKII and after 120 min (arrow) PKA was added at 2.4 μ g/ml (♦). At the indicated times aliquots of reaction mixtures were analyzed for quantification of phosphoprotein as described in the legend of Fig.2.

Table 1. Sequence analysis of the tryptic phosphopeptide of protein A1*

Cycle no.	Phenylthiohydantoin derivatives	Yield pmol
1	Ser	n.d.
2	Gly	340
3	Ser	n.d.
4	Gly	311
5	Asn	262
6	Phe	274
7	Gly	185
8	Gly	180
9	Gly	178
10	Arg	66

n.d. = not determined.

* 700 pmol of peptide was analyzed.

digested with trypsin and the resulting peptides were separated by RP-HPLC. The radioactivity profile revealed the presence of only one labeled peptide containing 88% of the total incorporated radioactivity (See Fig.4). The amino acid sequence of this peptide, reported in Table I, corresponds to residues 197–206 of the native protein sequence and contains two possible

Figure 4. Elution profile of tryptic phosphopeptides of protein A1. Tryptic digest of 32 P-labeled A1 was injected into an Aquapore RP-300 column equilibrated with 0.05% trifluoroacetic acid in water. The column was developed with a linear gradient of 0–35% acetonitrile in 0.05% aqueous TFA (thin line) in 80 min at 22°C with a flow rate of 1.5 ml/min. To identify fractions containing phosphorylated peptides radioactivity was determined by Cerenkov counting. Only one peak (*) containing the major part of radioactivity incorporated could be detected. This peak was then sequenced as described in Materials and Methods to identify SGSGNFGGGR as the phosphopeptide.

Figure 5. Phosphorylation of mutant protein A1. Increasing amounts (25, 50, 100 and 200 μ g/ml) of mutant A1 (lanes 1–4) and wild type A1 (lanes 5–8) were incubated, at standard conditions, with 2.4 μ g/ml of PKA (upper panel) or with 1.4 μ g/ml of CKII (lower panel) and the phosphoproteins were analyzed as indicated in Materials and Methods.

sites of phosphorylation: Ser 197 and Ser 199. Since phosphorylated serine is not recoverable from the sequencing instrument used in our experiment it is difficult to unequivocally identify the radioactive serine residue solely on the bases of these data although Ser 199 is the most likely candidate. This hypothesis is supported by the previously shown data on the lack of phosphorylation of recombinant N-terminal peptide (residues 1–198) and efficient phosphorylation of V8-generated C-terminal peptide (aa 186–320) (see also Fig. 1). Moreover Ser 199 lies in a PKA consensus sequence of the type RXXS* (where X is any amino acid) (see Fig.1 A). This hypothesis was in fact confirmed with the experiment described in the next section.

Figure 6. Effect of phosphorylation of protein A1 on strand annealing. Proteins from the phosphorylation as well as from the mock reaction were purified as indicated in the text and analyzed for reannealing activity as described in Materials and Methods. The amount of proteins used is indicated at the top of each lane. The migration positions of the ^{32}P -labeled 19 mer oligonucleotide and of the M13mp18 DNA-oligonucleotide hybrid are indicated in the margin.

In vitro phosphorylation of mutant A1

To determine whether Ser 199 is the site of phosphorylation a Ser $\rightarrow$ Pro mutation was introduced at this codon as described in Materials and Methods. The recombinant mutant A1 was then challenged with kinases under the conditions used for the wild type A1 protein. The results shown in Fig.5 indicate that the replacement of serine 199 by proline prevents phosphorylation of the protein by both PKA and CKII. These results are consistent with the previously reported indications and demonstrate that Ser 199 is the target of *in vitro* phosphorylation of protein A1. Moreover this experiment demonstrates that Ser 199 is indeed the site of CKII phosphorylation even if it does not belong to a consensus sequence (S*XXE) for this kinase. This is an intriguing finding if one considers that none of the serine residues, located in the four CKII consensus sequences present in the N-terminal domain of A1 (Ser 2, Ser 6, Ser 32, and Ser 182), is phosphorylated in our experimental conditions.

Phosphorylation of A1 inactivates the strand annealing activity

It was previously shown that the purified recombinant protein A1 (34 kDa) is a strong single-stranded nucleic acid binding protein organized in two discrete domains (N-terminal and C-terminal) each capable, as purified fragment, of binding to both single-stranded DNA and RNA (7). Furthermore, an additional property of A1 has emerged: i.e. the strong reannealing activity on both RNA and single-stranded DNA complementary strands (8–10). Such activity resides exclusively within the C-terminal domain of A1 since only the purified 14 kDa C-terminal fragment, but not the N-terminal fragment, is able to catalyze this reaction (9).

Since the C-terminal domain is the target of phosphorylation we checked whether this modification could alter the annealing activity of both the entire protein and its C-terminal purified peptide. A typical phosphorylation reaction was scaled up 500 fold and A1 (0.5 mg) was phosphorylated by PKA in presence of cold ATP (100 μM) for 30 min at 30°C. The reaction was then applied to a FPLC cation column (Mono S) and the elution was performed with a linear gradient as described in Materials and Methods. This column was used to remove PKA and other components of the kinase reaction because we observed that these components significantly interfere with the annealing assay. In effect protein A1 eluted at 450 mM NaCl while most of PKA eluted in the flowthrough and earlier in the gradient. The C-

Figure 7. Effect of phosphorylation of protein A1 on the binding activity. Proteins used in the assay were the same as in Fig.6 and the amounts used are indicated at the top. Positions of the ^{32}P -labeled 19 mer oligonucleotide and of the protein-oligonucleotide photocrosslinked product are indicated in the right margin; molecular weight standards are shown on the left.

Figure 8. *In vivo* radiolabeling of protein A1. Immunoprecipitates obtained with anti-A1 monoclonal antibody 4B10, as described in Materials and Methods, were run on SDS-PAGE. Labeled bands were identified by autoradiography of dried gels. +OK and -OK indicate okadaic acid treated and control HeLa cell cultures, respectively. The position of molecular weight markers is shown in the margin.

terminal domain fragment was phosphorylated and purified on the Mono S column using the same procedure. For both type of proteins a parallel mock reaction was carried out i.e. A1 and its C-terminal peptide fragment were incubated for the same time and in the same conditions but without added kinase.

Next we examined the activity of these modified proteins in an annealing assay that uses the M13 mp18 plus strand DNA and a base-pair complementary 19 mer oligonucleotide as described in Materials and Methods. Results presented in Fig.6 demonstrate that under our experimental conditions phosphorylation by PKA completely abolishes the reannealing activity of both A1 and of its C-terminal domain peptide.

Effect of phosphorylation on the nucleic acid binding activity of A1 and of its C-terminal peptide

Intact protein A1 as well as its N- and C-terminal fragments are able to bind single-stranded nucleic acid structures (4, 7, 22). Using the previously described binding assay based on UV-crosslinking to single-stranded oligonucleotides (18) we examined the effect of phosphorylation on the binding ability of A1 protein

and of its C-terminal fragment. The proteins, phosphorylated and purified as described in the previous section, were used to perform the UV-crosslinking binding experiment described in Fig. 7. As it can be seen, in this assay the binding capability of intact A1 to the substrate oligonucleotide was almost unaffected while the binding of the C-terminal domain was completely inhibited by phosphorylation. This observation is consistent with the idea that multiple intermolecular interactions are involved in the A1 binding to single-stranded nucleic acid molecules (8–10), and with the fact that phosphorylation by PKA does not affect the binding determinants in the N-terminal region of the protein (see above). Taken together, these data indicate that the phosphorylation of serine residue 199 in the C-terminal region of intact A1 is a specific modification that affects some of the *in vitro* properties of the protein and suggest a possible functional significance.

In vivo phosphorylation of A1

The finding that a certain protein is a good *in vitro* substrate for a ubiquitous protein kinase, indicates, but does not prove that the same protein might be phosphorylated *in vivo*. In fact no clearcut evidence of *in vivo* phosphorylation of protein A1 has been produced so far. However, it is possible that this protein is indeed phosphorylated in intact cells but is rapidly dephosphorylated in cell extracts by the multiple phosphatase activities present. To address this point, HeLa S3 human cells were metabolically labelled in medium containing [³²P] orthophosphate in the presence of okadaic acid as described in Materials and Methods. Okadaic acid is a potent inhibitor of protein phosphatases 2A and 1, two of the four major protein phosphatases in the cytosol of eukaryotic cells that dephosphorylate phosphoserine and phosphothreonine residues (23). Protein A1 was then immunoprecipitated with the monoclonal antibody 4B10 from total lysates of okadaic-treated and untreated cells. As shown in Fig. 8, treatment with okadaic acid enhanced at least 5 fold the amount of ³²P-radioactivity in the A1 band relative to the untreated cells. Therefore, alike other hnRNP proteins (12, 24, 25), also A1 appears to be a phosphoprotein, probably with a high dephosphorylation rate. Experiments are underway to define the site(s) of *in vivo* phosphorylation and the kinase(s) involved.

DISCUSSION

In this work we demonstrate that cAMP-dependent protein kinase and casein kinase II phosphorylate *in vitro* the A1 protein at Ser 199 and that, at least the modification introduced by PKA, results in a remarkable inactivation of the *in vitro* strand annealing activity of A1. Interestingly, the phosphorylation occurs in the C-terminal domain, a region of the protein that has been shown to support the cooperative binding of A1 to single stranded DNA and RNA and to be absolutely required for the annealing reaction (9). To explain such activity it has been suggested that the protein-protein binding capacity of this domain (4) might be responsible for an increase of the local concentration of A1-coated strands that can in turn lead to the rapid renaturation of complementary sequences (8, 9). In principle, phosphorylation may alter the carboxyl terminus of the protein in such a way that it interferes with the capacity of protein-protein interaction of A1. On the other hand, the fact that the C-terminal fragment alone is able to promote reannealing points to a direct binding of nucleic acid

to such domain, probably at the level of the flexible, weakly interacting, repeating structures (8). A direct interaction of the purified C-terminal domain has indeed been experimentally proven (7) and is further supported by the recent identification within this domain of a novel RNA binding motif designated 'RGG box' (26).

On these bases an alternative model for reannealing activity can be proposed whereby increased local concentration of complementary strands is attained by the simultaneous binding of nucleic acid molecules to the multiple binding sites of the protein (8–10). If this is the case it is conceivable that the modification of Ser 199 could directly inactivate one of the binding sites, for example a closely located 'RGG box' (a.a. 217–240 of intact A1) (26) or induce allosteric conformational changes with negative effects on the overall flexibility and binding geometry of the protein. The latter possibility is substantiated by the fact that the observed target of phosphorylation lies in the region of abrupt structural transition (27) where another *in vivo* modification has been located (N^ω, N^ω-dimethylarginine, at residue 196) (28). It should also be mentioned that such 'hinge' region of A1 is also particularly exposed to proteolysis (7, 22). We have indeed observed a significant decrease of V8 protease sensitivity upon phosphorylation of protein A1 by PKA and CKII (our unpublished results), suggesting that this modification can significantly alter the conformation of the protein.

Other hnRNP proteins, such as the C and U group proteins, are known to be phosphorylated both *in vivo* and *in vitro* (12, 25 and G.Dreyfuss personal comm.). In the course of this work we observed that also the recombinant C1 protein is able to promote renaturation of nucleic acid strands apparently with the same efficiency as A1 (our unpublished results). It will be interesting to determine the site(s) of phosphorylation of C1 and to analyze the effect of this modification on the reannealing activity of this protein.

Our demonstration that *in vitro* phosphorylation of A1 significantly alters its physico-chemical properties raises a number of questions as to the possible role of such modification *in vivo* in the light of the observation that A1 is a phosphoprotein in intact HeLa cells.

Several recent reports indicate that protein A1 could intervene along with other hnRNPs in the first steps of spliceosome assembly through binding to specific intron sequences (20, 29, 30) and interaction with components of the splicing machinery (31–33). Moreover, we have recently presented evidence for a possible role of A1 RNA reannealing activity in the first steps of spliceosome assembly mediated by the specific interaction between A1 and the U2 snRNP (20).

On the bases of our results and of the above considerations it is conceivable that phosphorylation of A1 might have a modulatory effect in pre-mRNA processing, for example, by facilitating and/or destabilizing RNA–RNA base pairing. If one considers that other hnRNP proteins and several splicing factors appear to be phosphorylated (34) it is tempting to propose that this modification could be an important regulatory switch in the RNA processing reactions. In support of this hypothesis it has recently been reported that addition of phosphatases inhibitors to HeLa nuclear extracts blocks both catalytic steps of pre-mRNA splicing (35). Finally, it should be mentioned that also the recently discovered shuttling of protein A1 from the nucleus to the cytoplasm during mRNA transport (36) could be influenced (or even mediated) by the state of phosphorylation of the protein.

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